RhNAC2 and *RhEXPA4* Are Involved in the Regulation of Dehydration Tolerance during the Expansion of Rose Petals[1][C][W][OA]

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Dehydration inhibits petal expansion resulting in abnormal flower opening and results in quality loss during the marketing of cut flowers. We constructed a suppression subtractive hybridization library from rose (*Rosa hybrida*) flowers containing 3,513 unique expressed sequence tags and analyzed their expression profiles during cycles of dehydration. We found that 54 genes were up-regulated by the first dehydration, restored or even down-regulated by rehydration, and once again up-regulated by the second dehydration. Among them, we identified a putative NAC family transcription factor (*RhNAC2*). With transactivation activity of its carboxyl-terminal domain in yeast (*Saccharomyces cerevisiae*) cell and Arabidopsis (*Arabidopsis thaliana*) protoplast, *RhNAC2* belongs to the NAC transcription factor clade related to plant development in Arabidopsis. A putative expansin gene named *RhEXPA4* was also dramatically up-regulated by dehydration. Silencing *RhNAC2* or *RhEXPA4* in rose petals by virus-induced gene silencing significantly decreased the recovery of intact petals and petal discs during rehydration. Overexpression of *RhNAC2* or *RhEXPA4* in Arabidopsis conferred strong drought tolerance in the transgenic plants. *RhEXPA4* expression was repressed in *RhNAC2*-silenced rose petals, and the amino-terminal binding domain of RhNAC2 bound to the *RhEXPA4* promoter. Twenty cell wall-related genes, including seven expansin family members, were up-regulated in Arabidopsis plants overexpressing *RhNAC2*. These data indicate that *RhNAC2* and *RhEXPA4* are involved in the regulation of dehydration tolerance during the expansion of rose petals and that *RhEXPA4* expression may be regulated by RhNAC2.

Drought or dehydration results in water deficit stress (WDS), one of the most important abiotic stresses to which plants are subjected. WDS can cause numerous morphological and developmental changes in plants, including reduced longevity, growth inhibition, enhancement of root growth, and early flowering (Xiong and Zhu, 2002). Roses (*Rosa* spp.) are the most important ornamental plants worldwide, and cut roses account for about 21% and 31% of all cut flowers traded in China and at the European auctions, respectively (Heinrichs, 2008). Commercially, roses are usually harvested at the open bud stage and are extremely susceptible to WDS during postharvest handling; WDS causes abnormal flower opening, flower wilting, wilting of the pedicel (bent neck), and failure to open (Jin et al., 2006; Xue et al., 2008). The effect of dehydration on flower opening and senescence in cut roses, therefore, has been studied at the physiological and biochemical levels for several decades (Kumar et al., 2008).

The effect of WDS on the transcriptome has been studied, using microarray technologies, in different plant tissues and species, including seedlings, leaves, and roots of Arabidopsis (*Arabidopsis thaliana*; Seki et al., 2002), seedlings, leaves, roots, and panicles of rice (*Oryza sativa*; Rabbani et al., 2003; Wang et al., 2011), leaves and roots of poplar (*Populus* spp.; Cohen et al., 2010), seedlings and leaves of barley (*Hordeum vulgare*; Talamè et al., 2007; Tommasini et al., 2008), and leaves of tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*; Schafleitner et al., 2007; Gong et al., 2010). The number of ESTs analyzed ranges from 1,000 in barley (Talamè et al., 2007) to tens of thousands in Arabidopsis and rice (Kawaguchi et al., 2004; Zhou et al., 2007). To date, several hundred genes have been reported to respond to drought stress and are classified into two major groups. The first group comprises regulatory proteins, including transcription factors and protein kinases (Seki et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). The second group comprises functional proteins, which are downstream effectors in the stress response pathway. WDS-induced transcriptomes differ significantly depending on species, developmental stage, and organs and tissues analyzed, but they also show strong overlapping of gene function, including many orthologs.

Transcription factors are important regulatory proteins controlling the differential expression of physiological and biochemical processes during plant growth and development. They function by binding to cis-

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elements in the promoters or enhancers of their target genes. There are about 1,600 genes encoding for independent transcription factors in the genome of Arabidopsis (Riechmann et al., 2000; Melzer and Theissen, 2011). Based on structural features or conserved domains, they are grouped into families, such as basic leucine zipper, WRKY, MYB, APETALA2/ethylene responsive element binding protein (AP2/EREBP), NAM, ATAF, and CUC (NAC), zinc finger, MADS, and heat shock factor family proteins (Zhou et al., 2007; Nakashima et al., 2009; Qiu and Yu, 2009; Seo et al., 2009; Jeong et al., 2010). Many transcription factor families have been implicated in the regulation of WDS responses in plants (Hussain et al., 2011).

Gene Ontology (GO) enrichment analysis has been a useful tool for identifying biological processes that may be involved in the response to WDS. An overview of functional groups involved in WDS responses has been obtained from several plant species, including Arabidopsis (Wilkins et al., 2010), poplar (Street et al., 2006; Cohen et al., 2010), and chickpea (*Cicer arietinum*; Molina et al., 2008). Biological functions and metabolic processes that are indicated by the GO analysis vary, depending on the organ or plant species exposed to WDS. To date, studies of changes in gene expression profiles during WDS have mostly focused on vegetative organs, including seedlings, leaves, and roots, of model and crop plants. Less information is available on floral organs, apart from a global transcriptomics study of the response to WDS in *Alstroemeria aurantiaca* (Wagstaff et al., 2010) and a suppression subtractive hybridization (SSH) study of desiccation in lily (*Lilium* spp.) anthers (Hsu et al., 2007). No such study has been conducted on roses, a multipetaled and dicotyledonous model flower that is also the most important commercial cut flower.

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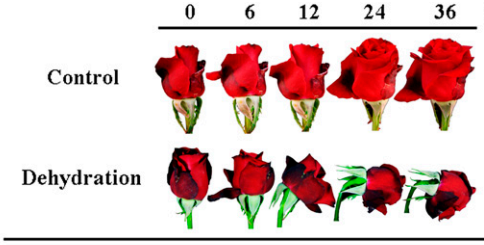

B

	Numbers	Average length (bp)
ESTs	7,071	403.7
Singletons	2,213	410.2
Contigs	1,300	917.9
UniESTs	3,513	598.1

Figure 1. Subtracted cDNA library of rose flowers under dehydration. A, Phenotypes of rose flowers under dehydration. Dehydrating flowers were held in air (25°C, 40%–50% relative humidity) under continuous light (140 μmol m^{-2} s^{-1}), and control flowers remained in water under the same conditions. Both were sampled at 6, 12, 24, and 36 h. B, Rose flower ESTs in the subtracted cDNA library prepared from the combined samples. cDNA prepared from flowers under dehydration was used as tester, and cDNA prepared from the control flowers was used as driver.

Plant-specific NAC (for NAM, ATAF, and CUC) transcription factors are characterized by highly conserved DNA-binding NAC domains located in the N-terminal region and a variable C terminus (Ooka et al., 2003; Jeong et al., 2010). Arabidopsis and rice contain approximately 117 and 151 *NAC* members, respectively (Nuruzzaman et al., 2010). There are at least five different NAC subgroups based on their target DNA-binding sites in Arabidopsis (Le et al., 2011). NAC transcription factors play important roles in regulating diverse biological processes, including development, senescence, growth, cell division, and responses to environmental stress stimuli (Olsen et al., 2005).

The involvement of NACs in the regulation of the Arabidopsis WDS response was suggested by the identification and functional analysis of the *AtNAC2*, *ANAC019*, *ANAC055*, *ANAC072*, and *ANAC102* genes under drought conditions (Tran et al., 2004; He et al., 2005; Christianson et al., 2009). Genome-wide transcriptomic analysis indicates that 20% to 25% of NAC genes in the whole genome of Arabidopsis and rice are responsive to drought stress (Tran et al., 2010). There are also a number of studies on abiotic stress-related NAC transcription factors in other plant species, such as that on *GmNACs* from soybean (*Glycine max*; Tran et al., 2009), *TaNAC69* from wheat (*Triticum aestivum*; Xue et al., 2011), and *AhNAC2* from groundnut (*Arachis hypogaea*; Liu et al., 2011). There has been no study, so far, of the possible role of NAC family genes in the response to WDS during petal expansion of flowers.

Expansins are plant cell wall-loosening proteins grouped into four subfamilies, designated as α-expansin, β-expansin, expansin-like A, and expansin-like B (Sampedro and Cosgrove, 2005). Expansin genes were first isolated from cucumber (*Cucumis sativus*) hypocotyls and play a primary role in cell wall extension (McQueen-Mason et al., 1992). Expansin proteins have been identified in widely different species and are involved in a variety of developmental processes requiring cell wall modification (McQueen-Mason and Cosgrove, 1995; Cosgrove, 2000). For example, they function in leaf and root growth (Wu et al., 2001; Shin et al., 2005), internode elongation (Choi et al., 2003), leaf development (Fleming et al., 1997; Pien et al., 2001), and floral development (Zenoni et al., 2004).

There is increasing evidence of a role for expansins in plant stress responses. Expansins are strongly regulated at the transcriptional level by WDS in maize (*Zea mays*; Wu et al., 2001; Zhu et al., 2007) and in a resurrection plant, *Craterostigma plantagineum* (Jones and McQueen-Mason, 2004). Heat tolerance, shade stress, low oxygen, and pathogens also up-regulate the expression of expansins in plants (Colmer et al., 2004; Xu et al., 2007; Ding et al., 2008; Fudali et al., 2008; Sasidharan et al., 2008). Overexpression of *TaEXPB23*, a wheat expansin gene, improves drought tolerance in tobacco (*Nicotiana tabacum*; Li et al., 2011). The transgenic plants showed improved tissue integrity during

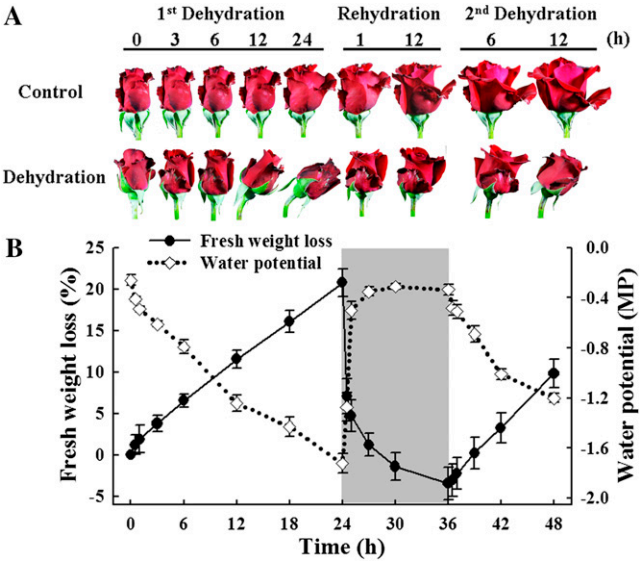

Figure 2. Morphological changes of rose flowers during cyclic dehydration. A, Flower phenotypes. Flowers were held dry in air for 24 h (first dehydration), returned to water for 12 h (rehydration), and then held dry in air for a further 12 h (second dehydration). Control flowers were held continuously in water. The flowers were held at 25°C and 40% to 50% relative humidity in continuous light (140 μmol m^{-2} s^{-1}). B, Fresh weight loss (solid line) and water potential (dotted line) for the flowers. Vertical lines represent SD; $n = 30$ for fresh weight loss and $n = 10$ for water potential.

WDS, suggesting that expansins increase the flexibility of the cell wall (Li et al., 2011).

In ornamental plants, α- and β-expansin were identified from opening and senescing flowers of *Mirabilis jalapa* (Gookin et al., 2003). Two expansin genes, *DcEXPA1* and *DcEXPA2*, are associated with petal growth and development during carnation (*Dianthus caryophyllus*) flower opening (Harada et al., 2011). In roses, *RhEXPA*, one of three α-expansin genes (*RhEXPA1–RhEXPA3*), is mainly involved in petal expansion (Takahashi et al., 2007; Yamada et al., 2009). However, information is very limited on the role of expansins in flower opening under WDS conditions.

