The *Arabidopsis* NFYA5 Transcription Factor Is Regulated Transcriptionally and Posttranscriptionally to Promote Drought Resistance [W]

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Nuclear factor Y (NF-Y) is a ubiquitous transcription factor composed of three distinct subunits (NF-YA, NF-YB, and NF-YC). We found that the *Arabidopsis thaliana NFYA5* transcript is strongly induced by drought stress in an abscisic acid (ABA)-dependent manner. Promoter:β-glucuronidase analyses showed that *NFYA5* was highly expressed in vascular tissues and guard cells and that part of the induction by drought was transcriptional. *NFYA5* contains a target site for miR169, which targets mRNAs for cleavage or translational repression. We found that miR169 was downregulated by drought stress through an ABA-dependent pathway. Analysis of the expression of miR169 precursors showed that miR169a and miR169c were substantially downregulated by drought stress. Coexpression of miR169 and *NFYA5* suggested that miR169a was more efficient than miR169c at repressing the *NFYA5* mRNA level. *nfya5* knockout plants and plants overexpressing miR169a showed enhanced leaf water loss and were more sensitive to drought stress than wild-type plants. By contrast, transgenic *Arabidopsis* plants overexpressing *NFYA5* displayed reduced leaf water loss and were more resistant to drought stress than the wild type. Microarray analysis indicated that *NFYA5* is crucial for the expression of a number of drought stress–responsive genes. Thus, *NFYA5* is important for drought resistance, and its induction by drought stress occurs at both the transcriptional and posttranscriptional levels.

INTRODUCTION

Drought stress is a major environmental factor limiting crop productivity worldwide. To reduce the adverse effects of drought stress, plants have evolved multifaceted strategies, including morphological, physiological, and biochemical adaptations (Ingram and Bartels, 1996; Xiong et al., 2002; Zhu, 2002; Shinozaki et al., 2003; Bohnert et al., 2006). Some of these strategies aim to avoid dehydration stress by increasing water uptake or reducing water loss, while other strategies seek to protect plant cells from damage when water is depleted and tissue dehydration becomes inevitable (Verslues et al., 2006). Changes in gene expression play an important role in plant drought stress response,

and many stress-induced genes are known or presumed to play roles in drought resistance. For many of these genes, the hormone abscisic acid (ABA) is a key signaling intermediate controlling their expression. This has been shown in large part by analysis of ABA-deficient and ABA-insensitive mutants in *Arabidopsis thaliana* (Koornneef et al., 1998).

A number of stress-regulated genes encode regulatory proteins, such as transcription factors that are important in regulating the expression of downstream genes (Singh et al., 2002). In *Arabidopsis*, members of the AP2/ERF, bZIP, NAC, HD-ZIP, and MYB/MYC families, as well as several classes of zinc finger domain proteins, are induced by drought stress (Shinozaki et al., 2003; Zhang et al., 2004). In several cases, it has been shown that altering the expression of a transcription factor can alter stress resistance by activating downstream target genes. Examples of this are the CBFs/DREBs, NACs, and RING-H2 zinc finger proteins (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Hu et al., 2006; Ko et al., 2006).

It is well known that regulation of gene expression at the transcriptional level plays a crucial role in the development and

physiological status of plants. With the discovery of small RNAs, increased attention has been focused on the importance of posttranscriptional gene regulation by small RNAs (Carrington and Ambros, 2003; Bartel, 2004; Tang, 2005). These small RNAs include 20- to 24-nucleotide microRNAs, 21-nucleotide trans-acting small interfering RNAs (siRNAs), ~24-nucleotide repeat-associated siRNAs and 21- or 24-nucleotide natural antisense transcript-generated small interfering RNAs (nat-siRNAs). MicroRNAs (miRNAs) are processed from hairpin precursors by the ribonuclease III–like enzyme Dicer and differ from siRNAs, which are not generated from long double-stranded RNAs.

Plant miRNAs are involved in various developmental processes, including flowering, leaf and root development, embryo development, and auxin signaling (Carrington and Ambros, 2003; Bartel, 2004; Allen et al., 2005; Jones-Rhoades et al., 2006). Recently, studies in *Medicago truncatula* found that symbiotic nodule development is regulated by miR169 (Combier et al., 2006). miRNAs also play important roles in plant responses to abiotic stresses, such as sulfate and phosphate nutrient deprivation, and oxidative stress (Jones-Rhoades and Bartel, 2004; Fujii et al., 2005; Sunkar et al., 2006; Sunkar and Zhu, 2007). nat-siRNAs were demonstrated to regulate salt tolerance and disease resistance in *Arabidopsis* (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). However, despite the importance of drought resistance, thus far no small RNAs have been reported to regulate drought stress responses.

Nuclear factor Y (NF-Y) is a ubiquitous transcription factor with high affinity and sequence specificity for the CCAAT box, a *cis*-element present in ~25% of eukaryotic gene promoters. NF-Y is a heterotrimeric complex composed of NF-YA (also known as CBF-B or HAP2), NF-YB (CBF-A or HAP3), and NF-YC (CBF-C or HAP5). In mammals, NF-YB and NF-YC tightly dimerize through a histone fold motif, then NF-YA associates prior to DNA binding, with the sequence-specific interaction of the trimer mediated by NF-YA (Mantovani, 1999). NF-YA and NF-YC subunits contain large domains rich in glutamines and hydrophobic residues that are important for activating transcription (Mantovani, 1999). In animals and yeast, each subunit of NF-Y is encoded by a single gene, whereas the *Arabidopsis* genome encodes 10 NF-YAs, 13 NF-YBs, and 13 NF-YCs (Gusmaroli et al., 2002). It has been demonstrated that *NFYB9* (*LEC1*) plays a pivotal role in embryo development (Lee et al., 2003). Recently, overexpression of *NFYB1* in *Arabidopsis* and maize (*Zea mays*) was shown to significantly improve drought resistance and yield under drought stress conditions (Nelson et al., 2007). However, the biological roles of most of the NF-Y family members in plants are not understood.

Here, we show that expression of *NFYA5*, a member of the *Arabidopsis* NF-YA family, is strongly induced by drought stress and ABA treatments. Promoter:β-glucuronidase (GUS) analysis suggested that part of this induction occurred at the transcriptional level; however, transcriptional regulation alone could not explain the high level of *NFYA5* transcript accumulation seen after stress or ABA treatment. We found that *NFYA5* contained a target site for miR169, and miR169 expression was downregulated by drought. When we analyzed the expression of miR169 precursors, we found that two of them, miR169a and miR169c, were downregulated by drought stress. Coexpression of miR169

and *NFYA5* mRNAs suggested that miR169a was more efficient than miR169c at downregulating the *NFYA5* mRNA.

Thus, the results suggest that downregulation of miR169a by drought stress contributes to the high level induction of *NFYA5* by drought and ABA. *NFYA5* was highly expressed in vascular tissues and guard cells, and analysis of *nfya5* knockout plants and miR169a or *NFYA5* overexpression lines showed that *NFYA5* was important in controlling stomatal aperture and drought resistance. Taken together, our results show that *NFYA5* is important for drought resistance, and it is regulated by drought stress at both transcriptional and posttranscriptional levels.

RESULTS

NFYA5 Expression Is Induced by Drought Stress and ABA Treatments

We investigated *NFYA5* initially because it is annotated to overlap with another gene (At1g54150) on the antisense strand in their 3′ untranslated region (UTR) regions to form a natural *cis*-antisense transcript (NAT) gene pair. Previously, we reported a new type of endogenous siRNA derived from a NAT pair formed by D1-pyrroline-5-carboxylate dehydrogenase and SRO5 and described its role in salt tolerance in *Arabidopsis* (Borsani et al., 2005). The *NFYA5* transcript was strongly induced by drought stress (Figure 1A). In the publicly available *Arabidopsis* eFP Browser microarray database (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), *NFYA5* expression was also strongly induced by osmotic (300 mM mannitol) or salt (150 mM NaCl) stress. By contrast, *NFYA5* expression was not induced by drought treatment (15-min exposure to an air stream of 18-d-old seedlings grown on rafts floating on liquid Murashige and Skoog [MS] medium), possibly because the brief air exposure was not severe enough to activate drought responsive genes.

