A Novel Non-coding RNA Regulates Drought Stress Tolerance in Arabidopsis thaliana

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In Partial Fulfillment of the Requirements
For the Degree of
Masters of Science

King Abdullah University of Science and Technology, Thuwal,
Kingdom of Saudi Arabia
May 2014
Examination Committee Approvals Form

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Drought (soil water deficit) as a major adverse environmental condition can result in serious reduction in plant growth and crop production. Plants respond and adapt to drought stresses by triggering various signalling pathways leading to physiological, metabolic and developmental changes that may ultimately contribute to enhanced tolerance to the stress. Here, a novel non-coding RNA (ncRNA) involved in plant drought stress tolerance was identified. We showed that increasing the expression of this ncRNA led to enhanced sensitivity during seed germination and seedling growth to the phytohormone abscisic acid. The mutant seedlings are also more sensitive to osmotic stress inhibition of lateral root growth. