NERF encodes a RING E3 ligase important for drought resistance and enhances the expression of its antisense gene NFYA5 in Arabidopsis

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ABSTRACT

NFYA5 is an important drought-stress inducible transcription factor gene that is targeted by miR169 in Arabidopsis. We show here that the cis-natural antisense transcript gene of NFYA5, NFYA5 Enhancing RING FINGER (NERF), can produce siRNAs from their overlapping region (OR) and affect NFYA5 transcripts by functioning together with miR169. The NERF protein functions as an E3 ligase for ubiquitination. Overexpression of NERF or OR cDNA leads to siRNA/NERF accumulation, miR169 repression, and NFYA5 transcript enhancement; knockdown of NERF transcripts by an artificial miRNA enhances miR169 abundance and reduces NFYA5 transcripts. Overexpression of NFYA5 does not affect the NERF mRNA level. Deep sequencing of the small RNA library from 35S::OR plants identifies 960 sequences representing 323 unique siRNAs that originate from OR; the sequences of some siRNA/NERF are similar/complementary to those of miR169. Overexpression of the 195- to 280-bp OR cDNA-containing siRNAs similar/complementary to miR169 also leads to the accumulation of NFYA5 transcripts. Analysis of NERF knock-down plants and NERF overexpression lines showed that, like NFYA5, NERF is important for controlling stomatal aperture and drought resistance. This regulatory model might apply to other natural antisense transcripts with positively correlated expression patterns.

INTRODUCTION

Small RNAs regulate gene expression through post-transcriptional gene silencing, translational inhibition or heterochromatin modification (1). Small RNAs can be classified into 20- to 24-nucleotide (nt) microRNAs and endogenous short interfering RNAs (siRNAs), such as 21-nt ta-siRNAs (trans-acting siRNAs), ~24-nt repeat-associated siRNAs, and 21- or 24-nt nat-siRNAs. miRNAs are processed from hairpin precursors by the ribonuclease III-like enzyme Dicer-like in plants. siRNAs differ from miRNAs in that they are generated from long, double-stranded RNAs, including transcribed repeat sequences, products of RNA-dependent RNA polymerase, and natural antisense transcripts (NATs) (2,3).

NATs are RNA molecules that are transcribed from the opposite DNA strand compared with other transcripts and overlap in part with sense RNA (4). There are two kinds of NATs: cis-NATs and trans-NATs. cis-NATs are formed by antisense transcripts at the same genomic locus, whereas the sense and antisense transcripts of trans-NATs are derived from different genomic loci (5). cis-NATs have been detected in most species analyzed, and the reported frequencies for overlapping gene pairs in different species range from 5 to 10%. In the human genome, 4 to 9% of all transcripts overlap, while >20% genes have been reported to be overlapping in Drosophila. In plants, cis-NATs have been analyzed in rice and Arabidopsis (1,5,6). In Arabidopsis, 1340 potential cis-NAT pairs have been predicted, and 957 cis-NATs have been confirmed to be sense and antisense transcripts by analysis of full-length cDNAs and massively parallel signature sequencing data (7).

cis-NATs mediate inhibition of microRNA function in humans (8). In plants, cis-NATs are thought to be important for the biogenesis of nat-siRNAs, and genome-wide analysis has demonstrated the widespread existence of nat-siRNAs in plants (9–11). nat-siRNAs could be the main modulators of gene regulation in plants as suggested by research on the NaCl-inducible nat-siRNA SRO5 (12), pathogen-related nat-siRNA ATGB2 (13), and sperm-specific nat-siRNA NAKPL (14). These nat-siRNAs usually cause silencing of the antisense transcript and lead to the anti-correlated expression pattern of NATs (7,9,11). However, independent researchers have reported that cis-NATs do not tend to produce nat-siRNAs when compared to non-
overlapping neighboring gene pairs, and anti-correlated expression of cis-NATs represent only a small number of the total cis-NATs (5,15,16). Thus, the mechanisms by which NATs regulate gene expression remain poorly understood.