ABA accumulation is required for some drought stress–induced upregulation of gene expression (Shinozaki and Yamaguchi-Shinozaki, 1996; Zhu, 2002). Thus, we tested the response of *NFYA5* to ABA treatment. *NFYA5* expression increased ~13-fold by 24 h after the application of 100 μM ABA. To verify that ABA is required for the drought-induced increase of *NFYA5* expression, we examined an ABA-deficient (*aba2-1*) mutant and an ABA-insensitive (*abi1-1*) mutant (Koornneef et al., 1998) In both the Columbia (Col-0) and Landsberg *erecta* (L*er*) wild types, the expression of *NFYA5* was strongly induced by withholding watering for 10 d; however, drought-induced accumulation of *NFYA5* mRNA was substantially reduced in *aba2-1* and *abi1-1* (Figure 1B). This result suggests that *NFYA5* expression is at least partly dependent on ABA signaling.

Transcriptional Level Induction by Drought Stress and ABA and Tissue Expression Pattern of *NFYA5*

Although the real-time PCR and RNA gel blot assays both showed a clear induction of *NFYA5* RNA accumulation in response to drought stress or ABA, such assays could not address whether this increase was caused by increased promoter activity
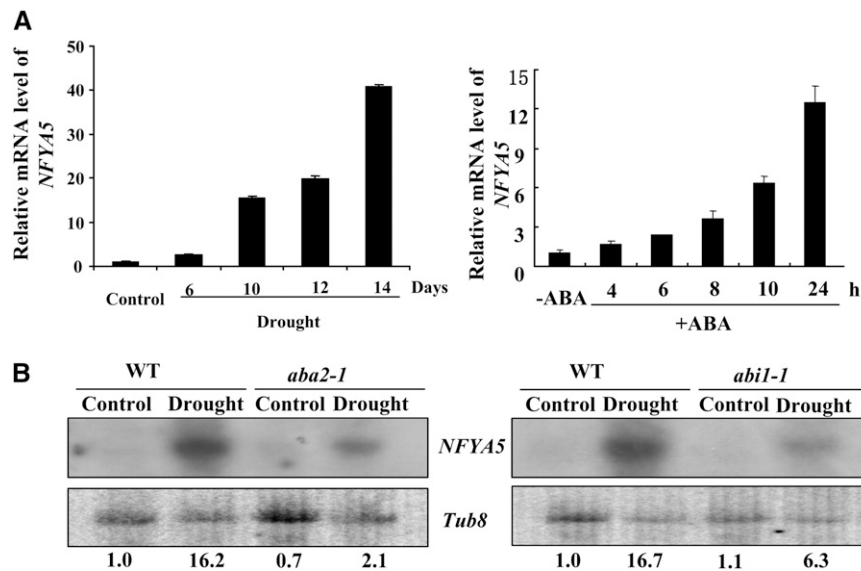
Figure 1. Regulation of *NFYA5* Expression by Drought Stress and ABA.

(A) Real-time PCR assay of the accumulation of *NFYA5* gene transcript in *Arabidopsis* plants in response to drought stress (withholding water from 3-week-old soil-grown plants for the indicated durations) and to ABA (2-week-old seedlings on agar medium). The expression levels were normalized to that of *Tub8*, and the level of *NFYA5* transcript in the controls was set at 1.0. Error bars represent SE for three independent experiments.
(B) Detection of *NFYA5* mRNA in ABA-deficient (*aba2-1*) or signaling (*abi1-1*) mutants. The wild type and mutants were grown with sufficient water for 3 weeks, and then water was withheld for 10 d. Twenty micrograms of total RNA from each sample was loaded and hybridized with a ^{32}P-labeled full-length *NFYA5* cDNA probe. *Tub8* was used as a loading control, and numbers below each lane indicate the expression level of *NFYA5* relative to *Tub8*.

or altered RNA stability. To address this question, we constructed a promoter:GUS fusion using a 1.7-kb fragment upstream from the initiation codon of the *NFYA5* gene. Analysis of GUS staining patterns in several transgenic lines showed that GUS staining increased in response to ABA treatment (Figure 2A). Drought treatment (water withholding for 10 d) increased the GUS activity in leaves from 280 ± 9 to 450 ± 25 (SE, $n = 4$). This was much less than the ~15-fold upregulation of *NFYA5* mRNA level found by quantitative PCR analysis (Figure 1A). Thus, in addition to transcriptional induction, posttranscriptional regulation of *NFYA5* may also play a role.

We also used the promoter:GUS transgenic lines to examine the expression pattern of *NFYA5*. *NFYA5* expression was high in leaf tissues, with prominent expression in both the leaf vascular system and, importantly, a high level of expression in guard cells (Figure 2B, panels I and II). GUS staining was also observed in floral tissues and the root vascular system (Figure 2B, panels III and IV). Quantitative PCR analysis of *NFYA5* mRNA levels in different tissues was consistent with the tissue pattern of GUS staining and showed that expression was highest in leaf and root tissues with significant expression also occurring in floral and stem tissues (Figure 2C).

In *Arabidopsis*, the A subunit of the NF-Y complex is encoded by a 10-member gene family. Though other regions of the proteins vary, the NF-YAs contain a highly conserved core region that consists of two functional subdomains: an NF-YB/NF-YC binding subdomain and a DNA binding subdomain, which are connected by a small linker (Romier et al., 2003; Wenkel et al., 2006; see Supplemental Figure 1 online). The Psort II program

predicted a nuclear localization of NFYA5 protein with 70% certainty. To confirm the subcellular localization of NFYA5 protein, a translational fusion between yellow fluorescent protein (YFP) and the C terminus of NFYA5 was transformed into *Arabidopsis*. Cells expressing the NFYA5-YFP fusion protein showed that the YFP signal appeared only in the nucleus (Figure 2D).

Drought Stress Downregulates a miRNA That Targets the 3′ UTR of *NFYA5*

Because the high level of *NFYA5* mRNA accumulation in *Arabidopsis* under drought stress could not be explained solely by the promoter activity of *NFYA5*, this strongly suggested the presence of another regulatory mechanism operating at the posttranscriptional level. One possibility is regulation by small RNAs. To investigate this possibility, we searched the *Arabidopsis* MPSS Plus Database (http://mpss.udel.edu/at/) and found two small RNA signatures (17 nucleotides) that matched the 3′ end of At1g54150 and are complementary to the 3′ UTR of *NFYA5*. The small RNA signatures in the *Arabidopsis* MPSS Plus Database were identical to part of ASRP1815 (19 nucleotides) in the ASRP small RNA database (http://asrp.cgrb.oregonstate.edu). Thus, we designed an oligonucleotide probe complementary to ASRP1815 (Figure 3A).

Using this oligonucleotide probe, we detected a 21-nucleotide small RNA in plants grown under normal growth conditions. In plants subjected to drought stress, the level of the ASRP1815 small RNA decreased substantially, to ~10% of the level in
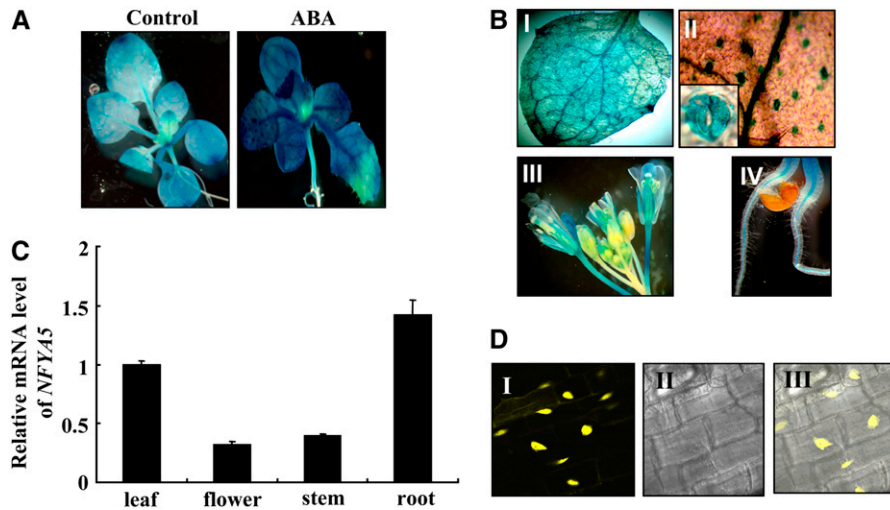
Figure 2. *NFYA5* Expression Pattern and Transcriptional Regulation.

(A) GUS activity in 2-week-old transgenic seedlings on MS-agar medium that were exposed to ABA treatment for 8 h or immersed in water for 8 h (control).