Our previous work has indicated that dehydration can inhibit petal expansion, resulting in abnormal flower opening in roses. It is known that petal expansion depends on three important processes: cell wall metabolism, changes in cell turgor pressure, and restructuring of the cytoskeleton (Cosgrove, 2005; Smith and Oppenheimer, 2005; Zonia and Munnik, 2007). Nevertheless, the molecular mechanism of the effect of dehydration on petal expansion is still unclear. In this work, we focused on RhNAC2, a transcription factor identified in a dehydration-response SSH library from rose (*Rosa hybrida*) flowers. Functional analysis of this gene suggested that it is involved in the regulation of dehydration tolerance during petal expansion. An up-regulated expansin gene, *RhEXPA4*, may be directly regulated by *RhNAC2*. Our findings indicate that *RhNAC2* and *RhEXPA4* play an important role in flower opening under water stress conditions.

RESULTS

Isolation of Unique ESTs in Response to Dehydration in Rose Flowers

To analyze changes in the transcriptome resulting from dehydration on flower opening in roses, we first constructed a subtracted complementary DNA (cDNA) library by SSH. For the dehydration treatment,

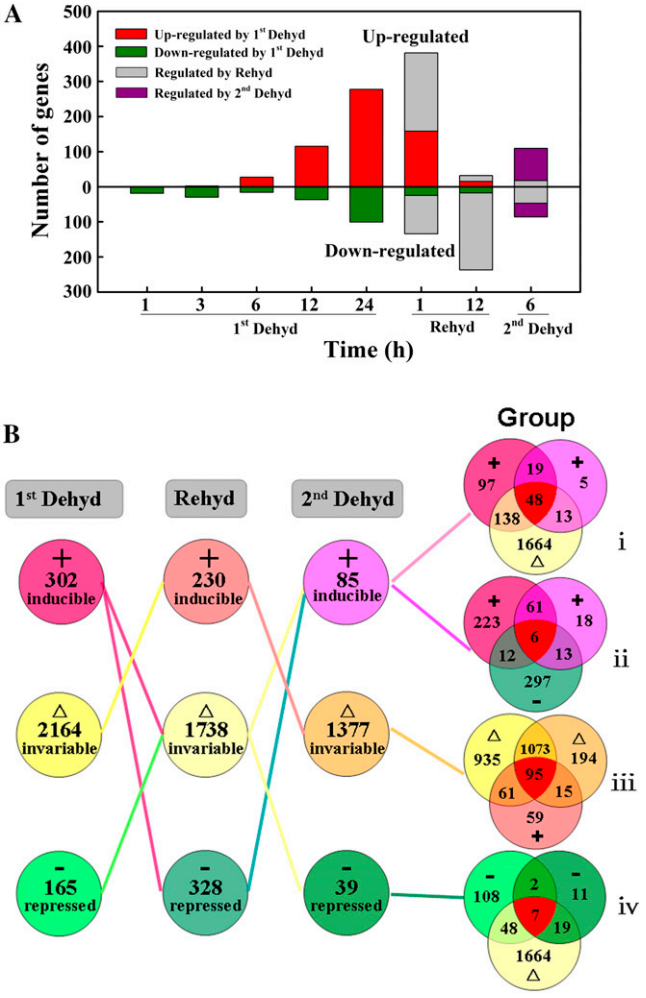

Figure 3. Differentially expressed genes in rose petals during cyclic dehydration. Flowers were held dry for 36 h (first dehydration), placed in water for 12 h (rehydration), and then held dry again for 8 h (second dehydration). Experiments were conducted under controlled conditions of 25°C, 40% to 50% relative humidity, and continuous light (140 μmol m^{-2} s^{-1}). Genes were considered induced or repressed by petal dehydration (Dehyd) and rehydration (Rehyd) when the ratio of their transcript abundance to that in control petals was 2 or greater or 0.5 or less, respectively ($P < 0.05$); other genes were considered invariable. A, Number of differentially expressed genes. B, Selected gene groups classified by their expression pattern during dehydration and rehydration. Groups are as follows: i, first Dehyd-induced, Rehyd-invariable, second Dehyd-induced genes; ii, first Dehyd-invariable, Rehyd-repressed, second Dehyd-invariable genes; iii, first Dehyd-invariable, Rehyd-induced, second Dehyd-invariable genes; iv, first Dehyd-repressed, Rehyd-invariable, second Dehyd-repressed genes. [See online article for color version of this figure.]

we held the cut flowers without water for 36 h, by which time the flowers showed considerable petal wilting and bent neck (Fig. 1A). We combined RNA samples isolated from the flowers at 6, 12, 24, and 36 h of dehydration as tester and from the mixture of RNA samples from the control flowers at the corresponding time points as driver. The forward subtractive PCR products were ligated into the pGEM T-Easy vector (Promega) and then transferred into *Escherichia coli*. We obtained 7,071 high-quality ESTs with an average length of 403 bp through random sequencing of bacterial colonies. A total of 3,513 uniESTs, including 1,300 contigs and 2,213 singletons, were identified by clustering and assembly analysis. Among the unique ESTs (uniESTs), 527 were putative full-length cDNAs based on the BLAST analysis using GenBank. The uniESTs had an average length of 598 bp (Fig. 1B). Ninety percent of the ESTs and the uniESTs were between 200 and 800 bp in length; the number of ESTs over 800 bp in length was greater for the contigs than for the singletons (Supplemental Fig. S1).

Classification of Genes Responding to Dehydration in Rose Petals

To evaluate the expression profiles of dehydration-regulated genes in rose petals, we amplified all uniESTs from the SSH library by PCR and spotted them on amino-silaned glass slides to make a cDNA microarray. Flowers were exposed to cyclic dehydration, namely dehydration for 24 h (first dehydration), rehydration for 12 h in water, then dehydration for 12 h (second dehydration). We recorded the phenotypes, water potential, and fresh weight of flowers during the treatment. During the first dehydration, flowers started to wilt after 12 h, and neck bending occurred at 24 h. The necks recovered after 1 h of rehydration (Fig. 2A). In the second dehydration, flowers wilted after 6 h and recovered after 12 h of rehydration (data not shown), indicating that the second dehydration resulted in greater stress. During the first dehydration, flowers lost fresh weight linearly, reaching approximately 20% loss after 36 h. Water uptake was very rapid in the first 1 h of rehydration, and the flowers reached their original fresh weight within 3 h. Changes in water potential reflected the changes in flower fresh weight (Fig. 2B).

Petal total RNA extracts were hybridized to the microarray chips; spot intensities were quantified using the Axon GenePix pro 6.0 software, and the Lowess strategy was applied to normalize the ratio values for each array using the Bioconductor marray R package (Yang et al., 2002). We first compared differentially expressed genes identified by SSH in rose flower with genes that were up-regulated (more than 2.0-fold change, corrected $P < 0.05$) during the first dehydration. We found up-regulation of approximately 54% of the uniESTs present in more than 10 copies in the SSH library. In contrast, only 5.5% of the singleton uniESTs in the SSH library were found to be up-regulated in the microarray assay (Supplemental Fig. S2).

We considered genes with an expression ratio of 2 or greater or 0.5 or less and corrected $P < 0.05$ at any time-course point in the cyclic dehydration treatment as dehydration-regulated genes. During the first dehydration, the number of such genes increased rapidly after 12 h of dehydration. After rehydration for 12 h, transcript abundance for these genes, especially those up-regulated by the first dehydration, was restored to control levels; a number of genes were concomitantly down-regulated. By 6 h of the second dehydration, the number of genes responding to the stress was similar to that seen in the first dehydration at 12 h (Fig. 3A).

To classify the genes on the basis of expression patterns in the cyclic dehydration treatment, we

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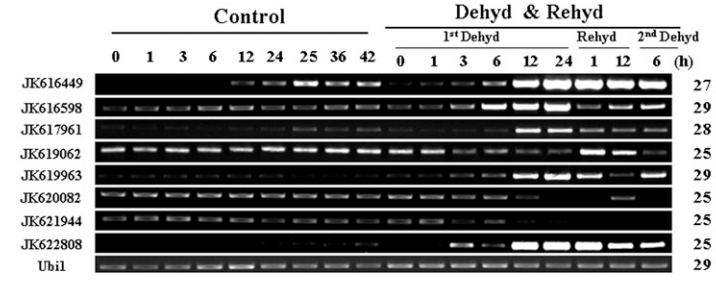

B

Accession No.	Annotation	1st Dehyd					Rehyd		2nd Dehyd
		1 h	3 h	6 h	12 h	24 h	1 h	12 h	6 h
JK616449	Iron-binding protein	1.0	1.5	1.8	4.8	20.4	22.0	2.8	7.6
JK616598	Expansin protein	1.0	1.5	2.5	5.2	9.0	0.1	0.5	1.0
JK617961	ATHB-12	0.9	0.9	1.2	2.1	4.3	1.7	0.6	0.6
JK619062	14-3-3b protein	1.0	0.8	0.9	0.5	0.4	1.9	1.5	0.5
JK619963	NAC domain protein	0.9	1.2	1.1	3.2	4.5	1.4	1.1	2.3
JK620082	Sterol desaturase	1.3	0.9	0.7	0.5	0.2	0.5	0.8	0.3
JK621944	Pollen allergen-like	1.3	1.0	0.7	0.4	0.1	0.1	0.4	0.2
JK622808	Early dehydration inducible protein	1.1	1.4	1.7	11.8	12.2	18.0	1.0	2.1

Figure 4. Verification of microarray results by RT-PCR. A, RT-PCR analysis of selected genes. *Ubi1* was used as the internal control. Numbers of PCR cycles are listed on the right. Dehyd, Dehydration; Rehyd, rehydration. B, Expression ratios of the selected genes derived from the microarray analysis. Data matching the RT-PCR results are underlined.

Table I. *Transcription factors up-regulated by dehydration in rose petals*

Significant differences (corrected $P < 0.05$ and expression ratio ≥ 2) in relative levels are shown in boldface.

Accession No.	Annotation	First Dehydration					Rehydration		Second Dehydration
		1 h	3 h	6 h	12 h	24 h	1 h	12 h	6 h
NAC family									
JK616545	NAC domain-containing protein	1.0	0.9	0.9	**2.0**	**4.2**	**3.0**	1.7	1.5
JK617046	NAC domain protein	0.8	0.9	1.3	**2.1**	**3.2**	**3.5**	1.1	1.7
JK617768	NAC domain protein	1.1	1.2	0.9	**3.0**	**3.6**	**3.0**	**2.1**	1.2
JK619963	NAC domain protein NAC2	0.9	1.2	1.1	**3.2**	**4.5**	1.4	1.1	**2.3**
JK620479	NAC domain transcription factor	1.0	1.0	1.1	**2.8**	**4.0**	**2.7**	1.4	1.5
Homeodomain family									
JK616983	HD domain transcription factor	0.9	1.1	1.3	**2.5**	**2.6**	1.3	1.1	1.9
JK617961	ATHB-12	0.9	0.9	1.2	**2.1**	**4.3**	1.7	0.6	0.6
BTB/TAZ family									
JK619064	BTB and TAZ domain protein4	0.9	0.8	0.8	1.6	**2.7**	**3.6**	1.2	1.0
JK622967	BTB and TAZ domain protein1	1.0	0.9	1.0	1.4	**2.4**	**4.4**	1.1	0.8
AP2 family									
JK619081	EREB protein	1.1	1.3	1.0	1.2	**2.2**	1.3	1.7	1.1
JK622912	ABR1 (ABA REPRESSOR1)	0.9	0.9	0.8	1.7	**3.7**	**16.7**	**0.5**	0.9
Other transcription factors									
JK617262	SET domain-containing protein	0.9	1.1	1.6	**2.5**	**3.1**	**3.0**	0.9	**2.7**
JK617756	Zinc-binding family protein	0.9	1.1	1.0	1.8	**3.4**	0.9	1.3	1.5
JK619813	DNA-binding protein S1FA	1.1	1.2	1.0	2.1	**2.4**	1.6	**2.7**	1.2
JK621262	WRKY65 transcription factor	1.0	1.1	1.4	1.6	**2.4**	**0.3**	1.1	1.6
JK622333	MYB transcription factor	1.1	1.5	1.7	1.7	**2.0**	**0.5**	1.4	1.8

regarded genes with an expression ratio of 2 or greater and corrected $P < 0.05$ at any time-course point as induced genes, genes with an expression ratio of greater than 0.5 to less than 2 as invariable genes, and genes with an expression ratio of 0.5 or less and corrected $P < 0.05$ as repressed genes. Genes that were induced, invariable, and repressed during the cyclic dehydration treatment (Fig. 3B) are listed in Supplemental Table S1. Using Venn diagram analysis, we analyzed the specificity and cross talk in gene expression in the responses to dehydration and rehydration. We classified them into 27 groups based on their gene expression patterns (Supplemental Fig. S3), and four of the 27 groups are shown in Figure 3B. We postulated that the genes most closely related to dehydration tolerance would be those whose transcript abundance was up-regulated during the first dehydration, restored to control abundance or unaffected during rehydration, and up-regulated again during the second dehydration. Groups i (induced-invariable-induced genes) and ii (induced-repressed-induced genes) meet these criteria (Fig. 3B). Group i comprises 48 genes and group ii comprises six genes, including genes known to be associated with drought

Table II. *Genes involved in the biological process of plant-type cell wall organization responsive to dehydration*

Significant differences (corrected $P < 0.05$ and expression ratio ≥ 2 or ≤ 0.5) in relative levels are shown in boldface.