Regulation of gene expression at the transcriptional level is crucial for normal development and physiology in plants (17,18). We previously reported that the transcription factor NFYA5 is important for drought resistance in Arabidopsis, and that drought stress up-regulates NFYA5 gene expression not only at the transcriptional level but also at the post-transcriptional level by down-regulating the expression of miR169a that targets NFYA5 transcript for cleavage (19). NFYA5 is annotated to overlap with NERF (NFYA5 Enhancing RING FINGER) on the antisense strand in their 3′ UTR regions to form a cis-NAT gene pair. In several cases, a change in the expression of the E3 ubiquitin ligase gene altered drought resistance and plant hormone action by regulation of downstream target genes. For example, overexpression of SDIR1 (salt- and drought-induced RING finger 1) led to ABA hypersensitivity and ABA-associated phenotype, such as salt hypersensitivity in germination, enhanced ABA-induced stomatal closing, and enhanced drought tolerance (20); and 35S::Rma1H1 (RING membrane-anchor 1 homolog 1) transgenic Arabidopsis became more resistant to drought stress via the down-regulation of plasma membrane aquaporin levels by inhibiting aquaporin trafficking to the plasma membrane and subsequent proteasomal degradation (21).

Here, we show that NERF functions as a RING finger protein that has ubiquitin E3 ligase activity. Transcriptional levels of NERF positively affect NFYA5 mRNA abundance by antagonizing miR169 expression through siRNA-NERF originating from the NERF and NFYA5 overlapping region (OR). Expression of NERF in vascular tissues and guard cells, and analysis of NERF knock-down plants and NERF overexpression lines show that, like NFYA5, NERF is pivotal in controlling stomatal aperture and drought resistance.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type (WT) and was the genetic background for transgenic plants. dcl2/3/4 and other mutants involved in the small RNA biogenesis pathway were maintained in our laboratory. These mutants were in the Col-0, Landsberg erecta (Ler), Nossen-0 (No), or C24 genetic backgrounds as indicated in the text and figures. Arabidopsis plants were grown under 16 h light/8 h dark at 23 ± 1°C. For drought treatment, plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for the indicated durations.

Constructs and generation of transgenic plants

The cDNAs of NERF with/without 3′ UTR, NFYA5 with 3′ UTR, and OR and ORC fragments were amplified by PCR. The corresponding products were introduced into the pENTR™/D-TOPO vector (Invitrogen) and cloned into pMDC32 by LR reactions (Invitrogen). The schematic diagrams of OR and ORC amplicons are provided in Supplementary Figure S1.

To generate NERF knockdown Arabidopsis plants, we engineered a gene-specific amiRNA by replacing the original miR319a sequence in the pRS300 plasmid as previously described (22). The constructs were cloned into the pCPB vector behind the cauliflower mosaic virus (CaMV) 35S promoter using the BamHI restriction site by In-Fusion reaction.

For the NERF promoter::GUS construct, a 1.4-kb fragment of the full inter-region between the stop codon of At1g54130 and the initiation codon of NERF was amplified and cloned into the pMDC164 vector following Gateway recombination.

A fusion of yellow fluorescence protein (YFP) to the C-terminal end of NERF was generated and introduced into the pEarleyGate101 vector by Gateway recombination. YFP images were collected on a Leica SP2 confocal microscope.

The plasmid was electroporated into Agrobacterium tumefaciens GV3101 and was transformed into Arabidopsis by the floral dip method (23). Transgenic plants were selected with the use of 35 µg ml⁻¹ hygromycin or 45 µM PESTANAL. T3 or T4 homozygous lines were used for all experiments. The sequences of the primer pairs used in the experiments are listed in Supplementary Table S1.

RNA analysis

Total RNA was extracted from WT and transgenic plants with Trizol reagent (Invitrogen). For enrichment of small RNAs, high molecular weight RNA was selectively precipitated by the addition of one volume of 20% PEG 8000/1 M NaCl and separated on 1.2% formaldehyde-MOPS agarose gels for northern blot. The probes were labeled with 32P-dCTP using a Ready-To-Go DNA Labeling Kit (Amersham Biosciences). Low molecular weight RNA was fractionated on 17% denaturing polyacrylamide gels, and small RNA northern blots were probed and washed as described (19) except for OR cDNA, which was labeled using similar methods to northern blot.