(B) *NFYA5p:*GUS expression pattern in various tissues. The staining was prominent in the vascular tissues (I) and guard cells (II) of leaves. The staining was also visible in floral tissues of the inflorescence (III) and root vascular system (IV).

(C) Tissue pattern of *NFYA5* transcript accumulation. Total RNA was isolated from various tissues of 4-week-old wild-type plants grown under long-day growth conditions. Real-time RT-PCR quantifications were normalized to the expression of 18S rRNA. Error bars represent SE for three independent experiments.

(D) Subcellular localization of *NFYA5*. The *NFYA5-YFP* fusion construct was expressed in transgenic *Arabidopsis* under the control of the cauliflower mosaic virus 35S promoter, and the plant roots were observed under a confocal microscope. The photographs were taken in the dark field for yellow fluorescence (I), in the bright field for the morphology of the cells (II), and in combination (III).

unstressed plants after 14 d (Figure 3B). In this same treatment, the expression of *NFYA5* increased substantially in response to the drought stress. This is consistent with the possibility that lower expression of ASRP1815 under drought may reduce the small RNA–directed degradation of *NFYA5* mRNA. Also consistent with ABA-induced *NFYA5* mRNA accumulation, we found that ABA treatment suppressed the level of ASRP1815 to ~16% of the control level (Figure 3B). In both the Col-0 and L*er* wild types, the expression of ASRP 1815 was strongly suppressed by drought stress; however, ASRP 1815 expression in the *aba2-1* and *abi1-1* mutants was not substantially affected by drought (Figure 3B). Therefore, drought stress–induced suppression of ASRP 1815 was dependent on ABA signaling.

The fact that *NFYA5* and At1g54150 form a NAT gene pair raised the possibility that they may generate a nat-siRNA that regulates *NFYA5*. The biogenesis of nat-siRNAs requires DCL2 or DCL1, RDR6, SGS3, and NRPD1a (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). To test whether ASRP1815 was a nat-siRNA, we examined its biogenesis in mutants defective in various proteins known to be required for biogenesis of specific types of small RNAs. ASRP1815 was still produced in *dcl3*, *rdr2*, *dcl4*, *dcl2*, *rdr6*, *sgs3*, or *sde4/nrpd1a* (Figure 3C). This suggested that it was not a heterochromatic siRNA, transacting siRNA, or nat-siRNA of the types described previously. Instead, ASRP1815 was absent in *hen1*, *dcl1-7*, and *hyl1* (Figure 3C). The requirement of these components suggests that ASRP1815 is probably a miRNA. Disruption of *NFYA5* expression had little effect on

ASRP1815 accumulation (see Supplemental Figure 2 online), consistent with the notion that ASRP1815 is not a nat-siRNA.

We tested the levels of *NFYA5* mRNA in the mutants where the ASRP1815 small RNA was absent. The level of *NFYA5* mRNA was higher in *hen1*, *dcl1-7*, and *hyl1* than that in the wild type (Figure 3D). This result suggested that ASRP1815 indeed downregulates *NFYA5* expression.

NFYA5 Is Mainly Regulated by miR169a

The requirement of HEN1, DCL1, and HYL1 suggested that we were detecting a miRNA with the ASRP1815 probe. Thus, we searched for sequences homologous to ASRP1815 in small RNA databases and found that ASRP1815 is homologous to sequences of the miR169 (21 nucleotides) family. Indeed, *NFYA5* is one of the predicted targets of the miR169 family (Jones-Rhoades and Bartel, 2004). The family of miR169 in *Arabidopsis* contains 14 members, and this miRNA family is conserved in *Oryza sativa* and *Populus trichocarpa* (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Sunkar et al., 2005). Based on sequence of the miRNA produced, the *MIR169a/b/c/h/i/j/k/l/m/n* family members are predicted to target *NFYA5* and can be divided into three subgroups. *MIR169a* represents the first subgroup, *MIR169b* and *MIR169c* form the second group, and the third group is made up of *MIR169h/i/j/k/l/m/n*. The main difference among the subgroups is the sequence at the 3' end: the last two nucleotides of *MIR169a* are G and A,

A

TAGCCAAGGATGACTTCCC

At1g54150 5' ▭—▭—▭—▭—▭ ↓ 3'

3' ▭—▭—▭ 5' *NFYA5(At1g54160)*

B

C

D
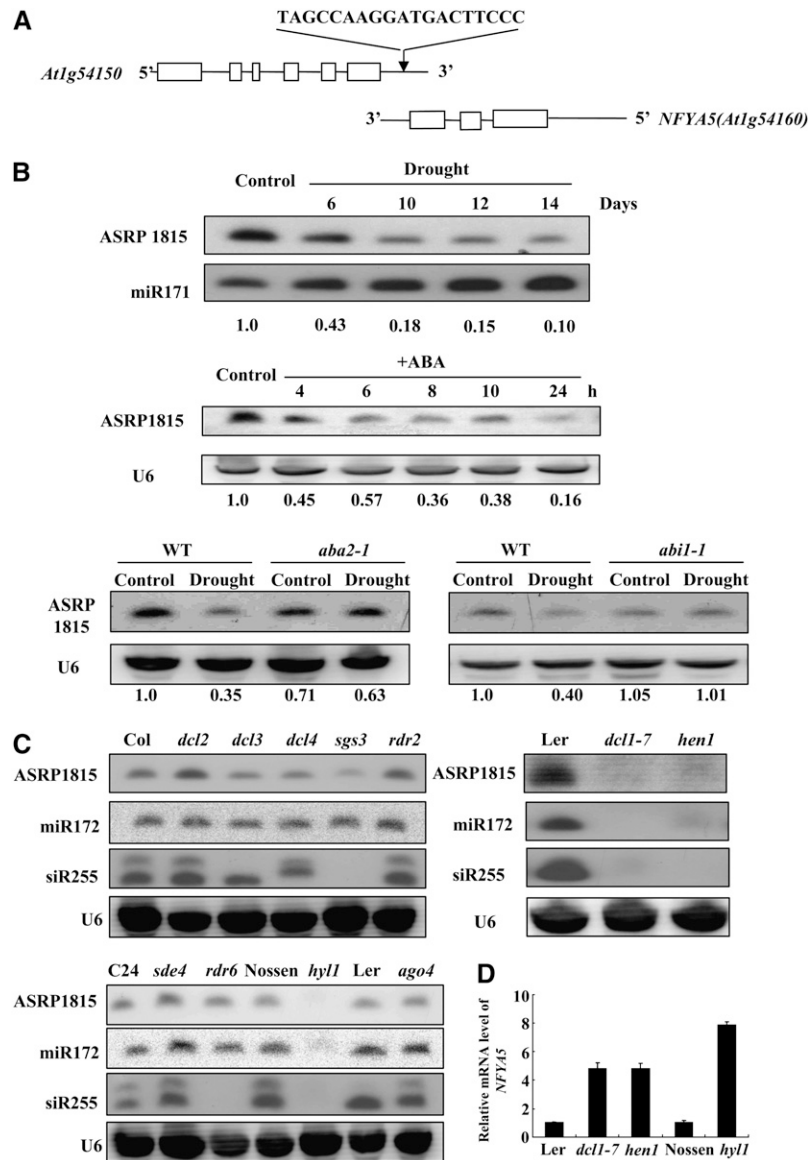
Figure 3. Drought Stress Downregulates a 21-Nucleotide Small RNA That Is Complementary to *NFYA5* mRNA.

(A) Diagram of the *cis*-antisense gene pair of *NFYA5* and At1g54150. Exons are boxed, and lines between boxes represent introns. Arrow indicates target position of the small RNA.

(B) Regulation of the small RNA by drought stress and ABA treatment. miR171 or U6 RNA was probed as a loading control. Numbers below each lane indicate relative expression.

(C) Accumulation of the small RNA in various RNA silencing mutants. Forty micrograms of small RNA from each sample was loaded per lane and hybridized with a ^{32}P-labeled oligonucleotide probe corresponding to the sequence of ASRP1815. miR172, siR255, and U6 were probed as loading controls.

(D) *NFYA5* mRNA levels in *dcl1-7*, *hen1*, and *hyl1* and their corresponding wild types. The expression levels were normalized to that of *Tub8*. Error bars represent SE for three independent experiments.

while those of *MIR169b/c* and *MIR169 h/i/j/k/l/m/n* are GG and UG, respectively. Also, the 5′ end of *MIR169 h/i/j/k/l/m/n* is UA, which is different from CA of other two subgroups.