Accession No.	Annotation	Dehydration			Rehydration
		6 h	12 h	24 h	1 h
JK616598	ATEXPA10	**2.5**	**5.2**	**9.0**	**0.1**
JK617470	Pro-rich cell wall protein	1.1	0.6	0.3	**0.3**
JK617691	ATEXPA9	1.6	1.2	0.9	**0.02**
JK617882	ATEXPA1	1.7	1.2	0.9	**0.04**
JK618171	Cellulase	1.6	**3.1**	**5.0**	**3.7**
JK618328	ATEXPA1	**2.4**	**3.1**	**2.5**	**0.1**
JK618813	Pectate lyase	1.4	1.0	**0.3**	**0.1**
JK619324	ATEXPA11	0.9	1.8	**4.4**	1.1
JK619325	ATEXPA8	1.3	1.2	1.2	**0.1**
JK619868	Cellulose synthase	0.7	0.6	**0.4**	0.8
JK620968	ATEXPA4	1.2	1.6	1.8	**2.3**
JK621413	ATEXPA1	**2.4**	**3.2**	**2.1**	**0.1**
JK622421	Pectin methylesterase31	1.5	1.8	1.2	**2.3**

stress (dehydrin, Suc synthase), transcription factors (NAC protein, zinc ion-binding protein), and cell wall-related genes (expansin, pectate lyase; Supplemental Table S2).

To confirm the microarray results, we conducted reverse transcription (RT)-PCR of eight genes selected from the dehydration-responsive genes identified in microarray analysis (Fig. 4A). Results from semiquantitative RT-PCR are in agreement with the microarray data for 55 out of 64 (86%) data points (Fig. 4B).

Transcription Factors Responding to Dehydration in Rose Petals

To understand transcriptional regulation during dehydration of rose petals, we first analyzed all transcription factors that were responsive to dehydration. From 101 annotated transcription factor unigenes in our microarray, we observed 41 unigene homologs that responded to dehydration and rehydration, comprising members of at least 17 transcription factor families (Supplemental Table S3). We focused on transcription factors up-regulated by dehydration, especially those from four major families: NAC, Homeodomain, Broad-Complex, Tramtrack, and Bric-a-Brac/Transcriptional Adaptor Zinc finger (BTB/TAZ), and AP2 (Table I). Some NAC family transcription factors were strongly up-regulated by dehydration; transcripts of a unigene, JK619963, increased 4.5-fold after 24 h of the first dehydration, decreased to 1.4-fold after 1 h of rehydration, and then increased to 2.3-fold after 6 h of the second dehydration. JK619963 is in group i of the Venn diagram (Fig. 3B; Supplemental Table S2).

Biological Processes in the Dehydration Response of Rose Petals

To understand the functional significance of the dehydration-responsive genes in rose petals, we classified differentially regulated genes into different categories using a set of plant-specific GO slims (http://www.geneontology.org/GO.slims.shtml). This analysis suggested that cyclic dehydration caused significant changes in a range of biological processes, including drought, defense, and stimulus responses (Supplemental Tables S4 and S5). Processes such as Pro biosynthesis, carbohydrate metabolism, and polysaccharide catabolism relate to osmotic adjustment. This suggests that the synthesis and breakdown of osmotic components is tightly linked to the dehydration response. Cytokinin metabolism was also very responsive to dehydration; two genes encoding cytokinin oxidase/dehydrogenases were highly up-regulated during dehydration, suggesting that endogenous cytokinin content of petals may be reduced during dehydration.

Interestingly, we found that the abundance of transcripts for enzymes associated with plant cell wall organization changed significantly during cyclic dehydration.

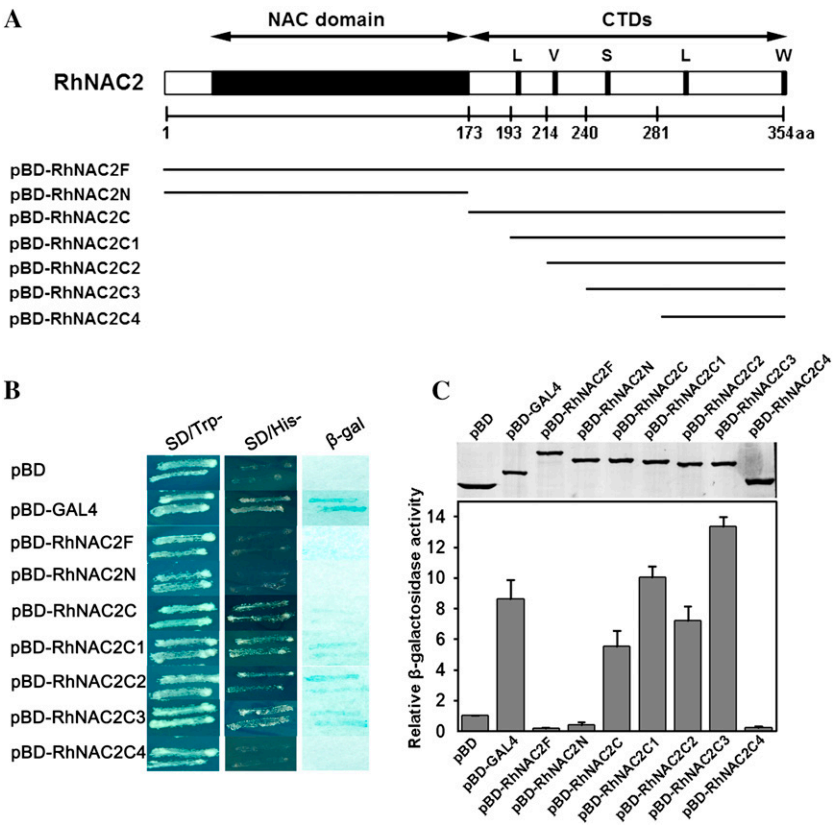

Figure 5. Truncation analysis of transactivation activity of RhNAC2 in yeast. A, Constructs used in the transactivation analysis. The regions shown as lines below the RhNAC2 diagrams were inserted into the expression vector pBD-GAL4 to create expression vectors comprising partial RhNAC2 proteins fused with the GAL4 DNA-binding domain in yeast. The numbers below the line indicate the positions of each truncated portion of RhNAC2. aa, Amino acids; CTDs, C-terminal domains; L, V, S, and W indicate highly conserved motifs common to NAC proteins. B, Transactivation activity of RhNAC2 in yeast. Expression vectors (pBD-X) were introduced into yeast YRG-2; pBD-GAL4 was used as the positive control and pBD vector was used as the negative control. The transformants were streaked on SD-Trp medium (left panel) and SD-His medium (middle panel) for examination of growth. C, β-Galactosidase activity was measured in the transformed yeast cells (bar graph). The top panel shows the expression of pBD-X proteins. Rabbit anti-GAL4 DNA-BD antibody was used western-blot analysis. Each data point represents the mean ± SD (n = 6). [See online article for color version of this figure.]

These genes were up-regulated at the first 6, 12, and 24 h of dehydration (Supplemental Table S4), down-regulated immediately after 1 h of rehydration (Supplemental Table S5), and up-regulated during the second 6 h of dehydration (Supplemental Table S4). These findings suggest that the assembly, disassembly, and rearrangement of constituent parts of the cellulose- and pectin-containing cell wall are important components of the dehydration response in rose petals.

We analyzed the effects of cyclic dehydration on all 41 of the cell wall-associated unigenes on the rose petal microarray. We found that 13 out of 41 unigenes were involved, comprising homologs to eight expansins, one cellulase, one cellulose synthase, one Pro-rich cell wall protein, one pectin methylesterase, and one pectate lyase (Table II). One of the 13 unigenes, JK616598, was rapidly and strongly up-regulated during the first dehydration and then was quickly down-regulated during rehydration. This unigene, therefore, was further evaluated to determine its role in rose flower opening during the cyclic dehydration treatment.

Characterization of *RhNAC2* and *RhEXPA4*

To characterize the functional role of JK619963 and JK616598 in dehydration tolerance of rose petals, we first cloned the two full-length cDNAs. JK619963 is 1,395 bp in length with a 227-bp 5′-untranslated region (UTR) and a 103-bp 3′-UTR; the gene encodes a polypeptide of 355 amino acids. Analysis of the deduced amino acid sequence indicates that the protein shares homology to known NAC family proteins and contains five conserved subdomains (A–E). In our previous work, a unigene induced by ethylene was identified and designated as *RhNAC1* (data not shown); therefore, JK619963 is designated as *RhNAC2* (Supplemental Fig. S4, A and B). Phylogenetic comparison with NAC proteins from Arabidopsis indicates that *RhNAC2* is a close homolog of embryo development-related genes CUC1 and CUC2 and also of secondary cell wall NACs VND6 and VND7 (Supplemental Fig. S4C).

JK616598 is 1,298 bp in length with a 43-bp 5′-UTR and a 499-bp 3′-UTR; the gene encodes a polypeptide of 252 amino acids. Phylogenetic analysis indicates that JK616598 is a homolog of α-type expansin proteins in Arabidopsis and is designated as *RhEXPA4* (Supplemental Fig. S5), since three other expansin genes (*RhEXPA1*, AB370116; *RhEXPA2*, AB370117; *RhEXPA3*, AB370118) have been described previously (Takahashi et al., 2007; Yamada et al., 2009).

We examined the expression patterns of the two genes during cyclic dehydration of rose petals using RT-PCR and found that they mirrored the pattern previously determined using the microarray. We also measured the expression levels of *RhNAC2* and *RhEXPA4* in different floral organs and leaves. During dehydration, both genes were strongly induced in petals, and *RhNAC2* was also clearly induced in other organs, but *RhEXPA4* expression showed no obvious changes in other floral organs or leaves (Supplemental Fig. S6). To determine subcellular localization of *RhNAC2*, we transiently expressed *p35S*::*GFP-RhNAC2* in onion (*Allium cepa*) epidermal cells. GFP fluorescence in the onion cells was detected exclusively in the nucleus, indicating that the gene encodes a nucleus-localized protein (Supplemental Fig. S7).