For real-time RT-PCR, 5 µg of total RNA isolated with the RNeasy plant mini kit was used for the first-strand cDNA synthesis using SuperScript III first-strand synthesis supermix (Invitrogen). The cDNA reaction mixture was diluted 50 times, and 3 µl was used as template in a 25-µl PCR reaction. PCR included a pre-incubation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 40 s, and extension at 72°C for 40 s. All the reactions were carried out in an ABI 7500 system (Applied Biosystems) using the SYBR Premix Ex Taq™ (perfect real time) kit (TaKaRa Biomedicals). Primer efficiencies were measured and calculated (24). The PCR products were loaded on 1.5% agarose gels and photographed after staining with ethidium bromide. Each experiment was replicated three times. The comparative Ct method was applied.
Small RNA library construction, sequencing and data analysis

The miRNAs were cloned as described by Sunkar and Zhu with some modifications (25). In brief, low molecular weight RNA was fractionated on 17% denaturing polyacrylamide gels. Small RNAs in the range of 16-30 nt were excised and eluted with 0.3 M NaCl. The RNA was dephosphorylated and ligated sequentially to 5' and 3' RNA/DNA chimeric oligonucleotide adaptors. Reverse transcription PCR was carried out, and the resulting PCR products were sequenced using Solexa sequencing technology (BGI, Shenzhen, China).

The raw reads were produced after excluding low quantity reads and 5' and 3' adaptor contaminants. The identical adaptor-trimmed sequences in the range of 1630 nt were grouped as unique sequences with normalized counts for the individual sequence reads. The unique small RNAs were aligned to the NERF and NFYA5 overlapping region.

5' RACE

To obtain cleavage fragments resulting from transcript processing by microRNAs, we used the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen). Total RNA was extracted from 10-day-old 35S::OR seedlings using the TRIZOL method described above. The oligo(dT) was then used for cDNA synthesis. Initial PCR was carried out using the 5' RACE Abridged Primer and gene specific outer primer (5'-GTA ATG CAA TTG TAC TCT CGA G-3'). Nested PCR was carried out using 1/100 of the initial PCR reaction as template, Universal Amplification Primer, and gene-specific inner primer (5'-AGA GAA TCG GAA GTT AAC AAA ATA G-3'). RACE fragments were cloned and sequenced after gel purification.

Ubiquitination assays

The coding region of NERF was cloned into the pGEX-4T-3 vector by BamHI and NotI sites and expressed in Escherichia coli. The recombinant fusion protein was purified with a B-PER® GST Spin Purification Kit (Thermo Scientific). Site-directed mutagenesis was performed to generate NERF with a mutated RING function domain with a QuickChange II Site-Directed Mutagenesis Kit (Stratagene). This fragment was sequenced to ensure that only the desired mutations were introduced. The sequences of the primer pair used for the preparation of the WT, H348Y and C367S mutants are listed in Supplementary Table S1.

The ubiquitination assays were performed in the presence of E1, E2 and flag-ubiquitin as described by Xie et al. (26). Approximately 500 ng of GST-NERF fusion protein was used in the mixture of 40 ng of rabbit E1 (Boston Biochemicals), 40 ng of human UbcH5b (Boston Biochemicals), and 1 μg of flag-ubiquitin (Boston Biochemicals). The reaction mixture was incubated in 50 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM MgCl2, and 2 mM DTT for 2 h at 30°C with agitation in an Eppendorf Thermomixer. Samples were then heated to 95°C in a buffer containing 2-mercaptoethanol before separation by SDS-PAGE and immunoblot analysis using anti-flag antibody.

Stomatal aperture analysis

For the determination of stomatal aperture responses to drought stress, rosette leaves were obtained from 3-week-old soil-grown plants at similar developmental stages that had been provided with/without sufficient water. The leaves were harvested in the morning 2 h after they were exposed to light. Leaves were immediately frozen in liquid nitrogen, and their guard cells were observed by environmental scanning electron microscopy (HITACHI, TM 1000). The width and length of stomatal pores were measured.

RESULTS

NERF protein functions as an E3 ligase for ubiquitination

Previously, Borsani et al. reported a new type of endogenous siRNA derived from a NAT pair and described its role in salt tolerance in Arabidopsis (12). This motivated us to investigate NERF (At1g54150) because it formed a cis-NAT with NFYA5 in their 3' UTR regions (Supplementary Figure S1), and because we have demonstrated the critical functions of NFYA5 in drought resistance in Arabidopsis (19). The C terminus of NERF contains a C3-H-C4 RING finger domain comprising conserved Cys and His residues (Figure 1A). To determine whether this RING finger protein has E3 ligase activity, we performed an in vitro self-ubiquitination assay. We expressed NERF in E. coli as a fusion protein with glutathione-S-transferase (GST) and subsequently obtained affinity purified GST-NERF (Figure 1B). In the presence of rabbit E1 and a human E2, polyubiquitinated proteins were formed (Figure 1C), verifying that this ubiquitination activity depends on the presence of GST-NERF as well as E1 and E2. To determine whether the RING domain is required for NERF E3 ligase activity, we constructed two single amino acid alleles by mutagenizing the conserved amino acid His-348 to Tyr (H348Y) and Cys-367 to Ser (C367S), which presumably disrupted the RING finger domain (Figure 1A). In vitro self-ubiquitination assay showed that neither mutant protein had significant ubiquitin ligase activity (Figure 1D), demonstrating that the RING domain is required for NERF E3 ligase activity.