Because of their sequence similarity, the *MIR169* family members cannot be differentiated in small RNA gel blots because of cross-hybridization. To determine which of the *MIR169* loci may regulate *NFYA5*, we performed real-time RT-PCR using miR169 locus-specific primers to determine if expression of any of the miR169 loci is regulated by drought stress. RNA was extracted from soil-grown *Arabidopsis* plants that had been subjected to water withholding for 10 d. Only *MIR169a* and *MIR169c* exhibited substantial changes in transcript abundance in response to

drought stress (Figure 4A). Both *MIR169a* and *MIR169c* were downregulated by drought stress, consistent with the downregulation of the mature miRNA by drought. Thus, we undertook further experiments to determine if *MIR169a* and *MIR169c* could downregulate *NFYA5* mRNA.

In many cases, it has been assumed that members of a miRNA family have mostly redundant functions; however, Sieber et al. (2007) recently provided evidence that closely related miRNAs that were predicted to target the same genes had in fact different functions during development. To test whether *MIR169a* or
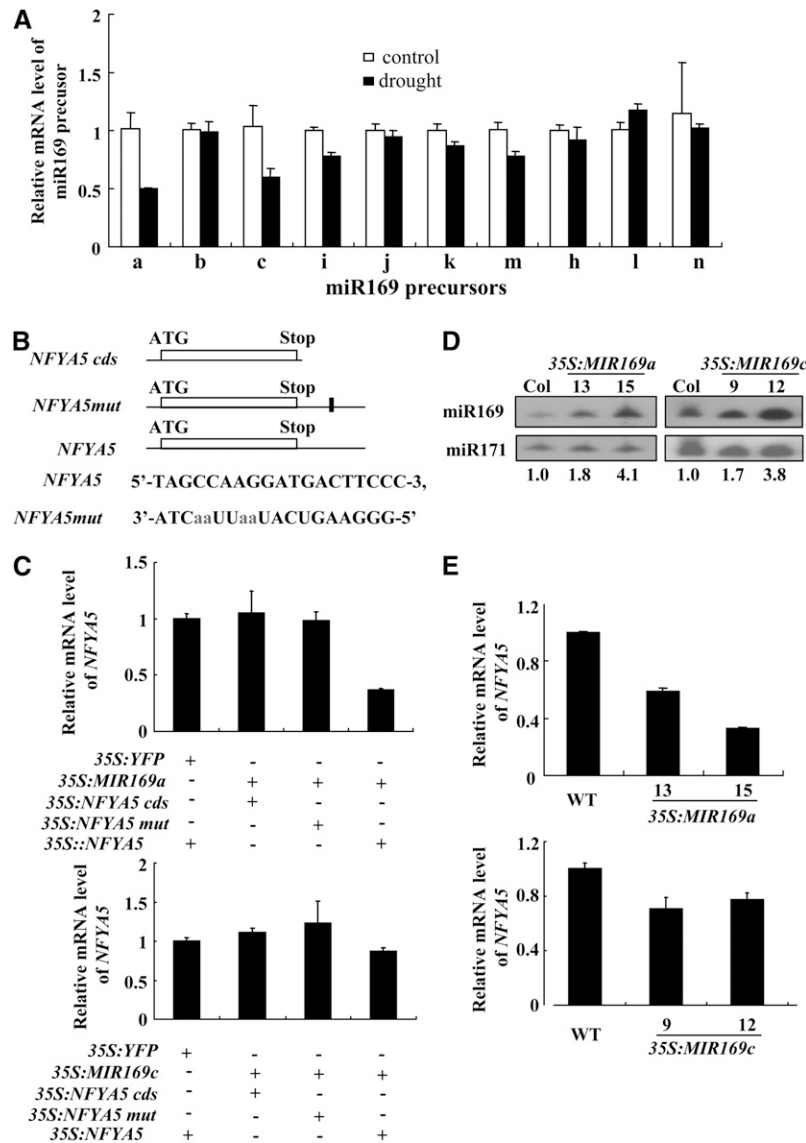
Figure 4. *NFYA5* Is Mainly Regulated by miR169a.

(A) Detection of precursor transcripts of the *MIR169* family in response to drought stress by real-time RT-PCR. Quantifications were normalized to the expression of *Tub8*. Error bars represent SE for three independent experiments.

(B) Diagram of *NFYA5* expression constructs. The introduced mutations in the target site of *NFYA5* are shown in lowercase letters. Black box in the 3′ UTR indicates the miRNA target site.

(C) Coexpression of various combinations of miR169 and *NFYA5* expression constructs in *N. benthamiana*. As a control, *NFYA5* was also coexpressed with an unrelated YFP construct. Real-time RT-PCR quantifications were normalized to the expression of 18S rRNA of tobacco. Error bars represent SE for three independent experiments.

(D) Overexpression of miR169a and miR169c in transgenic *Arabidopsis*. RNA gel blot analysis of miR169a and miR169c levels in the wild type and two representative transgenic lines. miR171 is shown as a loading control. Numbers below each lane indicate relative expression.

(E) Detection of corresponding *NFYA5* gene transcripts in *35S:MIR169* transgenic plant lines by real-time RT-PCR. Quantifications were normalized to the expression of *Tub8*. Error bars represent SE for three independent experiments.

MIR169c may play a specific role in regulating *NFYA5* expression, we performed transient coexpression assays in *Nicotiana benthamiana*. Because the target site of miR169 is located in the 3′ UTR of *NFYA5*, we tested three *NFYA5* constructs: a full-length *NFYA5* including the 3′ UTR, a construct without 3′ UTR, and another construct that was mutated in the 3′ UTR to introduce four mismatches between it and miR169 (Figure 4B). Both the *NFYA5* and miR169a or miR169c constructs were expressed under control of the *35S* promoter. After 2 d of coexpression in *N. benthamiana*, RNA was extracted and *NFYA5* expression analyzed by quantitative RT-PCR. mRNA levels from the constructs that lacked a functional miR169 target site, *NFYA5cds* and *NFYA5mut*, were not affected by coexpression with miR169 (Figure 4C). However, the level of *NFYA5* mRNA containing a miR169 target site was decreased significantly (37% of the control level) when coexpressed with *MIR169a* (Figure 4C). Interestingly, coexpression with miR169c caused only a 13% decrease in the level of *NFYA5* transcript. The results suggested that the degradation of *NFYA5* mRNA was mainly directed by miR169a. Small RNA gel blots prepared from the same samples and probed with oligonucleotides complementary to miR169 clearly showed that a 21-nucleotide small RNA was highly expressed in all of the coexpression samples (see Supplemental Figures 3A and 3B online). Thus, the failure of strong miR169c-directed cleavage of *AtNFYA5* was not due to a lack of miR169c expression.

To confirm these results, we also overexpressed the precursors of *MIR169a* and *MIR169c* in *Arabidopsis* and chose lines with similar miR169 expression levels to quantify the effect of miR169a and miR169c overexpression on *NFYA5* (Figure 4D). In agreement with the transient coexpression assay results, overexpression of miR169a caused a larger decrease in the level of *NFYA5* mRNA than overexpression of miR169c (Figure 4E). We also chose two other members of the miR169 family, miR169b and miR169 h, and overexpressed them in *Arabidopsis* (see Supplemental Figures 2B and 2C online). The relative mRNA levels of *NFYA5* did not change substantially despite the overexpression of the miRNA (see Supplemental Figures 2D and 2E online). These results again strongly suggest *MIR169a* as the major miRNA locus important for the regulation of *AtNFYA5* expression.