To examine the transactivation potential and the function of the different conserved motifs of RhNAC2, we performed a yeast (*Saccharomyces cerevisiae*) one-hybrid assay. We truncated and fused different portions of RhNAC2 to the GAL4 DNA-binding domain in the pBD vector (Fig. 5A). We tested the growth on synthetic dextrose (SD)-His plates of yeast bearing four different C-terminal domain truncations of RhNAC2. In comparison with the positive control (pBD-GAL4), the full length of RhNAC2 (pBD-RhNAC2F) and the NAC domain (pBD-RhNAC2N) showed no obvious β-galactosidase activity, while the C-terminal domain (pBD-RhNAC2C) retained 0.64-fold activity. One, pBD-RhNAC2C4, failed to grow; the other three grew well and showed higher relative β-galactosidase activity than colonies bearing the full C-terminal domain (Fig. 5, B and C). The abilities of RhNAC2C and RhNAC2C3 to activate transcription were also examined using the GAL4 transient expression system in Arabidopsis protoplasts. Compared with the GAL4-DBD negative control, both fusion proteins strongly activated the reporter GUS gene, and the relative GUS activities of RhNAC2C and RhNAC2C3 were 2.11- and 3.91-fold higher than the negative control, respectively (Supplemental Fig. S8). The results indicate that the 240 to 281 region of the polypeptide, with a putative S motif, may play an important role in the transactivation activity of RhNAC2.

Dehydration Tolerance in *RhNAC2*- or *RhEXPA4*-Silenced Rose Petals

To test the possible role of *RhNAC2* or *RhEXPA4* in dehydration tolerance in rose petals, we silenced each gene in rose petals using a virus-induced gene silencing (VIGS) approach. By infiltrating entire petals or petal discs with *Agrobacterium tumefaciens* containing tobacco rattle virus (TRV)-*RhNAC2*, we were able to partially or completely silence the *RhNAC2* gene (Fig. 6A). After 12 h of dehydration, the fresh weight of all the petals was about 70% of its initial value and petal width was about 83% of its initial value. During 24 h of rehydration, TRV control petals returned to 91% of their initial fresh weight, but it was still only 79% in the completely *RhNAC2*-silenced petals. Similarly, petal width returned to near the initial levels in the TRV controls but was still only 88% of the initial width in the completely *RhNAC2*-silenced petals (Fig. 6B; Supplemental Table S6).

The effects of gene silencing and dehydration were also tested in petal discs; the percentage of fully expanded discs provided a convenient rehydration

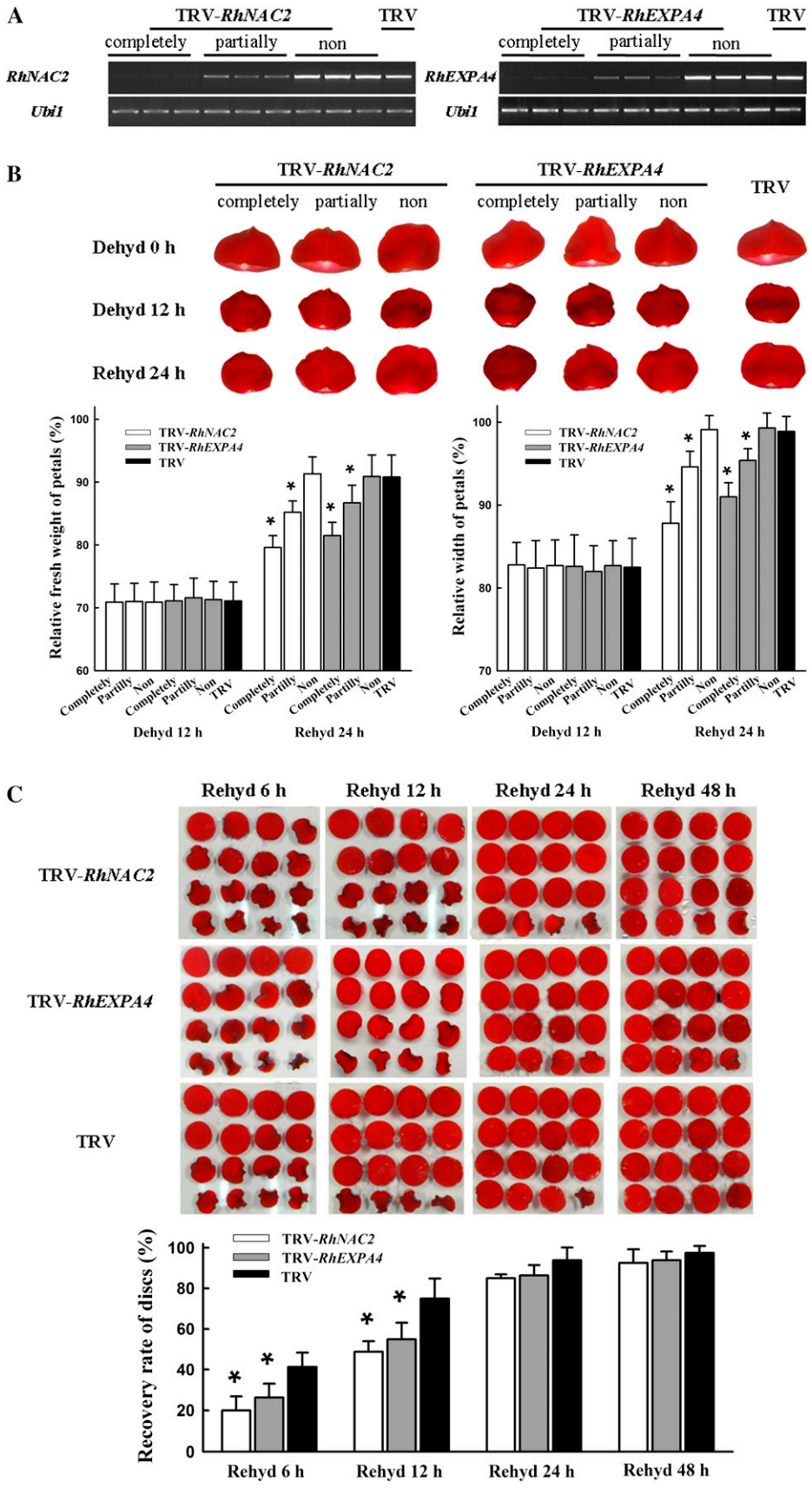

Figure 6. Silencing of *RhNAC2* or *RhEXPA4* in rose petals and discs by VIGS. Rose petals or discs were infiltrated with *A. tumefaciens* containing TRV control (TRV, pTRV1 + pTRV2), TRV carrying a *RhNAC2* fragment (TRV-*RhNAC2*, pTRV1 + pTRV2-*RhNAC2*), or TRV carrying a *RhEXPA4* fragment (TRV-*RhEXPA4*, pTRV1 + pTRV2-*RhEXPA4*). After the VIGS procedure, petals were dehydrated for 12 h and then rehydrated for 48 h to observe the recovery ability. Finally, the petals were sampled after 12 h of redehydration for the determination of VIGS efficiency by RT-PCR. Based on the expression level of VIGS-targeted genes, the samples were classified into three groups: completely silenced petals, partially silenced petals, and nonsilenced petals. A, RT-PCR analysis of *RhNAC2* or *RhEXPA4* expression in silenced petals. A total of 31, 27, and 29 PCR cycles were used for *RhNAC2*, *RhEXPA4*, and *Ubi1*, respectively. B, The phenotype of *RhNAC2*- or *RhEXPA4*-silenced petals and petal recovery in terms of width and fresh weight relative to initial values. Values are means ± SD ($n = 30$ for both groups of completely silenced petals and $n = 60$ for other groups; $^*P < 0.05$). C, Phenotype and recovery of *RhNAC2*- or *RhEXPA4*-silenced petal discs. After the VIGS procedure, petal discs were dehydrated for 9 h and then examined at intervals during 48 h of rehydration. Discs were scored as recovered if they were fully expanded. Values are means ± SD ($n = 5$, with 90 petal discs per repeat; $^*P < 0.05$). Dehyd, Dehydration; Rehyd, rehydration.

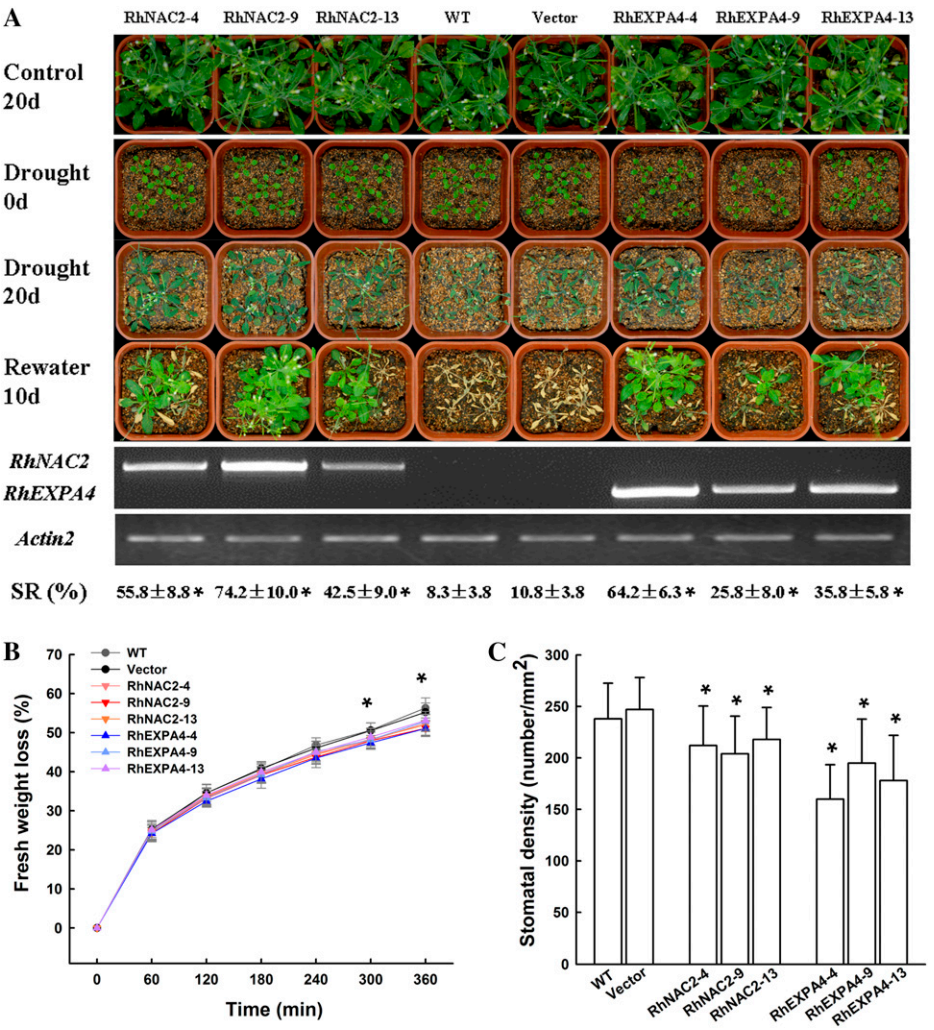

Figure 7. Tolerance of *RhNAC2*- and *RhEXPA4*-overexpressing Arabidopsis to water deficit stress. A, Drought tolerance and gene expression of *RhNAC2* and *RhEXPA4* overexpressors. T2 homozygous transformants were used in this experiment. Control 20d, Twenty-day-old plants growing under normal watering conditions; Drought 0d, 10-d-old well-watered plants; Drought 20d, 20 d after withholding water; Rewater 10d, 10 d after rewatering; SR, survival rate; RhNAC2-4, RhNAC2-9, and RhNAC2-11, three independent *RhNAC2*-overexpressing lines; RhEXPA4-4, RhEXPA4-9, and RhEXPA4-13, three independent *RhEXPA4*-overexpressing lines; WT, wild type. RT-PCR was conducted on fully expanded leaves of control plants. *Actin2* was used as the internal control. The *RhNAC2*, *RhEXPA4*, and *Actin2* genes were amplified for 28, 28, and 22 PCR cycles, respectively. Survival rate was calculated from three independent experiments (40 plants per line in one experiment; $*P < 0.05$). B, Water loss assay of aerial parts during dehydration. Four-week-old plants were derooted and held in air. Asterisks denote significant differences ($P < 0.05$). Vertical lines represent SD ($n = 15$). C, Leaf stomatal density. Vertical lines represent SD ($n = 20$).

assay. Discs were dehydrated for 9 h and then rehydrated for 48 h. By 6 h of rehydration, 41% of the discs in the TRV controls had recovered compared with only 20% in the *RhNAC2*-silenced discs (Fig. 6C). The difference between TRV controls and *RhNAC2*-silenced discs was still significant at 12 h of rehydration but disappeared after 24 h. These results are consistent with those obtained from intact petals and indicate that *RhNAC2*, a transcription factor gene, is involved in dehydration tolerance of rose petals.