Expression patterns of NERF

Semi-quantitative RT-PCR indicated that NERF was expressed in various tissues, including roots, stems, flowers and leaves (Figure 2A). We also constructed promoter::GUS transgenic lines to examine the expression patterns of NERF. Analysis of GUS staining patterns in several transgenic lines showed that the promoter was active in cotyledons, root vascular tissue and root tips of 8-day-old seedlings (Figure 2B, panels I and II). GUS staining was also observed in flowers and siliques (Figure 2B, panels III and IV), confirming that the NERF promoter, like NFYA5, was active at all developmental stages. Importantly, the NERF expression was detected in guard cells (Figure 2B, panels V), which is also consistent with the location of NFYA5 (19).

The Psort program predicted a membrane localization of NERF protein, with 83% certainty for its location in the mitochondrial inner membrane or 60% certainty for its location in the plasma membrane (http://psort.ims.u-tokyo.
ac.jp/form.html). To confirm the subcellular localization of NERF protein, we transformed Arabidopsis with a translational fusion between YFP and the C terminus of NERF under the control of the CaMV 35S promoter. In contrast to the prediction by the Psort program, root cells expressing the NERF fusion protein showed that the YFP signal mainly appeared in the nucleus as NFYA5 and was only weakly evident outside the nuclear compartment (Figure 2C).

To determine whether NERF expression was also regulated by water stress, as is the case with NFYA5 (19), we subjected 20-day-old Arabidopsis seedlings to dehydration treatments and then isolated the corresponding RNA for RNA gel blot analysis. The transcripts of NERF were induced by the dehydration treatment. The relative level of NERF expression was about 3-times greater after 6 h of dehydration treatment than in the control, suggesting that NERF is involved in stress responses (Figure 2D).

Transcriptional levels of NERF positively affect NFYA5 mRNA abundance

The similar expression patterns for NERF and NFYA5 prompted us to investigate whether the expression of the two genes is correlated. We first searched the publicly available T-DNA collections and obtained a T-DNA insertion line in NERF from the Arabidopsis Biological Resource Center, SALK_096716. 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 NERF (Supplementary Figure S2). However, the NERF transcripts in WT and SALK_096716 were similar (Supplementary Figure S2). Thus, an artificial miRNA targeting NERF mRNA was designed following the strict base-pairing rules described by Schwab (22) and using the designing tool at http://wmd3.weigelworld.org/cgi-bin/webapp.cgi. Artificial miRNA was then introduced into Arabidopsis (Figure 3A), and its effectiveness in silencing NERF expression was verified by real-time RT-PCR. Two independent amiNERF lines were chosen for further analysis (Figure 3A). Like NERF transcripts, the NFYA5 transcripts were significantly decreased in amiNERF transgenic plants, i.e., the level of NFYA5 mRNA was ~40% lower in amiNERF plants than in the WT (Figure 3A).

To further characterize the possible relationship between NERF and NFYA5, we generated transgenic Arabidopsis plants overexpressing the full-length cDNA of NERF and tested the NFYA5 transcripts in the corresponding transgenic lines (Figure 3B). Contrary to the down-regulation of NFYA5 transcripts in amiNERF plants, the mRNA abundance of NFYA5 significantly increased in 35S::NERF transgenic plants, and the NFYA5 level was positively correlated with the expression of NERF (Figure 3B). NFYA5 transcripts did not accumulate in 35S::NERF-cds (without 3′-UTR) transgenic plants, suggesting that 3′-UTR or the OR is essential for the accumulation of NFYA5 in 35S::NERF transgenic plants (data not shown). To test this hypothesis, we constructed transgenic plants only overexpressing OR cDNA, and quantified the effects of OR overexpression on NFYA5 transcripts (Figure 3B). The NFYA5 transcripts were significantly enhanced in 35S::OR transgenic plants (Figure 3B), demonstrating that OR is essential for the accumulation of NFYA5 transcripts in 35S::NERF transgenic plants.
Figure 2. NERF expression patterns. (A) Semi-quantitative RT-PCR analysis revealed the constitutive expression of NERF in various tissues, including root (lane 1), flower (lane 2), stem (lane 3) and leaf (lane 4). The Tub4 gene was used as a positive control. No bands were detected in the minus RT controls (lane 5). (B) NERFp::GUS expression patterns in various tissues. The staining was prominent in leaf and root vascular tissues (I), root tips (II), inflorescence (III), siliques (IV) and guard cells (V). (C) Subcellular localization of NERF. NERF-YFP fusion construct was expressed in transgenic Arabidopsis under the control of the CaMV 35S promoter, and the plant roots were observed with a confocal microscope. The roots were photographed in the dark field for yellow fluorescence (I), in the bright field for the morphology of the cells (II), and in combination (III). (D) Regulation of NERF mRNA abundance by dehydration shock. A 2μg quantity of total RNA from each sample was loaded and hybridized with 32P-labeled full-length NERF probe. Tub4 was used as a loading control. Numbers below each lane indicate the expression level of NERF relative to Tub4.