35S:MIR169a and *nfya5* Loss-of-Function Mutant Plants Are Hypersensitive to Drought Stress

Drought-responsive gene regulation and ABA signaling are crucial for drought resistance (Pei et al., 1998; Zhu, 2002). The drought- and ABA-inducible expression of *NFYA5* and its strong expression in guard cells prompted us to analyze its potential role in drought resistance. First, we tested plants overexpressing miR169a (*35S:MIR169a*, line #15), in which the level of *NFYA5* mRNA was ~33% of the wild type (Figure 4E). Wild-type and *35S:MIR169a-15* plants were grown for 3 weeks in soil and were then subjected to water withholding for 8 d. *35S:MIR169a-15* plants showed leaf rolling and the leaves became purple, whereas the wild-type plants were still turgid and their leaves remained green (Figure 5A). This result suggested that *35S: MIR169a-15* plants may have depleted the soil water more rapidly than the wild type and thus wilted more quickly. To

investigate this possibility, leaves from *35S:MIR169a-15* and wild-type plants grown in soil were used for stomatal aperture measurements. The stomatal aperture index of *35S:MIR169a-15* leaves was 0.25, which was ~20% greater than that of the wild type (Figure 5B). Consistent with these results, detached leaves of *35S:MIR169a-15* plants consistently lost water more quickly than those of the wild type (Figure 5C), suggesting that the more rapid appearance of wilting after water withholding in *35S: MIR169a-15* could be attributed at least in part to an inability of these plants to efficiently close their stomata and reduce transpiration. Because ABA is a regulator of stomatal aperture and transpiration, this phenotype is consistent with the ABA-induced expression of *NFYA5* and suggests that *NFYA5* may be important for ABA response in guard cells. Another indicator of stress sensitivity is the accumulation of the purple flavonoid pigment anthocyanin in leaves. The anthocyanin levels in *35S:MIR169a-15* plants after withholding water for 8 d was 19.4 μg g^{-1} fresh weight, which was ~3 times as much as that of the wild type, again supporting that *35S:MIR169a-15* was more sensitive to drought stress. The results suggest that adequate expression of *NFYA5* is required for drought resistance.

We searched the publicly available T-DNA collections and obtained a T-DNA insertion mutant (SALK_042760 in the Col background) from the ABRC to further investigate the function of *NFYA5*. Plants homozygous for the T-DNA insertion were identified by PCR, and sequencing of the T-DNA flanking region confirmed the insertion site in the promoter region of *NFYA5* (Figure 6A). RNA gel blot analysis showed that the *NFYA5* transcript was absent in the T-DNA line designated as *nfya5* (Figure 6A). In agreement with the phenotypes of *35S:MIR169a-15* plants, *nfya5* knockout mutant plants were also hypersensitive to drought stress (Figure 6B). The stomatal aperture index of *nfya5* leaves was 0.27, which was 42% greater than that of wild-type leaves (Figure 6C). Consistent with these results, detached leaves of *nfya5* lost water more quickly than those of wild-type leaves (Figure 6D). The anthocyanin levels in *nfya5* leaves after withholding water for 8 d was 4 times as much as that of the wild type, again supporting that *nfya5* plants were more sensitive to drought stress. These results show that *NFYA5* is necessary for drought resistance.

Overexpression of *NFYA5* Improves Drought Resistance

To further characterize the function of *NFYA-5* in drought resistance, we generated transgenic *Arabidopsis* plants overexpressing the gene (without the 3′ UTR) under control of the constitutive cauliflower mosaic virus *35S* promoter. Three transgenic lines (#2, 3, and 5) were chosen for further analysis based on their high level of *NFYA5* expression (Figure 7A). To evaluate the effects of *NFYA5* overexpression, 3-week-old soil-grown wild-type and *35S:NFYA5* plants were subjected to water withholding for 14 d. At the 14th day of water withholding, most of the wild-type plants appeared dehydrated, but the *35S:NFYA5* plants appeared less dehydrated than the wild type (Figure 7B). In contrast with *nfya5*, the stomatal aperture of *35S:NFYA5-3* was smaller than that of the wild type (Figure 7C). Detached leaves of *35S:NFYA5-3* lost water more slowly than those of the wild type (Figure 7D), and the anthocyanin levels in *35S:NFYA5-3* after withholding water for
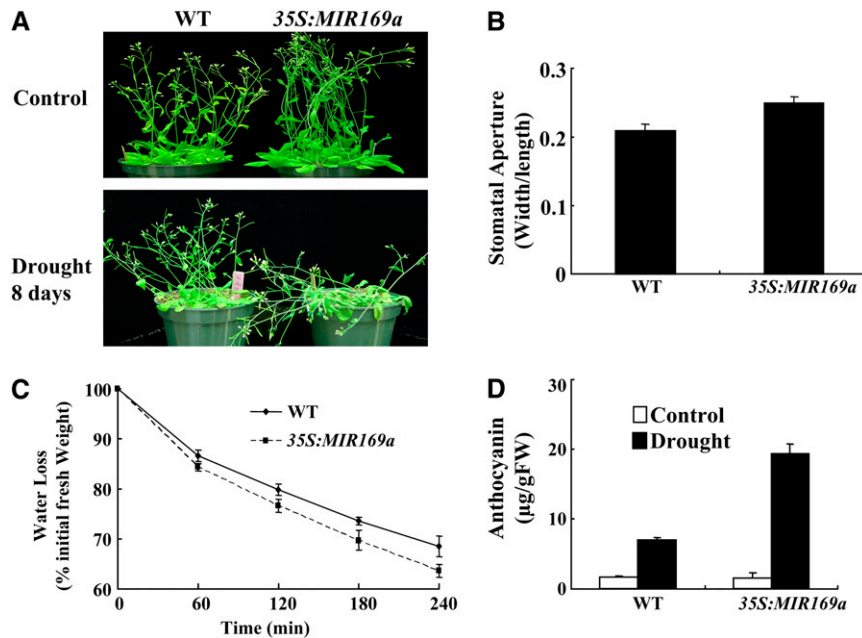
Figure 5. *35S:MIR169a* Plants Are More Sensitive to Drought Stress.

(A) *35S:MIR169a*-overexpressing *Arabidopsis* plants are more sensitive to drought stress. Wild type (Col) and *35S:MIR169a* plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for 8 d. A representative picture is shown. Control, without water withholding.

(B) Measurement of stomatal aperture in wild-type and *35S:MIR169a* plants. Data are mean ratios of width to length ± SE of three independent experiments (*n* = 40 to 50).

(C) Water loss from detached leaves of wild-type and *35S:MIR169a* plants. Water loss is expressed as the percentage of initial fresh weight. Values are means from 10 leaves for each of four independent experiments.

(D) Anthocyanin content in leaves of *Arabidopsis* with or without drought treatment for 8 d. Error bars represent SE for four independent experiments. FW, fresh weight.

14 d were much lower (Figure 6E). These results show that overexpression of *NFYA5* improves plant drought resistance.

NFYA5 Regulates the Expression of Stress-Responsive Genes

The nuclear localization and DNA binding domain of NFYA5 suggest that the protein may act in regulating the expression of other genes important for drought resistance. To test this possibility, we performed microarray experiment using Affymetrix *Arabidopsis* ATH1 Genechips. Approximately 130 genes showed statistically significant changes in expression in *35S:NFYA5* compared with wild-type seedlings under nonstress conditions (see Supplemental Table 1 online). Out of the affected genes, 28 showed a twofold or more change in expression (17 increased and 11 decreased) in the transgenic plants (Table 1). Most of these genes have known or presumed function associated with abiotic stress responses, and a number of them appear to be involved in oxidative stress (e.g., a subunit of cytochrome b6-f complex, glutathione *S*-transferase [GST], peroxidases, and an oxidoreductase family protein). Out of the genes affected by *NFYA5* ectopic expression, most of them contain the CCAAT motif in their promoter regions (Table 1), as expected since this short sequence motif can be found in a substantial fraction of gene promoters in general. Using the AlignACE program (Hughes

et al., 2000), we found a consensus *cis*-regulatory element, TX(C/A)TTXGX(C/A)CAXT, that contains the CCAAT motif in the promoters of a subset of the genes showing increased expression in the *NFYA5* overexpression plants (Table 1). It is possible that these genes are the direct targets of NFYA5.

We confirmed the microarray results by real-time RT-PCR. In agreement with our microarray data, the real time RT-PCR assay showed that At4g15210 (cytosolic β-amylase), At2g37870 (protease inhibitor), At1g17170 (glutathione transferase), At2g42530 (cold-responsive protein), and At2g42540 (COR15A) were expressed at higher levels in *35S:NFYA5* plants under normal conditions (see Supplemental Figure 4 online), suggesting constitutive expression of stress-responsive genes in *35S:NFYA5*. Under dehydration conditions, these genes were strongly induced in the wild type and *35S:NFYA5*; however, dehydration-induced accumulation of the majority of these genes was substantially reduced in *nfya5*, suggesting that, for many of these genes, NFYA5 is required for optimal induction by dehydration stress.