We used TRV-*RhEXPA4* to silence the *RhEXPA4* gene both in entire petals and in petal discs. After 24 h of rehydration, the fresh weight and petal width of *RhEXPA4*-silenced petals were significantly less than in the TRV controls (Fig. 6B; Supplemental Table S6). Recovery of petal discs after a 9-h dehydration confirmed this response. *RhNAC2*-silenced discs showed significantly higher recovery than TRV controls at 6 and 12 h of rehydration (Fig. 6C). These results indicate that *RhEXPA4*, a functional gene, is involved in dehydration tolerance of rose petals, but it has less effect on the tolerance than *RhNAC2*.

Drought Tolerance of Arabidopsis Plants Overexpressing *RhNAC2* or *RhEXPA4*

To further examine the function of *RhNAC2* and *RhEXPA4* in dehydration tolerance, we overexpressed *RhNAC2* and *RhEXPA4* in Arabidopsis. Based on the expression level of the transgene, we chose three each from 14 *35S*::*RhNAC2* transgenic lines and 12 *35S*::*RhEXPA4* transgenic lines to test their drought tolerance. Overexpression of both transgenes resulted in reduced plant height (Supplemental Fig. S9), but there were no changes in leaf shape, bolting time, silique size, root number, and architecture (Supplemental Fig. S10).

After 20 d without water, control plants (wild type and vector only) were severely wilted, while plants overexpressing *RhNAC2* or *RhEXPA4* were only slightly wilted (Fig. 7A). Ten days after watering resumed, over 60% of the transgenic plants had recovered, compared with only about 10% of the control plants (Fig. 7A). Improvement in survival rate was closely related to *RhNAC2* or *RhEXPA4* expression levels in the overexpressing lines (Fig. 7A). For a given level of gene expression, plants

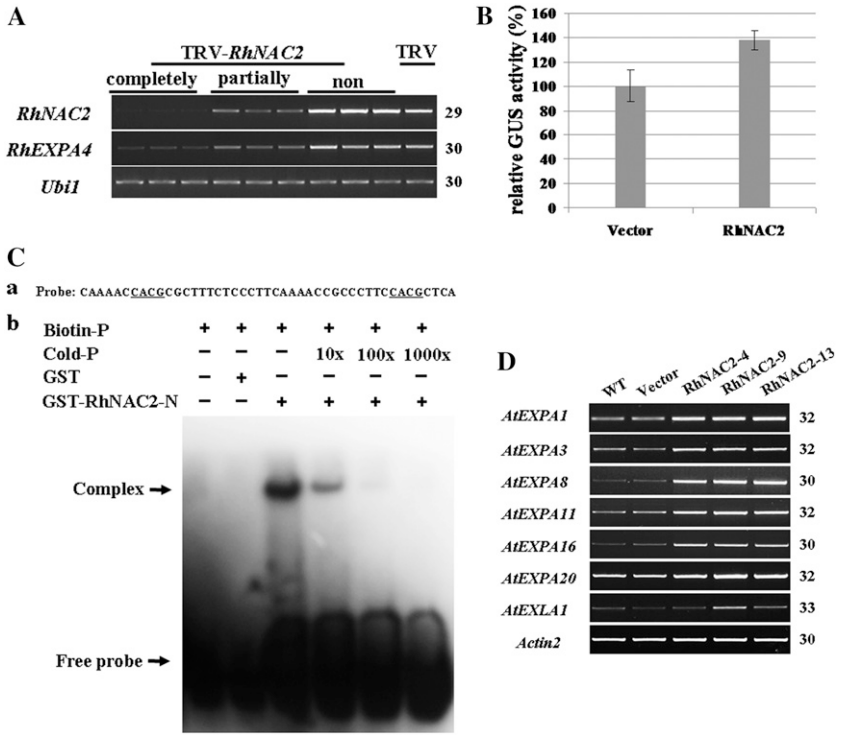

Figure 8. Analysis of the interaction between RhNAC2 and expansins in rose and Arabidopsis. A, *RhEXPA4* expression in *RhNAC2*-silenced rose petals. The first-strand cDNA was generated from 1 μg of total RNA from petals where *RhNAC2* was completely, partially, or not silenced. B, Transactivation of $Pro_{RhEXPA4}$::GUS gene expression by *RhNAC2* in Arabidopsis protoplasts. A pUC19-RhNAC2::GFP construct (RhNAC2) and pUC19-GFP (Vector) as a control were transformed into Arabidopsis mesophyll protoplasts isolated from 4-week-old $Pro_{RhEXPA4}$::GUS transgenic leaves. Nine independent transformation experiments were performed. C, Gel-shift analysis of RhNAC2 N-terminal binding to the *RhEXPA4* promoter. a, Oligonucleotide sequence of the −216 to −171 region of the *RhEXPA4* promoter. Underlined letters indicate the core sequences of NAC protein-targeted promoters. b, Interaction between GST-RhNAC2-N^{1-173} and biotin-labeled probe on native PAGE. Purified protein (2 μg) was incubated with 0.2 pmol of biotin probe. For the competition test, nonlabeled probe at different concentrations (from 10 to 1,000 times) was added to the reaction mixture. D, Transcript abundance of *AtEXPA* genes is enhanced in *RhNAC2* overexpressors. The first-strand cDNAs from 4-week-old transgenic Arabidopsis leaves were used for RT-PCR with *Actin2* as the internal control. WT, Wild type. PCR cycles are listed on the right side in A and D.

overexpressing *RhNAC2* had a higher survival rate than those overexpressing *RhEXPA4*.

Rosette leaves of *RhNAC2*- and *RhEXPA4*-overexpressing Arabidopsis plants lost water more slowly than those of wild-type plants (Fig. 7B). This was associated with a lower leaf stomatal density (Fig. 7C), but there was no obvious difference in stomatal aperture under normal growth conditions or in response to abscisic acid (ABA) treatment (Supplemental Fig. S11). It was reported that higher drought tolerance was also caused by lower stomatal density in an Arabidopsis mutant (Yoo et al., 2010). These data suggest that drought tolerance in the Arabidopsis *RhNAC2* or *RhEXPA4* overexpressors may be partially due to lower stomatal density.

Role of RhNAC2 in the Regulation of Expansin Gene Expression in Rose Petals and Arabidopsis

Our results showed that *RhNAC2* and *RhEXPA4* exhibited similar expression patterns under dehydration, similar phenotypes when silenced in petals or petal discs, and similar effects in extending drought tolerance when overexpressed in Arabidopsis. To test the hypothesis that *RhEXPA4* might be a candidate downstream gene of RhNAC2 in rose petals, we first determined the effect of silencing *RhNAC2* on the expression of *RhEXPA4*. The expression of *RhEXPA4* was obviously repressed in *RhNAC2*-silenced petals, and the degree of repression was correlated with the level of silencing of *RhNAC2* (Fig. 8A).

To test the possibility that the RhNAC2 protein activates the *RhEXPA4* promoter, we transformed Arabidopsis plants with a construct comprising the promoter for *RhEXPA4* driving the gene for glucuronidase ($Pro_{RhEXPA4}$::GUS). When mesophyll protoplasts isolated from 4-week-old $Pro_{RhEXPA4}$::GUS transgenic leaves were transformed with a construct inducing transient expression of *RhNAC2* (pUC19-RhNAC2::GFP), GUS activity in the protoplasts was nearly 40% higher than in protoplasts transformed with pUC19-GFP, the empty vector (Fig. 8B).

We also used electrophoresis mobility shift assay (EMSA) to investigate whether the RhNAC2 protein binds to the *RhEXPA4* promoter. We selected a fragment from the −216 to −171 region of the *RhEXPA4* promoter containing two core sequences common to NAC protein-targeted promoters (Olsen et al., 2005) as probe and the DNA-binding domain of RhNAC2 labeled with glutathione *S*-transferase (GST; GST-RhNAC2-N^{1-173}) as target. DNA-binding bands were detected when GST-RhNAC2-N^{1-173} was combined with the labeled probe but not in the GST control (Fig. 8C). When unlabeled probe was added to the reaction mixture, the DNA-binding band was abolished (Fig. 8C). These data suggest that the N-terminal binding domain of RhNAC2 binds to the promoter of *RhEXPA4* and support the hypothesis that *RhEXPA4* may be a direct downstream gene of RhNAC2.

To investigate the potential gene network related to cell expansion regulated by RhNAC2, we performed a microarray experiment with the GeneChip ATH1 Arabidopsis genome array (Affymetrix; http://www.affymetrix.com/index.affx) using aerial parts of transgenic 35S::*RhNAC* and vector control Arabidopsis plants. A total of 108 genes were up-regulated at least 2-fold by RhNAC2 overexpression; among them, there are 20 cell wall-related genes (Supplemental Table S7). Seven expansin family genes, especially *AtEXPA1*, a close homolog of *RhEXPA4*, were up-regulated by RhNAC2. The microarray data were confirmed using semiquantitative RT-PCR (Fig. 8D).

DISCUSSION

Expression Profiles of Genes in Response to Dehydration in Rose Petals

Water deficit is recognized as one of the main environmental constraints restricting natural and agricultural ecosystem productivity. The genetic basis of drought tolerance has been extensively studied by targeting relevant genes through transcriptomic techniques (Seki et al., 2007). In rose, a model for multipetaled flowers, several small-scale EST sequencing projects have resulted in a total of approximately 9,300 ESTs available in public databases, mainly genes involved in volatile biosynthesis and flowering control (Debener and Linde, 2009). Interestingly, around 30% of rose transcripts did not match any annotated sequence in the common databases (Channelière et al., 2002; Guterman et al., 2002), indicating that a number of rose petal genes are either unique or too divergent from the genes from other taxa to be identified by sequence homology. Our study comprehensively explored the temporal expression patterns of dehydration-induced genes in rose petals through SSH and cDNA microarray technology. We obtained a total of 3,513 uniESTs and detected 1,006 transcripts differentially expressed under the cyclic dehydration treatment, accounting for around 30% of total transcripts on the cDNA microarray.

Among the differentially expressed genes responsive to WDS, genes encoding transcription factors are considered to be the most interesting category because of their potential involvement in regulating the expression of downstream genes (Trewavas and Malho, 1997; Bray, 2004). It is known that there are more differences than similarities in transcription factor families based on their classification among plant species. The role of different transcription factor families in plant growth and development differs widely among different plant species. The most highly conserved are the changes observed in AP2/EREBP transcription factors responding to WDS in Arabidopsis, rice, and chickpea. However, even in this family, different members show different expression patterns in response to WDS (Molina et al., 2008; Narsai et al., 2010; Wang et al., 2011). Types and numbers of transcription factors responding to WDS vary from species to species (Tommasini et al., 2008), and some also show tissue- or development stage-specific features (Zhou et al., 2007; Wang et al., 2011).

We have identified 41 transcription factor genes that are differentially regulated by cyclic dehydration treatment in rose petals, accounting for approximately 40% of the total transcription factor transcripts on our cDNA microarray. The ratio is much higher than the 5% seen in genome-wide gene expression profiling in Arabidopsis or rice with Affymetrix microarrays (Narsai et al., 2010), because our rose EST library was derived by subtractive hybridization of ESTs from dehydrated petals. The differentially regulated transcription factor genes belong to a diverse range of transcription factor families, including NAC, Homeobox, AP2, and BTB/TAZ (Table I; Supplemental Table S3), and most have been reported as being responsive to drought stress in other plant species such as Arabidopsis and wheat (Olsen et al., 2005; Ergen et al., 2009). It seems that transcriptional regulation of the dehydration response in rose petals shares features of that in other plants.