In Arabidopsis and Medicago truncatula, the expression of NFYA5 is mainly regulated by miR169 at the post-transcriptional level (19,27). To determine whether the down-/up-regulation of NFYA5 in amiNERF, 35S::NERF and 35S::OR transgenic plants is due to the variation in miR169 expression, we isolated small RNA from amiNERF, 35S::NERF and 35S::OR transgenic plants. Compared to its expression in WT, the expression of miR169 was significantly up-regulated in amiNERF plants, and significantly down-regulated in 35S::NFYA5 transgenic plants. The changes in the levels of NFYA5 transcripts in amiNERF, 35S::NERF and 35S::OR transgenic plants were at least partly due to the variation of miR169 abundance.

Overexpression of NFYA5 does not increase NERF expression

Significant variations of NFYA5 mRNA abundance in amiNERF, 35S::NERF and 35S::OR transgenic plants prompted us to test whether NFYA5 transcripts could affect the expression of NERF. We generated transgenic Arabidopsis plants overexpressing NFYA5 with 3′ UTR (Figure 4A). Overexpression of NFYA5 did not affect the expression of NERF (Figure 4A). Correspondingly, we did not observe obvious variation in miR169 accumulation between WT and 35S::NFYA5 transgenic plants (Figure 4B), suggesting that it is the special strand in OR between NERF and NFYA5 that affects the expression level of miR169.

siRNANERF biogenesis

When the OR cDNA was used as a probe and the hybridization temperature was increased to 45°C to improve the specificity of the signal, a small RNA signal around 21 nt was detected in WT and transgenic plants and significantly accumulated in 35S::NERF transgenic plants (Figure 5A). Because the stem-loop structure, which is the basic criterion for identification of plant microRNAs (28), did not exist in OR predicted by Mfold (29), we deduced that the small RNAs originated from OR were siRNAs, and we designated them siRNANERF. The siRNANERF level was also significantly enhanced in 35S::MIR169a transgenic plants compared to the WT (Supplementary Figure S3), indicating that siRNANERF and miR169 might have similar sequences.

To confirm that siRNANERF can regulate NFYA5 expression, we carried out modified 5′ RACE using mRNA from 35S::OR transgenic plants in order to map the potential cleavage sites of NFYA5 (Figure 5B). Although it was detected at only a low frequency (4 of 20), one site between
Figure 3. *NFYA5* mRNA abundance was positively affected by *NERF* transcripts. (A) *NFYA5* mRNA levels decreased in *amiNERF* transgenic plants. Small RNA gel blot analysis of artificial-miRNA in *amiNERF* transgenic plants. U6 was used as a loading control. The corresponding *NERF* and *NFYA5* expression levels were analyzed by real-time RT-PCR. Quantifications were normalized to that of *Tub4*. Error bars represent standard errors (n = 3). LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01. (B) *NFYA5* mRNA levels increased in 35S::*NERF* and 35S::*OR* transgenic plants. The *NERF*, *OR*, and *NFYA5* expression levels were analyzed by real-time RT-PCR. Quantifications were normalized to that of *Tub4*. Error bars represent standard errors (n = 3). LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01. (C) Expression analysis of miRNA169 accumulation in 35S::*NERF*, 35S::*OR* and *amiNERF* transgenic plants. A 20-μg quantity of small RNA from each sample was loaded per lane and hybridized with 32P-labeled probe corresponding to the sequence of miR169. *miR171* was used as a loading control. Numbers below each lane indicate the expression level of miR169 relative to *miR171*.

nucleotides 10 and 11 of *miR169* was identified, demonstrating that *miR169* can direct *NFYA5* mRNA cleavage. To our surprise, four additional cleavage sites in the *NFYA5* mRNA were identified, and one of these was the most frequent cleavage site (10 of 20). No other conserved miRNAs except for *miR169* from the *Arabidopsis* small RNA database were predicted to target *NFYA5* ([http://mpss.udel.edu/at_sRNA/](http://mpss.udel.edu/at_sRNA/)), indicating that siRNA-NERF does exist in *OR* and can direct the cleavage of *NFYA5* mRNA.