DISCUSSION

Gene regulation under drought stress is mediated by multiple transcriptional cascades (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). In each of these cascades, a transcription factor gene is induced, which in turn activates or represses
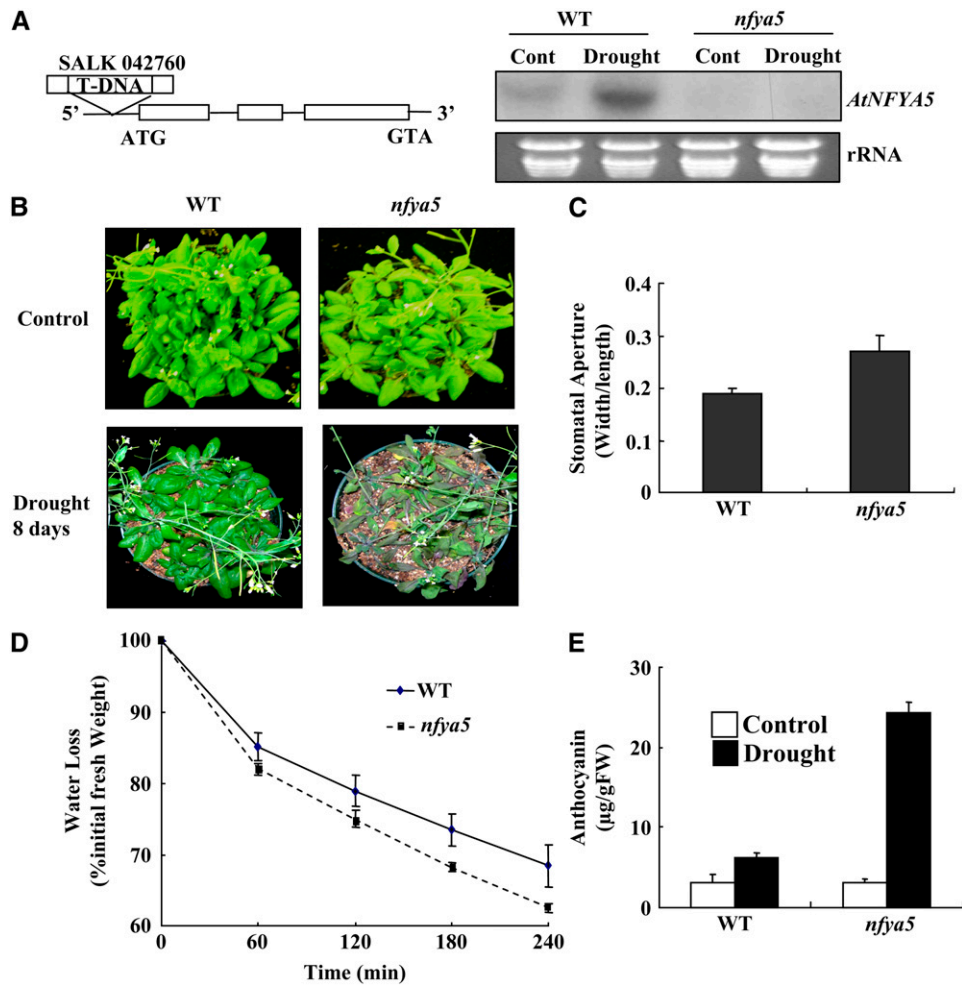
Figure 6. *nfya5* Mutant Plants Are More Sensitive to Drought Stress.

(A) Schematic diagram of the T-DNA insertion site in the *NFYA5* locus and detection of *NFYA5* mRNA by RNA gel blot analysis. Exons are boxed, and lines between boxes represent introns. Twenty micrograms of total RNA from each sample was loaded and hybridized with ^{32}P-labeled full-length *NFYA5* probe. The corresponding ethidium bromide–stained rRNA is shown as a loading control.

(B) *nfya5* mutant plants are more sensitive to drought stress. Wild-type (Col) and *nfya5* plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for 8 d. A representative picture is shown.
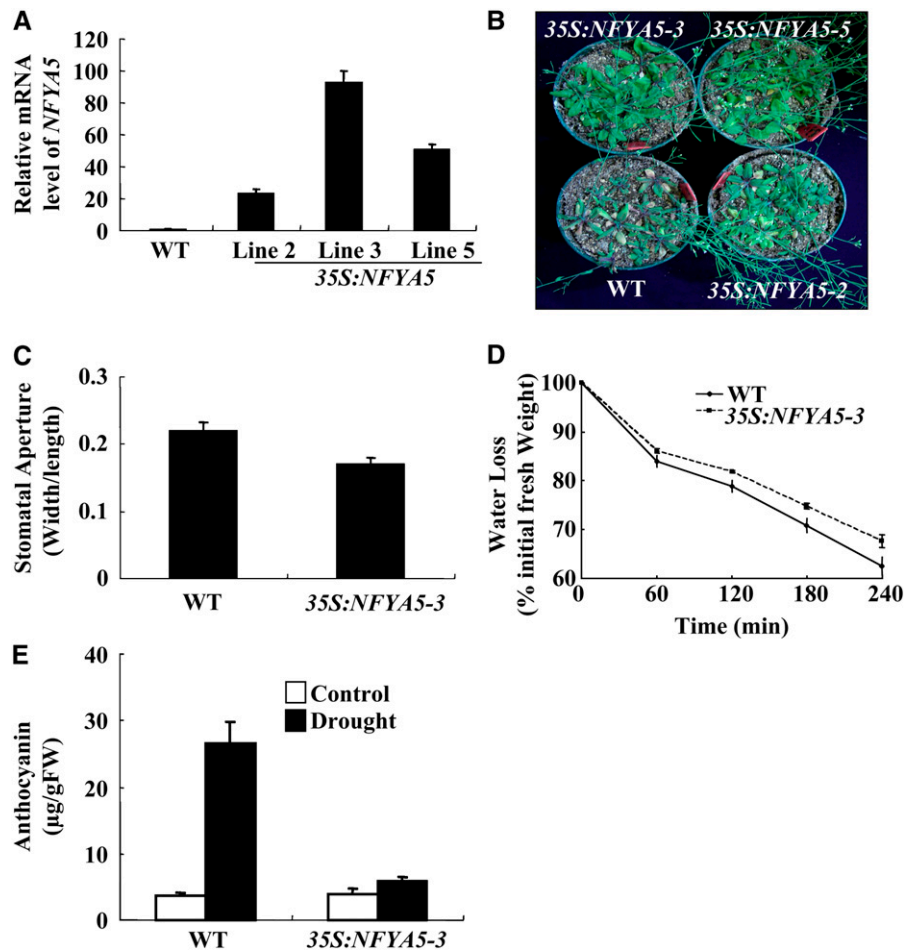
(C) Measurement of stomatal aperture in wild-type and *nfya5* mutant plants. Data are mean ratios of width to length $\pm$ SE of three independent experiments (*n* = 40 to 50).

(D) Water loss from detached leaves of wild-type and *nfya5* mutant plants. Water loss was expressed as the percentage of initial fresh weight. Values are means from 10 leaves for each of four independent experiments.

(E) Anthocyanin content in leaves of *Arabidopsis* with or without drought treatment for 8 d. Error bars represent SE for four independent experiments.

downstream target genes important for drought resistance. *NFYA5* may define one of these drought stress–responsive transcriptional cascades. This transcriptional cascade is critical for drought resistance because *35S:MIR169a* and *nfya5* mutant plants are hypersensitive to drought stress, whereas overexpression of *NFYA5* improves drought resistance. A related transcription factor, *NFYB1*, was recently reported to confer drought tolerance not only in *Arabidopsis* but also in maize, when overexpressed. Field test results showed the utility of *NFYB1* overexpression in stabilizing crop yield under drought conditions (Nelson et al., 2007). The physiological pathway by which *NFYB1*

improves drought tolerance is not known; however, its target genes appear largely different from that of *CBF4*, another drought-induced transcription factor. Another At NFYB family member, *LEC1*, is known to be essential for embryo development and dessication tolerance (Lotan et al., 1998). Our results suggest that part of the role of *NFYA5* in drought resistance involves its expression in guard cells and control of stomatal aperture. In addition, *NFYA5* is broadly expressed in various tissues. In non-guard cells, *NFYA5* is likely important for dehydration tolerance via its role in activating target stress-responsive genes, such as genes involved in oxidative stress responses. The

Figure 7. Improved Drought Resistance in *35S:NFYA5* Plants.

(A) Detection of *NFYA5* mRNA in *35S:NFYA5* transgenic *Arabidopsis*. Real-time RT-PCR quantifications were normalized to the expression of *Tub8*. Error bars represent SE ($n = 3$).