GO functional categories are useful tools for analyzing changes in the transcriptome during growth and development and in response to stress in model and crop plants (Street et al., 2006; Rodriguez et al., 2010; Wilkins et al., 2010; Wang et al., 2011). Genes grouped in the functional categories of "response to stresses" and "response to biotic and abiotic stimuli" were identified in the Arabidopsis response to drought (Wilkins et al., 2010). In rice, WDS caused tissue-specific down-regulation of genes in distinct categories, including photosynthesis-related genes in leaves and genes involved in cell membrane biogenesis and cell wall modification in the roots and in the young panicle (Wang et al., 2011). In poplar, genes involved in the maintenance of cellular homeostasis and in GA response and signaling were up-regulated by drought (Street et al., 2006). In *C. plantagineum*, GO analysis of the 500 most variable unigenes revealed metabolic processes that are hallmarks of the stages of dehydration and rehydration (Rodriguez et al., 2010).

GO analysis of the cyclic dehydration response in rose petals indicated a primary role for three processes: osmotic adjustment, cytokinin metabolism, and cell wall metabolism (Supplemental Tables S4 and S5). Osmotic adjustment can help to maintain cell turgor and stabilize cell proteins under stress conditions (Seki et al., 2007). Recent reports have described the involvement of cytokinins in stress responses of vegetative organs of plants (Rivero et al., 2007; Tran et al., 2007; Werner et al., 2010; Nishiyama et al., 2011). However, there is little information on the role of cytokinins in the dehydration response of reproductive organs, particularly petals. In rose petals, higher expression levels of cytokinin oxidase/dehydrogenase genes accompanied cyclic dehydration (Supplemental Table S4), which may lead to a decrease in endogenous cytokinin levels and, therefore, better adaptation to stresses. Our results in rose share the common feature with other plants that reduction of active cytokinin content by stimulation of cytokinin degradation contributes to increased drought stress tolerance (for review, see Ha et al., 2012). Changes in cell wall organization are also strongly implicated in the dehydration response of rose petals. This confirms results from other studies; for example, the processes of cell wall modification, cell wall loosening, cell wall organization and biogenesis, and plant-type cell wall organization are associated with the drought responses of poplar (Street et al., 2006), *C. plantagineum* (Rodriguez et al., 2010), and chickpea (Molina et al., 2008).

Our analyses suggest that rose petal dehydration is accompanied by a number of other biological processes commonly reported as plant responses to WDS, including response to water, defense response, and response to stimulus (Supplemental Tables S4 and S5). It appears that dehydration may trigger defense reactions that enhance petal cells' ability to restrict damage and tolerate stress. Our data suggest that roses not only share similar biological processes with model and crop plants responsive to the WDS but also have species-, stage-, or tissue-specific GO categories.

RhNAC2 and *RhEXPA4* Increase Tolerance to Dehydration in Rose Petals

The indicators of WDS tolerance reported so far include the survival rate of seedlings (Tran et al., 2004; Lu et al., 2007), wilting degree (Gao et al., 2010), maintenance of green leaves (Huang et al., 2011), biomass of roots and leaves (Nelson et al., 2007; Yu et al., 2008), transpiration rate (Yoo et al., 2010), and photosynthesis rate (Nelson et al., 2007). For cut flowers, a consumer quality requirement is that the flowers open normally, gradually, and sufficiently from the harvested bud stage after postharvest handling. In this study, dehydration tolerance of rose flowers is defined as the ability of the dehydrated flowers to open fully during rehydration. Since flower opening depends mainly on petal expansion, we tested the dehydration tolerance of rose petals by examining the expansion of intact petals or petal discs after rehydration. In VIGS experiments, we used the width and length of petals as tolerance indicators for intact petals and the disc expansion as a tolerance indicator for petal discs.

NAC proteins, one of the largest families of plant-specific transcription factors, have been identified and functionally characterized in Arabidopsis, rice, soybean, and wheat (Tran et al., 2010). Based on sequence alignments and phylogenetic analyses, NACs can be classified into subgroups with different functions in plant growth, development, and senescence (Tran et al., 2004; Olsen et al., 2005; Le et al., 2011). Previous reports have provided a close match between amino acid sequence and function for a range of NAC proteins (Fang et al., 2008; Zhang et al., 2008), so phylogenetic analysis of amino acid sequences can be used systematically to predict the functions for new NAC members (Le et al., 2011).

One group of NACs targets a DNA-binding site termed a drought-responsive NAC recognition sequence, which contains a core CACG motif. This group clusters in the stress-responsive clades identified by phylogenetic analysis. Group members responsive to drought or dehydration were identified in Arabidopsis (Tran et al., 2004), rice (Hu et al., 2006, 2008; Nakashima et al., 2007), cotton (*Gossypium hirsutum*; Meng et al., 2009), and soybean (Le et al., 2011).

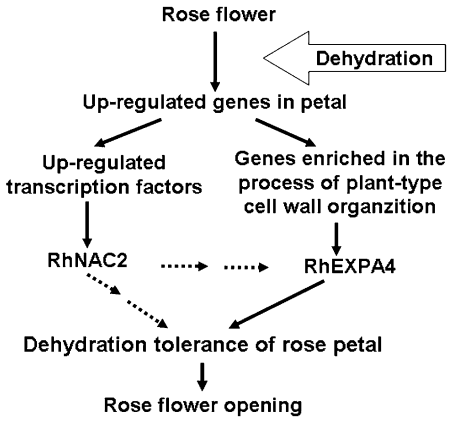

Figure 9. Schematic model describing the involvement of RhNAC2 and RhEXPA4 in dehydration tolerance of rose petals. The up-regulated genes in rose petals under dehydration are obtained by SSH and microarray hybridization. Dehydration causes the inhibition of petal expansion and results in abnormal flower opening. Rose petals adapt and tolerate dehydration through regulating the dehydration-related gene expression and biological processes. Among the up-regulated transcription factor genes, increased *RhNAC2* expression improves petal expansion. A biological process, plant type cell wall organization, is closely involved in petal expansion, and RhEXPA4 plays a key role in this process. RhNAC2 may function as a potential regulator gene of RhEXPA4 in response to dehydration during petal expansion. In a word, two development-related proteins, RhNAC2 and RhEXPA4, are involved in dehydration tolerance of rose petal during flower opening.

Overexpression of WDS-responsive NACs conferred drought tolerance and resulted in morphological, physiological, and molecular adjustments to stress conditions in transgenic Arabidopsis, rice, and wheat (Tran et al., 2004; Hu et al., 2006; Zheng et al., 2009; Jeong et al., 2010; Xue et al., 2011). Our study indicates a similar role for *RhNAC2* in the dehydration response of rose petals (Fig. 4). Silencing this gene resulted in a significant increase in sensitivity to WDS and reduced petal expansion (Fig. 6); overexpressing it in Arabidopsis conferred significant drought tolerance (Fig. 7). Unlike other NACs involved in stress responses (Le et al., 2011), RhNAC2 appears to be a member of a development-related clade of the phylogenetic tree. Members of this clade and homologs of *RhNAC2*, such as *CUC* in Arabidopsis, *NAM* in petunia (*Petunia hybrida*), *CUP* in snapdragon (*Antirrhinum majus*), and *NAM/CUC* in tomato, play an evolutionarily conserved role mainly in shoot apical meristem function and organ separation (for review, see Hasson et al., 2011).

Genome-wide analysis of direct targets of a *VASCULAR-RELATED NAC DOMAIN* transcription factor, VND7, reveals that this protein directly activates the expression of a number of genes involved in secondary wall biosynthesis and cell wall modification (Zhong et al., 2010). Based on this relationship between dehydration tolerance and cell wall development, we hypothesize that RhNAC2 is involved in dehydration tolerance as one of key regulators in the process of cell wall metabolism under stress conditions.

Expansins are cell wall proteins that induce pH-dependent cell wall extension and stress relaxation in a characteristic and unique manner by disrupting hydrogen bonds between cellulose microfibrils and matrix polymers, thus increasing the flexibility of the cell wall (Sampedro and Cosgrove, 2005). Expansin proteins play a role in cell enlargement, pollen tube invasion of the stigma, cell wall disassembly during fruit ripening and softening, organ abscission, leaf organogenesis, and response to environmental stimuli (Cosgrove, 2000; Li et al., 2003). In ornamental crops, expansins have been reported to be involved in development-related processes, such as opening and senescence of flowers of *M. jalapa* (Gookin et al., 2003), expansion of all floral tissues and leaves in gladiolus (*Gladiolus gandavensis*; Azeez et al., 2010), and petal growth and development during carnation flower opening (Harada et al., 2011). Three α-expansin genes (*RhEXPA1–RhEXPA3*) in roses are reported to be mainly involved in expansion growth of rose petals (Takahashi et al., 2007; Yamada et al., 2009).

Increasing evidence suggests the involvement of expansins in WDS tolerance in plants such as maize (Wu et al., 2001; Zhu et al., 2007), *C. plantagineum* (Jones and McQueen-Mason, 2004), and wheat (Li et al., 2011). Generally, WDS does harm to the cells through increasing the rigidity of the cell wall shrinkage in plants. Under drought conditions, cell wall extensibility in Arabidopsis leaves increased. This was a response that was thought to prevent damage that might result from the folding of rigid cell walls as cell volume decreases (Jones and McQueen-Mason, 2004). Expansins, as well as glucanase and pectin-modifying enzymes, confer this capacity to remodel cell wall composition and to maintain cell wall flexibility during WDS (Lu and Neumann, 1998; Rodriguez et al., 2010; Li et al., 2011). We identified eight expansin genes up-regulated during cyclic dehydration of rose petals (Table II). The results of silencing and overexpression experiments indicate that *RhEXPA4*, like *RhNAC2*, is involved in dehydration tolerance of rose petals.

Since *RhNAC2* is a homolog of *SND1* and *VND7*, which are the master regulator genes of secondary wall biosynthesis involved in cell wall modifications (Zhong et al., 2010), we wondered if RhNAC2 was a regulator of cell wall-related genes in rose petals. We noted that *RhNAC2* was strongly up-regulated (after 12–24 h of dehydration) and that *RhEXPA4* expression levels also increased quickly (Fig. 4). Although *RhEXPA4* expression increased earlier than that of *RhNAC2*, this may be attributed to other putative regulators of *RhEXPA4*, whose promoter (accession no. JN903506) contains other cis-elements in addition to the NAC protein-targeted core sequence. Silencing *RhNAC2* in rose petals reduced the expression of *RhEXPA4* (Fig. 8A). *RhEXPA4* is a close homolog of *AtEXPA1* and *AtEXPA10* in Arabidopsis (Supplemental Fig. S5), and *RhNAC2* overexpression in Arabidopsis enhanced the expression of cell wall-related genes, including seven expansin family genes. We obtained additional evidence of the link between *RhNAC2* and *RhEXPA4* through an assay of GUS activity in protoplasts and EMSA (Fig. 8). Taken together, these data are strong evidence that *RhNAC2* and *RhEXPA4* are involved in petal cell expansion and confer the dehydration tolerance in rose petals and that RhNAC2 acts as a potential regulator of *RhEXPA4*.

Concerning functional mechanisms of *RhNAC2* and *RhEXPA4* in rose leaf, RhNAC2 probably does not regulate *RhEXPA4* expression under dehydration, because the *RhNAC2* gene was induced by dehydration in rose leaf but *RhEXPA4* was not (Supplemental Fig. S6). However, *35S:RhEXPA4* transgenic Arabidopsis showed enhanced drought tolerance at the vegetative stage (Fig. 7), suggesting that *RhEXPA4* may play roles in drought stress response in Arabidopsis young seedlings. Thus, our data here are not sufficient to clearly illustrate the roles of *RhNAC2* and *RhEXPA4* in rose leaf under dehydration.

EXPANSIN proteins could disrupt hydrogen bonds between cellulose microfibrils and matrix polymers (Sampedro and Cosgrove, 2005). Thus, overexpression of EXPANSIN may break the elaborate microtubule arrays, cellulose depositions, and cell wall thickenings that are required for the development of stomatal pores and their adjacent cells during stomatal morphogenesis (for review, see Nadeau and Sack, 2002). RhEXPA4 perhaps influenced stomatal development through affecting cell wall structures. Our results showed that the constitutive RhEXPA4 overexpression

contributed to lower stomatal density in the 35S:RhEXPA4 transgenic plants than 35S:RhNAC2 plants (Fig. 7C). At present, it is not clear that the lower stomatal density found in 35S:RhEXPA4 transgenic plants may be contributed by the different expression levels of EXPANSIN in these two transgenic plants or may be due to stronger potency of RhEXPA4 in *35S:RhEXPA4* transgenic plants than its Arabidopsis counterparts in the *35S:RhNAC2* plants. We also could not rule out the possibility that this may be caused by another mechanism independently of RhNAC2.