To determine which pathway components might be involved in the biogenesis of siRNA-NERF, we checked the expression of siRNA-NERF in mutants defective in various proteins known to be required for biogenesis of specific types of small RNAs; the attempt failed (Supplementary Figure S4), perhaps because of cross-hybridization between the siRNA-NERF and miRNA169 (Supplementary Figure S3) or because of the low abundance of the siRNA-NERF. The generation of small RNAs depends on different DICER-like (DCL) proteins in plants. DCL2 is believed to generate viral siRNAs (30); DCL3 forms heterochromatic siRNAs (30); and DCL4 is required for ta-siRNA biogenesis (31,32). To investigate the possible roles of DCLs in the generation of siRNA-NERF, we overexpressed *OR* in *dcl2/3/4* triple mutants (Supplementary Figure S5). The mRNA level of *NFYA5* did not substantially change despite overexpression of *OR* in the *dcl2/3/4* triple mutant (Figure 5C). As expected, the accumulation of siRNA-NERF observed in 35S::NERF transgenic plants was blocked in *dcl2/3/4* 35S::OR plants, and the expression of *miR169* did not significantly differ among *dcl2/3/4* and transgenic plants (Figure 5A and D). These results suggested that
Figure 4. *NFYA5* overexpression did not affect *NERF* abundance. (A) Detection of *NFYA5* and *NERF* transcripts in 35S::*NFYA5* transgenic plants by real-time RT-PCR. Quantifications were normalized to the expression of *Tub4*. Error bars represent standard errors (n = 3). LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01. (B) Accumulation of miR169 in 35S::*NFYA5* transgenic plants. A 20-μg quantity of small RNA from each sample was loaded per lane and hybridized with 32P-labeled probe corresponding to the sequence of miR169. miR171 was used as a loading control. Numbers below each lane indicate the expression level of miR169 relative to miR171.

Figure 5. siRNANERF biogenesis. (A) siRNANERF accumulated in 35S::*NERF* transgenic plants. A 20-μg quantity of small RNA from each sample was loaded per lane and hybridized with 32P-labeled *OR* cDNA probe. miR171 was used as a loading control. Numbers below each lane indicate the expression level of siRNANERF relative to miR171. (B) *NFYA5* mRNA cleavage sites detected by 5′ RACE. Numbers indicate the frequency of cleavage at each site. Target site of miRNA 169 was underlined. (C) Detection of *NFYA5* gene transcripts in *dcl2/3/4* 35S::*OR* transgenic plants by real-time RT-PCR. Quantifications were normalized to the expression of *Tub4*. Error bars represent standard errors (n = 3). LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01. (D) Detection of siRNANERF and miR169 in *dcl2/3/4* 35S::*OR* transgenic plants. A 20-μg quantity of small RNA from each sample was loaded per lane and hybridized with 32P-labeled probe corresponding to the sequence of *OR* cDNA or miR169. miR171 was used as a loading control.
DCL2, DCL3, or/and DCL4 should affect the biogenesis of siRNANERF.

siRNANERF produced from OR

To avoid the interference of artificial miRNAs generated in amiNERF transgenic lines, we generated a small RNA library from 35S::OR transgenic plants and sequenced the library by Solexa high-throughput sequencing technology. The small RNA library yielded more than 11 million raw reads. Approximately 95% of the raw reads remained after 3′ and 5′ adaptor trimming, and more than 8 million reads could be perfectly mapped to the Arabidopsis genome (the Arabidopsis Information Resource 9). Sequences that could not be mapped to the Arabidopsis genome were discarded, and only those that perfectly mapped were analyzed further. Of these, 960 sequences representing 323 unique siRNANERF could be perfectly mapped to OR (Supplementary Table S2). Among the total sequence reads matching OR, 21-nt small RNAs were dominant (Supplementary Figure S6), which was in consistent with the small RNA molecular weight detected by OR CDNA.