(B) Drought resistance of *35S:NFYA5* plants (lines 2, 3, and 5). Wild-type and *35S:NFYA5 Arabidopsis* plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for 14 d. A representative picture is shown.

(C) Measurement of stomatal aperture in wild-type and *35S:NFYA5-3* transgenic plants. Data are mean ratios of width to length $\pm$ SE of three independent experiments ($n = 40$ to 50).

(D) Water loss from detached leaves of wild-type and *35S:NFYA5-3* plants. Water loss was expressed as the percentage of initial fresh weight. Values are means from 10 leaves for each of four independent experiments.

(F) Anthocyanin content in leaves of *Arabidopsis* with or without drought treatment for 14 d. Error bars represent SE for four independent experiments.

candidate target genes of At NFYB1 do not have obvious associations with stress tolerance, and some of them appear to be related to polysaccharide metabolism (Nelson et al., 2007). The lack of substantial overlap between the target genes of *NFYA5* and At *NFYB1* indicates that the two transcription factors may be involved in separate gene regulons.

Our microarray analysis showed that *NFYA5* overexpression caused only moderate changes in the expression of a relatively small number of genes under the conditions tested. It is possible that the NFYA5 protein may need to be modified under drought stress, or additional factors such as other NF-Y subunit genes may have to be overexpressed together, to have a more pronounced effect on target genes. Alternatively, substantial

changes in target gene expression in certain tissues or cells may have been masked by our analysis of expression changes using whole seedlings.

Transcriptional induction may explain part of *NFYA5* transcript accumulation under drought stress. ABA is involved in the transcriptional regulation since it is required for *NFYA5* transcript accumulation and it activates *NFYA5* promoter activity. Using the PlantCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare), two ABA-responsive element sequences could be found in the 1.7-kb promoter region of At *NFYA5*. Although the upstream transcription factor controlling *NFYA5* transcription under drought stress is not known, it might be one of the ABA-responsive element binding proteins.

Table 1. Genes with Expression Changes (pfp < 0.05) of at Least Twofold in the *35S:NFYA5* Transgenic Plants from Microarray Analysis

Affy ID	Gene ID	Description	Number of CCAAT Motifs	Number of Novel Elements	Fold Change (OX/WT)[a]
263158_at	AT1G54160	NFYA5	0	0	12.72
245275_at	AT4G15210	Cytosolic β-amylase expressed in rosette leaves and inducible by sugar	1	0	3.34
244966_at	ATCG00600	Cytochrome b$_6$f complex, subunit V	2	1	3.33
262517_at	AT1G17180	Glutathione transferase belonging to the tau class of GSTs	4	0	3.24
267565_at	AT2G30750	Putative cytochrome P450	0	0	3.08
261021_at	AT1G26380	FAD binding domain-containing protein	1	0	3.02
266098_at	AT2G37870	Protease inhibitor/seed storage/lipid transfer protein family protein	2	1	2.81
260568_at	AT2G43570	Chitinase, putative	5	1	2.77
262518_at	AT1G17170	Glutathione transferase belonging to the tau class of GSTs	1	2	2.46
247224_at	AT5G65080	MADS domain protein	5	1	2.38
267101_at	AT2G41480	Peroxidase	0	0	2.29
244993_s_at	ATCG01000 ATCG01130	[ATCG01000]hypothetical protein [ATCG01130]hypothetical protein	44	0	2.28
249481_at	AT5G38900	DSBA oxidoreductase family protein	3	1	2.23
263497_at	AT2G42540	COR15A	1	0	2.20
263495_at	AT2G42530	Cold-responsive protein	7	0	2.14
247718_at	AT5G59310	Member of the lipid transfer protein family	3	0	2.01
254232_at	AT4G23600	CORI3	4	0	2.00
246375_at	AT1G51830	ATP binding/kinase/protein Ser/Thr kinase	2	0	0.50
251226_at	AT3G62680	PRP3; structural constituent of cell wall	2	0	0.48
254828_at	AT4G12550	AIR1; lipid binding	1	0	0.48
264577_at	AT1G05260	RCI3; peroxidase	1	0	0.47
258338_at	AT3G16150	L-Asparaginase, putative/L-Asn amidohydrolase, putative	3	0	0.47
254044_at	AT4G25820	XTR9	2	0	0.46
266353_at	AT2G01520	Major latex protein-related	3	0	0.46
258473_s_at	AT3G02620, AT3G02610	[AT3G02620] acyl-desaturase, putative/stearoyl-ACP desaturase, putative; [AT3G02610]acyl-desaturase	31	0	0.45
254644_at	AT4G18510	CLE2; receptor binding	4	0	0.42
253767_at	AT4G28520	CRU3; nutrient reservoir	3	0	0.41
249082_at	AT5G44120	CRA1; nutrient reservoir	0	0	0.11

[a] OX/WT, overexpression line/wild type.

An interesting feature of *NFYA5* regulation under drought stress is the involvement of a miRNA. Our results suggest that the accumulation of *NFYA5* transcript is suppressed by miR169. Drought stress downregulates miR169 expression, thus relieving miR169 repression of *NFYA5*. miR169 is encoded by many loci. Only two of the loci, *MIR169a* and *MIR169c*, are substantially downregulated by drought stress. Based on the sequence read frequency data at the *Arabidopsis* ASRP small RNA database (http://asrp.cgrb.oregonstate.edu/db/microRNA.html?fid=12/), miR169a constitutes ~90% of the total miR169 population. Therefore, a substantial downregulation of miR169a would result in a reduction in overall miR169 level. Our data indicate that miR169a rather than miR169c plays a major role in repressing *NFYA5* transcript accumulation, despite the fact that both miRNAs have three mismatches with *NFYA* mRNA. ABA is required for the downregulation of *MIR169a* and *MIR169c* by drought stress. Therefore, ABA is involved in both the transcriptional and posttranscriptional regulation of *NFYA5*. The downregulation of *MIR169a* and *MIR169c* by ABA and drought stress

likely involves transcriptional repression at the two loci. It has been proposed that the induction of miRNAs and siRNAs leads to the downregulation of negative regulators of stress tolerance, whereas the suppression of miRNAs and siRNAs allows certain positive regulators to accumulate and function under stress (Sunkar et al., 2007). Consistent with this hypothesis, our results here support that the downregulation of miR169 contributes to the transcript accumulation of *NFYA5*, a critical positive regulator of drought stress resistance.

The importance of transcriptional regulation for plant stress resistance has been abundantly documented (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Much less is known about posttranscriptional regulation of stress-responsive genes. *NFYA5* is regulated by drought stress not only transcriptionally but also posttranscriptionally via a miRNA. This dual regulation is consistent with the critical importance of *NFYA5* for drought resistance. Both *NFYA5* and miR169 are highly conserved in rice (Jones-Rhoades and Bartel, 2004), so the dual modes of regulation of *NFYA5* may also apply to other plants.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana mutants, *rdr2-1*, *dcl2-1*, *dcl3-1*, and *dcl4-1*, were kindly provided by James Carrington (Center for Gene Research and Biotechnology, Oregon State University). *dcl1-7* and *hen1-1* were kindly provided by Xuemei Chen (University of California, Riverside). *rdr6* and *sde4/nrpd1a* were kindly provided by David Baulcombe (John Innes Center for Plant Science Research, Sainsbury Laboratory, UK). *sgs3* was kindly provided by Herve Vaucheret (Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, Versailles, France). *hyl1* was a gift from Nina Federoff (The Huck Institute of Life Science, Pennsylvania State University). These mutants were in the Col-0, L*er*, Nosssen-0, or C24 genetic backgrounds as indicated in the text and figures. *aba2-1*[CS156], *abi1-1*[CS22], and the T-DNA insertion mutant of *NFYA5* (SALK_042760) were obtained from the ABRC. *Arabidopsis* seedlings in MS nutrient agar medium were grown under continuous light (70 μmol m^{-2} s^{-1}) at 23 $\pm$ 1°C. Soil-grown (Metromix 350) *Arabidopsis* and *Nicotiana benthamiana* plants were grown under a 16-h-light/8-h-dark photoperiod at 23 $\pm$ 1°C. For dehydration treatment, 2-week-old seedlings were pulled out of agar medium and left to dry on Whatman 3MM paper on a laboratory bench for durations as indicated. For drought treatment, plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for durations as indicated.