We also noticed that *RhNAC2* overexpressors with higher stomatal density showed stronger tolerance than *RhEXPA4* overexpressors (Fig. 7). This may be because RhNAC2, as a transcription factor, could regulate some stress-related genes (Supplemental Table S7). These genes may contribute to the higher drought tolerance in RhNAC2 overexpressors from a broad range of morphological and physiological mechanisms. For the analysis reported here, we selected RhNAC2 and RhEXPA4 from a number of NAC and EXPANSIN genes that were up-regulated during rose petal dehydration (Fig. 3B). There may be functional redundancy among NAC family members (Taoka et al., 2004; Ko et al., 2007; Zhong et al., 2007; Yoon et al., 2008) and expansins (Choi et al., 2006). On the other hand, mutant phenotypes sometimes result from the loss of function of key NAC genes (Kim et al., 2006; Mitsuda et al., 2007) or expansins (Cho and Cosgrove, 2000). It would be worthwhile in future studies to examine the contributions of the other NAC and EXPANSIN genes we identified in the response of rose petals to dehydration.

Based on the results described above, we propose a hypothetical model for the functions of *RhNAC2* and *RhEXPA4* during dehydration of expanding rose petals (Fig. 9). Dehydration inhibits petal expansion and results in abnormal flower opening. Rose petals adapt to dehydration through regulating the expression of dehydration-responsive genes. Overexpression of *RhNAC2*, one of the up-regulated transcription factors, improves petal expansion. *RhEXPA4* is closely involved in petal expansion, and *RhNAC2* may function as one of the regulators of *RhEXPA4* gene expression in the dehydration response.

MATERIALS AND METHODS

Plant Materials

Cut rose flowers (*Rosa hybrida* 'Samantha') were harvested at opening stage 2 of flower from a local commercial greenhouse and placed immediately in water. Flower-opening stages were defined as described by Ma et al. (2005). The flowers were delivered to the laboratory within 1 h of harvest. Their stems were recut to 25 cm under water, and the flowers were held in deionized water until needed.

Dehydration Treatment of Rose Flowers

Flowers were placed horizontally on the bench for 6, 12, 24, or 36 h in a climate-controlled room at 25°C, 40% to 50% relative humidity, and a continuous light with intensity of 140 μmol m^{-2} s^{-1}. The sepals, petals, stamens, gynoecia, and receptacle of flowers were taken separately and frozen into −80°C for RNA isolation and construction of the cDNA library.

For microarray hybridization, the flowers were subjected to a cyclic dehydration treatment in the same climate-controlled room. The flowers were held in air for 24 h (first dehydration), recut, removing 1 cm from the base under water, and placed in water for 12 h (rehydration), then removed from the water and held in air for a further 12 h (second dehydration). Control flowers remained in water throughout the experiment. The outermost whorl of petals was collected and frozen to −80°C for RNA isolation.

Measurement of Fresh Weight and Water Potential

The weight of each harvested flower was measured at the start and at intervals throughout the cyclic dehydration experiment. The water potential of cut flowers was measured at intervals using a pressure chamber as described by Jin et al. (2006).

Construction of a Subtracted cDNA Library

To construct the subtracted cDNA library, we used RNA from dehydrated flowers as "tester" and the control flowers as "driver." Flowers were separated into five distinct parts: sepals, petals, stamens, gynoecia, and receptacles. Total RNA of sepals, petals, stamens, and receptacles was extracted using the hot borate method described by Ma et al. (2005), and total RNA from gynoecia was extracted using the hot phenol method described by Xue et al. (2008). We purified mRNA from total RNA using an Oligotex mRNA Purification Kit (Qiagen).

The subtracted cDNA library was constructed using the PCR-Select cDNA Subtraction Kit (Clontech) following the manufacturer's instructions. The tester cDNAs were reverse transcribed from 2 μg of mRNA mixed from five distinct parts of dehydrated rose flowers in the ratio of 4 (petal):1 (sepal):1 (receptacle):2 (gynoecia):2 (stamen). The RNAs of different parts of rose flower were mixed equally from 6-, 12-, 24-, and 36-h dehydration samples. The driver cDNAs were obtained from control flowers using the same method. Both tester and driver cDNAs were digested with *Rsa*I, and the tester cDNAs were then ligated to the adapters for forward subtraction. Two rounds of hybridization and PCR amplification were conducted to normalize and enrich the library with differentially expressed cDNAs. Products of the secondary PCR were directly inserted into pGEM T-Easy Vector (Promega) and transformed into *Escherichia coli* DH5α cells. Recombinant colonies were collected to establish the forward subtracted cDNA library. Colonies were randomly picked from the subtractive cDNA library for sequence analysis. A total of 7,071 independent clones were successfully sequenced. All 7,071 sequences have been submitted to GenBank with accession numbers JK616121 to JK623191. EST assembly was performed to obtain uniESTs using the iAssembler program (http://bioinfo.bti.cornell.edu/tool/iAssembler) with a minimum overlap of 40 bp and a minimum identity of 97% compared with the National Center for Biotechnology Information and 454 sequence libraries (J. Gao, N. Ma, and H. Pei, unpublished data).

Preparation of cDNA Microarray Slides

Bacterial clones containing all uniESTs from the subtracted cDNA library were selected and distributed individually onto 96-well plates for cDNA amplification by PCR. The nested PCR primer 5′-TCGAGCGGCCGCCCGGGCAGGT-3′ was used as the forward primer and 5′-AGCGTGGTCGCGGCCGAGGT-3′ was used as the reverse primer. The 50-μL reaction contained 34 μL of sterile water, 5 μL of 10× Taq buffer, 2 μL of each primer (10 μM each), 4 μL of deoxyribonucleotide triphosphate mix (2.5 mM each), 5 units of Taq polymerase (TaKaRa), and 1 μL of bacterial culture as template. PCR was performed as follows: at 95°C for 5 min, then 35 cycles at 95°C for 30 s, 68°C for 30 s, and 72°C for 1 min, then a final extension at 72°C for 10 min. PCR products were precipitated by the addition of 100 μL of anhydrous ethanol and resuspended in 40 μL of sterile water for preparation of the array. One-microliter aliquots of the resuspended products were analyzed by electrophoresis on 1% agarose gels to verify the quality and quantity of the cDNA. The remaining PCR products were spotted onto aminosilaned glass slides (CapitalBio). All uniESTs were spotted two times onto the microarray in different subgrids.

Preparation of Labeled DNA, Hybridization, and Scanning

The petal RNA samples were used to generate Cy3- and Cy5-labeled cDNA probes using the SuperScript direct cDNA Labeling System (Invitrogen) following the manufacturer's instructions. Cy3 and Cy5 probes were mixed in equal quantities and purified on spin columns (Minipore) to a final volume of

20 μL. Prehybridization, hybridization, and posthybridization washes were carried out according to the UltraGAPS coated slides instruction manual (Corning). After the washes, the slides were scanned using an Axon Genepix 4000B scanner (Molecular Devices) at a resolution of 10 μm. Laser and photomultiplier tube voltages were adjusted manually to minimize background and the number of spots that had saturated signal values. For biological repeats, the RNA samples used for preparations of labeled cDNAs were prepared from six replicate untreated and treated petal samples.

Data Processing and Analysis

Spot intensities were quantified using GenePix pro 6.0 (Axon Instruments). Spots with mean signal intensities less than local background intensities plus 2 SD from the local background in both channels were regarded as empty spots and not included in the downstream statistical analysis. A Print-tip Lowess Normalization strategy was applied to normalize the ratio values for each array using the Bioconductor marray R package (Yang et al., 2002). The significance of gene expression changes between stressed and control plants was identified using the Patterns from Gene Expression package (Grant et al., 2005). Genes with corrected $P < 0.05$ and fold change no less than 2 were considered to be differentially expressed genes. Identification of highly enriched GO terms and functional classification of differentially expressed genes were performed using the Plant MetGenMAP system (http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi; Joung et al., 2009). All microarray data were deposited at the Gene Expression Omnibus-National Center for Biotechnology Information with the following accession numbers: GSE33217 and GPL14787.

Silencing of RhNAC2 and RhEXPA4 in Rose Petals by VIGS

The silencing of *RhNAC2* and *RhEXPA4* in petals by VIGS was performed according to the procedures described by Ma et al. (2008) with some modifications. For the construction of the *RhNAC2* VIGS vector, we used a 351-bp fusion fragment combining 249 bp from the 5′ end of the gene with 102 bp from its 3′ end. For the construction of the *RhEXPA4* VIGS vector, we used a 302-bp fragment from the 3′ end of the gene. All primers used are listed in Supplemental Table S8. The two resulting products were cloned into pTRV2 to form the pTRV2-*RhNAC2* and pTRV2-*RhEXPA4* constructs, respectively. The two constructs, as well as the pTRV1 and pTRV2 vectors, were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation. The transformed *A. tumefaciens* lines were grown at 28°C in Luria-Bertani medium supplemented with 10 mM MES, 20 mM acetosyringone, and 50 mg L^{-1} kanamycin for about 24 h. *A. tumefaciens* cells were harvested and suspended in the infiltration buffer (10 mM $MgCl_2$, 200 mM acetosyringone, and 10 mM MES, pH 5.6) to a final optical density at 600 nm of around 1.5. A mixture of *A. tumefaciens* cultures containing empty or silencing pTRV1 and pTRV2 vectors in a ratio of 1:1 (v/v) was placed at room temperature for 4 h before vacuum infiltration into petal tissues.

Rose petals were collected from the outermost whorl of the flowers at flower stage 2. One-centimeter-diameter discs were taken from the center of the petals with a hole punch. For vacuum infiltration, rose petals or discs were placed into the bacterial suspension solution and infiltrated under vacuum at 0.5 MPa for 15 s. After release of the vacuum, petals and discs were washed in deionized water and kept in deionized water for 3 d at 8°C, then at 23°C for 1 d. Petals were dehydrated for 12 h and then placed in deionized water for rehydration for 48 h. The width, length, and fresh weight of all petals were determined at intervals. The petals were sampled after the 12-h dehydration to determine the VIGS efficiency using RT-PCR. We classified VIGS-silenced petals into three groups, completely silenced petals, partially silenced petals, and nonsilenced petals, based on the expression level of the VIGS-targeted gene determined by RT-PCR. Widths and fresh weights of petals in each group were determined according to image records or measurements of individual petals at intervals.

After the VIGS procedure, petal discs were dehydrated for 9 h and then rehydrated for 48 h. The discs were monitored by using a digital camera (Nikon D200). The discs were regarded as recovered when they expanded fully. The percentage of recovered discs was determined at intervals. Silencing experiments with petal discs were repeated five times using at least 90 discs per gene in each repetition. Student's *t* test ($P < 0.05$) was used for statistical analysis of the recovery data.

Semiquantitative RT-PCR Analysis

To verify microarray results, eight dehydration-responsive genes were selected and subjected to semiquantitative RT-PCR analysis. Total RNAs were isolated from rose petals under the cyclic dehydration process as well as under control conditions following the procedures described by Xue et al. (2008). cDNAs were synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). The rose *Ubiquitin1* gene (*Ubi1*; GenBank accession no. JK622648) was used as the internal control. PCRs were performed with 25 to 29 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, using 1 μL of cDNA as the template. PCR products were analyzed on 1.0% agarose gels and imaged using the AlphaImager 2200 (α Innotech).

To determine the expression levels of *RhNAC2* and *RhEXPA4* in silenced petals, we isolated total RNAs from the petals using the hot borate method (Wan and Wilkins, 1994). cDNA synthesis and RT-PCR were performed as described above. The primers were designed outside the region used for VIGS to avoid amplification of RNA from the silencing vectors (Supplemental Table S8). The rose *Ubi1* gene was used as the internal standard.