siRNANERF could originate from both positive and negative strands, and about 58% of them were from the negative strand. They were scattered along the OR, and two active loci producing siRNANERF (200–230 bp and 250–280 bp) were identified (Supplementary Figure S1). Interestingly, one of the two loci (200–230 bp) partially overlapped with the miR169 binding sites in NFYA5 (Supplementary Figure S1). The sequences of some siRNANERF that originated from the positive strand of OR are similar to those of miRNA169, especially at the 5′ end of mature miR169 (Figure 6A). We also found that some siRNANERF were partially complementary to miR169 (Supplementary Figure S7). To further characterize the function of these siRNANERF, we cloned the 195- to 280-bp cDNA in OR containing the two loci and overexpressed it in Arabidopsis (Supplementary Figure S1). Two transgenic lines designated as 35S::ORC (OR Core) were chosen for further analysis (Figure 6B). siRNANERF abundance and NFYA5 transcripts also increased in 35S::ORC transgenic plants, and correspondingly, the miR169 expression levels decreased (Figure 6B), which was similar to the phenomenon observed in 35S::NERF and 35S::OR transgenic plants. These results suggested that the functions of ORC containing siRNAs analogous/complementary to miR169 and OR are at least partially similar to that of OR.

Overexpression of NERF, like overexpression of NFYA5, increases drought resistance of Arabidopsis

The expression of NERF in guard cells (Figure 2B) and accumulation of NFYA5 transcripts in 35S::NERF and 35S::OR transgenic plants prompted us to analyze the role of NERF in drought resistance in Arabidopsis. Five transgenic lines (#15, #17, #20, #21 and #24) were chosen for further analysis based on their expression level (Figure 7A). WT and 35S::NERF plants were grown for 3 weeks in soil and were then subjected to water withholding for 14 days. Most WT plants wilted and their leaves became purplle, while the 35S::NERF plants remained turgid and their leaves remained green (Figure 7B). The results suggested that 35S::NERF plants wilt more slowly than WT plants because the former plants depleted soil water more slowly. To investigate this possibility, stomatal apertures were measured in leaves of 35S::NERF-20 and WT plants grown in soil. When water was not withheld, the stomatal aperture index of 35S::NERF-20 leaves was 0.34, which was nearly 30% smaller than that of the WT (Figure 7C). When water was withheld for 14 days, the stomatal aperture index of 35S::NERF-20 leaves declined to 0.24, which was ~32% smaller than that of the WT. These data indicated that the ability of the transgenic plants to remain turgid when water was withheld could be attributed at least in part to an increased ability to close stomata and reduce transpiration.

amiNERF plants are hypersensitive to drought stress

In contrast to 35S::NERF transgenic plants, amiNERF plants were hypersensitive to drought stress (Figure 8). After water had been withheld for 9 days, most of the amiNERF plants appeared more dehydrated than the WT (Figure 8A). The stomatal aperture index of WT leaves declined from 0.48 to 0.39, which was ~20% smaller than that of the amiNERF plants (Figure 8B). The results suggest that adequate expression of NERF is required for drought resistance and that NERF and NFYA5 have similar functions with respect to stomatal closure and drought stress.

DISCUSSION

Gene regulation under abiotic stress is mediated by multiple cascades of transcription factors (33). In each of these cascades, a transcription factor gene is induced or activated, which in turn activates or represses downstream target genes important for abiotic resistance. NFYA5 regulates the expression of stress-responsive genes (e.g. the genes that encode subunit of cytochrome b6-f complex, GST, peroxidases and oxidoreductase family proteins), and contributes to one of these abiotic stress-responsive transcriptional cascades (19). The transcriptional levels of NFYA5 were positively related to NERF mRNA abundance, and 35S::NERF transgenic- and amiNERF knock-down plants showed similar drought response phenotypes as NFYA5 overexpression lines or nfyA5 loss-of-function mutants in drought responses. These results suggest that NERF and NFYA5 are involved in the same gene regulon and that NERF acts upstream in this regulon.