Constructs and Generation of Transgenic Plants

Site-directed mutagenesis was performed to generate *NFYA5* mutated in the region complementary to the small RNA by the QuickChange II site-directed mutagenesis kit (Stratagene). This fragment was sequenced to ensure that only the desired mutations were introduced. *NFYA5* with and without its 3′ UTR was amplified with the primers indicated (with/without 3′ UTR forward 5′-CACCATGCAAGTCTTTCAAAGGAAAG-3′, with 3′ UTR reverse 5′-GTAATGCAATTGTACTCTCGAG-3′, and without 3′ UTR reverse 5′-TCAAGTCCCTGACATGAGAGCTGAGG-3′). These constructs were cloned into the plant expression GATEWAY destination vector pMDC32.

To generate pMDC32:miR169 constructs, a 200-bp fragment surrounding the miRNA sequence including the fold-back structure was amplified from genomic DNA with the following primers: miR169a forward 5′-CACCTGGGTATAGCTAGTGAAACGCG-3′ and reverse 5′-CCTTA-GCTTGAGTTCTTGCGA-3′, miR169b forward 5′-CACCCCCAACGGAG-TAGAATTG-3′ and reverse 5′-CTCATACGGTCGATGTAATCCGT-3′, miR169c forward 5′-CACCTCGTCCATTATGAGTATT-3′ and reverse 5′-CTAAT-ATGATATGAATATGGATGA-3′, miR169h forward 5′-CACCTCATATAA GAGAAAATGGTG-3′ and reverse 5′-CCAAAAAAGAGAAATGTGAATGAG-3′. The amplified fragments were introduced into the pENTR/D-TOPO vector (Invitrogen) and cloned into pMDC32 by LR reactions (Invitrogen).

For the *NFYA5* promoter:GUS construct, a 1.7-kb fragment upstream from the initiation codon was amplified with the forward primer 5′-CAC-CTGTATGACATATTCTGTGTGGAG-3′ and reverse primer 5′-TGCAA-ATTGGGTATTGGCTATG-3′ and cloned into the pMDC164 vector following Gateway recombination.

A fusion of YFP to the C-terminal end of NFYA5 was generated and introduced to pEarleyGate 101 vector by Gateway recombination. YFP images were collected on a Leica SP2 confocal microscope.

RNA Analysis

Total RNA was extracted from the wild type, mutants, and transgenic plants with Trizol reagent (Invitrogen). For enrichment of small RNAs, high molecular weight RNA was selectively precipitated by the addition of 1 volume of 20% PEG-1 M NaCl (Llave et al., 2002). High molecular weight RNA was separated on 1.2% formaldehyde-MOPS agarose, and low molecular weight RNA was fractioned on 17% denaturing polyacrylamide gels. The blots were probed and washed as described (Borsani et al., 2005).

For real-time RT-PCR, 5 μg of total RNA isolated with the RNeasy plant mini kit was used for the first-strand cDNA synthesized using SuperScript III first-strand synthesis supermix (Invitrogen). The cDNA reaction mixture was diluted three times, and 5 μL was used as template in a 25-μL PCR reaction. PCR was performed after a preincubation at 95°C for 3 min and was followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s. All the reactions were performed in the iQ5 real-time PCR detection system using iQ SYBR green supermix (Bio-Rad). Primers specific for the precursor of miR169 were used to detect expression levels of miR169 (see Supplemental Table 1 online). Primers were also designed to detect the transcription level of *NFYA5*. Each experiment was replicated three times. The comparative Ct method was applied. The primers used in this experiment were listed in Supplemental Table 2 online.

Transient Expression in *N. benthamiana*

Site-directed mutagenesis constructs, with and without 3′ UTR constructs, were transformed into *Agrobacterium tumefaciens* strain 3301. Overnight cultures were harvested and mixed at a 1:1 ratio with various combinations. After 1 h of incubation at room temperature in 10 mM MgCl$_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone, *Agrobacterium* suspension was coinfiltrated into 3-week-old *N. benthamiana* leaves. Leaves were harvested 2 d after the infiltration and small RNA extraction and blotting performed as described above.

Stomatal Aperture Analysis

Rosette leaves from 3-week-old soil-grown plants at similar developmental stages were harvested. Leaves were frozen immediately in liquid nitrogen and observed for guard cells by environmental scanning electron microscopy (Hitachi; TM 1000). Width and length of stomatal pores were measured for statistical analysis (Lemichez et al., 2001).

Water Loss Measurement

For water loss measurement, six leaves per individual of mutant and wild-type plants growing under normal conditions for 3 weeks were excised, and fresh weight was determined at designated time intervals. Four replicates were done for each line. Water loss was represented as the percentage of initial fresh weight at each time point.

Anthocyanin Content Measurement

Anthocyanin contents were measured as described by Rabino and Mancinelli (1986) and Sunkar et al. (2006). The pigments were extracted with 99:1 methanol:HCl (v/v) at 4°C, and the OD$_{530}$ and OD$_{657}$ for each sample were measured and OD$_{530}$ − 0.25 × OD$_{657}$ was used to compensate for the contribution of chlorophyll and its products to the absorption at 530.

GUS Activity Assay

Histochemical localization of GUS staining was performed by incubating the transgenic plants in 1 mg mL^{-1} 5-bromo-4-chloro-3 indolyl β-D-glucuronic acid, 0.1 M Na$_2$HPO$_4$ buffer, pH 7.0, 0.5 mM K$_3$[Fe[CN]$_6$], and 10 mM EDTA overnight at 37°C, followed by clearing with 70% ethanol. GUS activity was assayed according to the procedure of Jefferson (1987). One hundred milligrams of frozen tissues were homogenized in 100 μL of

extraction buffer (50 mM NaPO$_4$, pH 7.0, 1 mM Na$_2$EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] sodium lauryl sarcosine, and 10 mM DTT) and centrifuged for 10 min at 4°C at 13,000 rpm. The fluorogenic assay was incubated in a 0.5-mL volume extraction buffer supplied with 1 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma-Aldrich) for 2 h and then stopped by 0.2 M Na$_2$CO$_3$. Protein concentration was determined according to the Bio-Rad protocol provided with the protein assay kit. GUS activity was calculated as picomoles MU per minute per milligram of protein.

Microarray Analysis

For Affymetrix GeneChip array analysis, wild-type and *35S:NFYA5* seedlings were grown on MS plates for 15 d at 22°C with a cycle of 16 h light and 8 h darkness. Total RNA was extracted using an RNeasy plant mini kit (Qiagen) and was then used for preparation of biotin-labeled complementary RNA targets. Microarray analysis was performed as described by Breitling et al. (2004). Two biological replicates were used for each genotype. We normalized expression profiles with the RMA method (Irizarry et al., 2003). A list of genes with statistically significant changes in expression between the genotypes was generated by the RankProd method in which multiple testing was taken into account by the use of pfp (percentage of false prediction) (pfp < 0.05) (Gentleman et al., 2004; Hong et al., 2006). For predicting the consensus novel *cis*-regulatory element, we used the AlignACE program (Hughes et al., 2000). We applied the program to 1-kb upstream promoter sequences of up- or downregulated genes. Out of several candidate consensus elements, we chose one that contains a weak CCAAT consensus motif that was found within promoters of upregulated genes.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *NFYA5* (At1g54160), *MIR169a* (At3g13405), *DCL3* (At3g43920), *RDR2* (At4g11130), *DCL4* (At5g20320), *DCL2* (At3g03300), *RDR6* (At3g49500), *SGS3* (At5g23570), HEN1 (At4g20910), *DCL1* (At1g01040), *HYL1* (At1g09700), *SDE4* (At1g63020), *AGO4* (At2g27040), *nfya5* (SALK_042760), *aba2-1* [CS156], and *abi1-1* [CS22]. The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE12029 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12029).

Supplemental Data

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

Supplemental Figure 1. Sequence Alignment of the Conserved Domains in *Arabidopsis* NFYA Family Members.

Supplemental Figure 2. Analysis of miR169 and *NFYA5* mRNA Levels in *nfya5*, *35S:MIR169b*, and *35S:MIR169h* Transgenic Plant Lines.

Supplemental Figure 3. Coexpression of Various Combinations of miR169 and *NFYA5* Constructs in *N. benthamiana*.

Supplemental Figure 4. Analysis of Transcript Levels in the Wild-Type and *35S:NFYA5* Transgenic Plants.

Supplemental Table 1. List of Genes with Significant Expression Changes in the *35S:NFYA5* Transgenic Plants form Microarray Analysis.

Supplemental Table 2. List of Primers Used in This Study.

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