To determine the expression levels of *RhNAC2*, *RhEXPA4*, and Arabidopsis (*Arabidopsis thaliana*) expansin genes in transgenic Arabidopsis plants, we extracted total RNA from leaves collected from 4-week-old *RhNAC2*- and *RhEXPA4*-overexpressing transgenic plants (T2 generation) and wild type (Columbia ecotype) plants using the Trizol agent (Invitrogen). cDNA synthesis and RT-PCR were performed as described above. The *Arabidopsis Actin2* gene (GenBank accession no. NM_112764) was used as the internal control. PCR amplification of the *RhNAC2*, *RhEXPA4*, and *Actin2* genes was performed using 28, 28, and 22 cycles, respectively. All primers are listed in Supplemental Table S8.

Transactivation Assay of RhNAC2 in Yeast Cells

For transactivation assay in yeast (*Saccharomyces cerevisiae*), different portions of RhNAC2 to be examined were PCR amplified using forward primers with the *Sal*I site at the 5′ end and reverse primers with the *Pst*I site at the 5′ end. The amplified fragments were digested with *Sal*I and *Pst*I and inserted in frame into the *Sal*I and *Pst*I sites of pBD (Clontech) to make expression vectors. The proteins fused with pBD-GAL4 are as follows: pBD-RhNAC2F (1–354 of RhNAC2), pBD-RhNAC2N (1–173 of RhNAC2), pBD-RhNAC2C (173–354 of RhNAC2), pBD-RhNAC2C1 (193–354 of RhNAC2), pBD-RhNAC2C2 (214–354 of RhNAC2), pBD-RhNAC2C3 (240–354 of RhNAC2), and pBD-RhNAC2C4 (281–354 of RhNAC2). The primers are listed in Supplemental Table S8.

According to the protocol of the manufacturer (Stratagene), expression vectors (pBD-X), with pBD-GAL4 as the positive control or pBD vector as the negative control, were introduced into the yeast host strain YRG-2, containing the His-3 and LacZ reporter genes with three and four GAL4-binding elements in the promoters, respectively.

All transformants were streaked on SD-Trp and SD-His plates. The transcriptional activation activities were evaluated by growth of the colonies. Transfected yeast cells were also transferred onto filter paper and incubated at 30°C for 0.5 to 8 h in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid to check the β-galactosidase activity by monitoring the generation of blue color. Quantitation was performed using *o*-nitrophenyl β-D-galactopyranoside to test β-galactosidase activity as described in the yeast protocols handbook (PT3024-1; Clontech). Three transformants from each transfection were selected randomly and subjected to the *o*-nitrophenyl β-D-galactopyranoside assay. To monitor fusion protein production in yeast, total protein (0.3 mL of yeast culture equivalent) was extracted and size fractionated by 10% SDS-PAGE. Electrophoresed protein samples were transferred to nitrocellulose membrane and blocked with 3% (w/v) nonfat dry milk before incubation with rabbit anti-GAL4 DNA-BD antibody (Sigma-Aldrich) to detect pBD-X fusion proteins. Anti-rabbit IgG alkaline phosphatase (Sigma) was used as the secondary antibody and detected using alkaline phosphatase substrate.

Arabidopsis Transformation and Drought Tolerance Determination

To transform Arabidopsis with the *RhNAC2* and *RhEXPA4* genes, we first amplified the open reading frames of *RhNAC2* and *RhEXPA4* using gene-specific primers providing *Xba*I and *Sac*I sites (Supplemental Table S8). The PCR products were digested with *Xba*I and *Sac*I and inserted into the pBI121 vector. The transformed vectors were introduced into *A. tumefaciens* strain GV3101. Arabidopsis plants were transformed using the flower dip method described by Clough and Bent (1998).

Seeds were surface sterilized using 5% NaClO for 10 min, washed off and stratified at 4°C for 3 d, and then sown on Murashige and Skoog (MS) medium

to allow germination. Eight-day-old seedlings were then transplanted into 7-cm pots filled with a 1:1 mixture of peat and vermiculite in a controlled room at 23°C ± 1°C, 16/8-h day/night period, and 80 to 100 μmol m^{-2} s^{-1} illumination. The plants were watered every 4 d. The T0 seeds collected from transformed plants were germinated on MS medium containing 50 mg L^{-1} kanamycin to screen for kanamycin resistance. T1 seeds obtained from individual T0 plants were planted to obtain T2 seeds. To ensure homozygous T2 seedlings, the T2 seeds obtained from individual T1 seedlings were further sown on the MS medium containing 50 mg L^{-1} kanamycin, and only lines that germinated completely and grew normally were chosen. The homozygous T2 seeds were stored for further use. Based on the overexpression levels of *RhNAC2* and *RhEXPA4* in Arabidopsis leaves, we chose three lines each for further analysis. The morphological phenotypes of *RhNAC2*- or *RhEXPA4*-overexpressing plants were observed under normal growth conditions. Primary root length and lateral root number of 10-d-old seedlings were determined on MS medium. Water loss assay of aerial parts of 4-week-old *RhNAC2*- and *RhEXPA4*-overexpressed Arabidopsis was conducted on the laboratory bench at 23°C to 25°C, 30% to 40% relative humidity, and 40 μmol m^{-2} s^{-1} light intensity. The percentage loss of fresh weight was calculated on the basis of the initial weight of the plants. Leaf stomatal density was observed with a light microscope using the leaf surface imprint method (Yoo et al., 2010).

For the evaluation of drought tolerance, 8-d-old T2 transgenic and control Arabidopsis seedlings were transplanted into 7-cm pots filled with 100 g of substrate and five seedlings per pot and grown under the conditions described above. The 10-d-old plants were watered fully, then water was withheld for 20 d. The plants were then rewatered to determine recovery. The phenotype of the plants was monitored by digital photography (Nikon), and survival of the plants was scored 10 d after rewatering. All experiments were repeated at least three times, and more than 40 plants from at least three lines were used in each comparison.

Purification of Recombinant Protein and EMSA

The N terminus ($N^{1–173}$) of RhNAC2, which contains the DNA-binding domain, was fused in frame with GST and expressed in *E. coli* BL21. The fusion protein was induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside, and the cultures were incubated at 28°C for 6 h. The recombinant protein was purified by GST-agarose affinity chromatography. The EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce; 20148) according to the manufacturer's instructions. The fragment derived from the −216 to −171 region of the *RhEXPA4* promoter, 5′-CAAAACCACGCGCTTTCTCCCTT-CAAAACCGCCCTTCCACGCTCA-3′, was biotin labeled as a probe; it contains two (underlined) target binding sequences for NAC proteins (Olsen et al., 2005). The same unlabeled DNA fragment was used as a competitor in the assay. The probes were incubated with the fusion protein at room temperature for 25 min in a binding buffer (10× concentration: 100 mM Tris, 500 mM KCl, and 10 mM dithiothreitol, pH 7.5). Each 20-μL binding reaction contained 0.2 pmol of biotin probe and 2 μg of fusion protein; the reaction was supplemented with 1 μg of poly(dI·dC) to minimize nonspecific interactions. The reaction products were analyzed by 5% native PAGE. Electrophoresis was performed at 120 V for approximately 35 min at 4°C in 0.5× Tris-borate/EDTA buffer. DNA fragments were transferred from the gel to nitrocellulose membrane with 0.5× Tris-borate/EDTA at 380 mA (approximately 100 V) for 30 min at 4°C. After cross linking, the membrane was incubated in the blocking buffer for 15 min with gently shaking and then transferred to conjugate/blocking buffer by mixing 16.75 μL of stabilized streptavidin-horseradish peroxidase conjugate with 5 mL of blocking buffer. The membrane was washed four times, each for 5 min, with a 1× washing buffer. Biotin-labeled DNA was detected by the chemiluminescence method according to the manufacturer's protocol.

Construction of the RhEXPA4 Promoter-GUS Fusion and Transient Expression Assays

The 1.6-kb promoter fragment (accession no. JN903506) upstream of the RhEXPA4 coding region was amplified from rose genomic DNA and cloned into a modified pBI121 binary vector to replace the cauliflower mosaic virus 35S promoter. Arabidopsis mesophyll protoplasts were transformed with the resulting vector (pBI121-$Pro_{RhEXPA4}$::GUS). The open reading frame of *RhNAC2* was amplified and cloned into a modified pUC19 containing GUS. The primers used are listed in Supplemental Table S8. The resulting pUC19-RhNAC2::GUS was used as an effector plasmid, and the pUC19-GUS plasmid was used as a control. Isolation of Arabidopsis mesophyll protoplasts from pBI121-$Pro_{RhEXPA4}$::GUS-transformed plants, transformation of protoplasts, and GUS activity assays were carried out as described previously (Jefferson, 1987; Sheen, 2001).

ATH1 Microarray Analysis

Total RNAs were prepared from the aerial portion of 3-week-old Arabidopsis plants of two overexpressing lines, RhNAC2-4 and RhNAC2-9, using the Trizol agent (Invitrogen). The vector-only plant was used as a control. Twenty micrograms of total RNA was used for microarray analysis. We used the Affymetrix ATH1 arrays (Affymetrix), and probe labeling, hybridization, and scanning for microarray analysis were conducted according to the manufacturer's instructions (Affymetrix; http://www.affymetrix.com). The raw microarray data were normalized (GeneChip Robust Multiarray Averaging) and analyzed using the Genespring GX package (Agilent Technologies). Seven expansin genes were confirmed by RT-PCR: *AtEXPA1* (The Arabidopsis Information Resource [TAIR] accession no. AT1G69530), *AtEXPA3* (TAIR accession no. AT2G37640), *AtEXPA8* (TAIR accession no. AT2G40610), *AtEXPA11* (TAIR accession no. AT1G20190), *AtEXPA16* (TAIR accession no. AT3G55500), *AtEXPA20* (TAIR accession no. AT4G38210), and *AtEXLA1* (TAIR accession no. AT3G45970).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JN857363 (*RhNAC2*) and JN857364 (*RhEXPA4*).

Supplemental Data

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

Supplemental Figure S1. Length distributions of rose flower ESTs and assembled sequences.

Supplemental Figure S2. Differentially expressed genes identified by SSH and microarray analysis.

Supplemental Figure S3. All 27 groups of genes classified by their expression pattern during dehydration and rehydration.

Supplemental Figure S4. Analysis of the RhNAC2 protein sequence.

Supplemental Figure S5. Analysis of the RhEXPA4 protein sequence.

Supplemental Figure S6. Expression of *RhNAC2* and *RhEXPA4* in different floral organs and leaves.

Supplemental Figure S7. Nuclear localization assay of RhNAC2.

Supplemental Figure S8. Transcriptional activation of RhNAC2 fragments in Arabidopsis protoplast.

Supplemental Figure S9. Morphological phenotypes of Arabidopsis plants overexpressing *RhNAC2* or *RhEXPA4*.

Supplemental Figure S10. Root growth in MS medium of Arabidopsis plants overexpressing *RhNAC2* or *RhEXPA4*.

Supplemental Figure S11. Effect of different concentrations of ABA on stomatal aperture of wild-type and transgenic Arabidopsis plants overexpressing *RhNAC2* and *RhEXPA4*.

Supplemental Table S1. List of genes regulated by dehydration and rehydration in rose petals.

Supplemental Table S2. Genes comprising the induced-repressed-induced and induced-invariable-induced groups responding to cyclic dehydration.

Supplemental Table S3. Transcription factors regulated by dehydration in rose petals.

Supplemental Table S4. GO terms significantly overrepresented for UniProt identifiers under biological processes with up-regulated genes in response to dehydration in rose petals.

Supplemental Table S5. GO terms significantly overrepresented for UniProt identifiers under biological processes with down-regulated genes in response to dehydration in rose petals.

Supplemental Table S6. Petal recovery ability in width and fresh weight of *RhNAC2*- or *RhEXPA4*-silencing petals.

Supplemental Table S7. Up-regulated genes in Arabidopsis transgenic plants overexpressing *RhNAC2*.

Supplemental Table S8. Primers used in RT-PCR analysis and vector construction.

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