The similar expression pattern (leaf and root vascular systems, guard cells, and flowers) and nuclear localization of NERF and NFYA5 suggest the basic prerequisites for the formation of double-stranded DNA to produce siRNAs. Deep sequencing of a small RNA library constructed from 35S::OR transgenic plants verified the existence of the siRNAs that originated from OR. Because of cross-hybridization with miRNA169, we were unable to identify the biogenesis pathway of siRNA that originated from OR. We further deduced that these siRNAs belonged to nat-siRNA. Unlike NaCl-inducible nat-siRNA5SRO5 and pathogen-inducible nat-siRNAATGB2, which have specific sequences that regulate corresponding target genes (12,13), the siRNAs in our small RNA library had different se-
sequences. They were scattered along the OR and did not arrange in phase as did the small RNAs of nat-siRNASRO5 (12). Zhang et al. reported that the generation of 20- to 22-nt nat-siRNAs depended on DCL1 and that generation of the 23- to 28-nt nat-siRNAs depended on DCL3 (11). The biogenesis of sperm-specific nat-siRNAKPL was also demonstrated to be processed by the DCL1–HYL1 complex (14). In our research, the accumulation of siRNANERF and the induction of NFYA5 in 35S::NERF transgenic plants were blocked in del2/3/4, which strongly indicated that DCL1 was not the major effector regulating siRNANERF production and that DCL2, DCL3, DCL4 or some combination, is involved in the biogenesis of siRNANERF.

Figure 6. siRNANERF originated from OR. (A) Diagram of siRNANERF that originated from OR and had a sequence similar to that of miR169. (B) NFYA5 mRNA levels also increased in 35S::ORC transgenic plants. Detection of ORC and NFYA5 transcripts in 35S::ORC transgenic plants by real-time RT-PCR. Quantifications were normalized to the expression of Tub4. Error bars represent standard errors (n = 3). For analysis of siRNANERF and miR169 accumulation in 35S::ORC transgenic plants, 20 μg quantity of small RNA from each sample was loaded per lane and hybridized with 32P-labeled probe corresponding to the sequence of miR169 or OR cDNA. miR171 was used as a loading control. Numbers below each lane indicate the expression level of miR169/siRNANERF relative to miR171. LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01.

Figure 7. Increased drought resistance in 35S::NERF transgenic plants. (A) Detection of NERF mRNA in 35S::NERF transgenic plants by real-time RT-PCR. Quantifications were normalized to the expression of Tub4. Error bars represent standard errors (n = 3). LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01. (B) Drought resistance of 35S::NERF plants (#15, #17, #20, #21 and #24). WT and 35S::NERF plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for 14 days. Photographs of representative plants are shown. (C) Measurements of the stomatal apertures in WT and 35S::NERF transgenic plants. Values are mean ratios of width to length ± SE of three independent experiments (n = 60–80). LSD test was used to test differences between treatments. Means with the same letter were not significantly different at P < 0.01.
Interestingly, the sequences of some siRNANERF were similar to that of miR169, especially at the 5′ end of mature miR169. Pairing to the miRNA 5′ region was crucial for miRNA-guided cleavage of target genes (34,35). In humans, BACE1 antisense transcript and miR-485–5p ncRNAs competed for binding to the exonic region of BACE1 mRNA, and covering of the miRNA binding site by BACE1 antisense transcript eliminated the miRNA-induced translational repression and BACE1 mRNA decay (8). The siRNANERF with similar sequence to that miR169 might also compete for NFYA5-targeting miR169 in Arabidopsis. The inference is based on: (1) four additional cleavage sites in addition to that of miR169 in the NFYA5 mRNA were identified; and (2) miR169 expression was not affected when siRNANERF were blocked in dcl2/3/4 triple mutants. Unexpectedly, we also detected that some siRNANERF were partially complementary to miR169. When the sequence complementarity between a siRNA and its target is extensive, the inhibition that results is said to involve ‘target mimicry’. For example, a 23-nt motif in IPS1, At4 and other members in this family has extensive sequence complementarity with miR399, and this leads to substantial inhibition of miR399 activity (36). In contrast, the siRNANERF that originated from the negative strand of NERF has only partial complementarity with miR169. This partial complementarity did not affect the expression of miRNA169 according to the results obtained with 35S::NFYA5 transgenic plants with UTR. Determining the physiological functions and regulation of these siRNAs therefore requires further research.

NFYA5 transcripts accumulated and miR169 expression down-regulated in 35S::NERF, 35S::OR and 35S::ORC transgenic plants, while the opposite were true for amiNERF knock-down mutants, which strongly indicated that siRNANERF interacts with miR169. We propose the following model for the co-functioning of siRNANERF and miR169. Like BACE1-AS in human, siRNANERF originating from OR between NERF and NFYA5 could mask of and/or compete with the NFYA5-targeting miR169 and thereby impair the miR169-guided cleavage of NFYA5, leading to NFYA5 accumulation under drought stress and, thereby to enhance drought tolerance in Arabidopsis. This regulatory model might be applied to other natural antisense transcripts with positively correlated expression patterns.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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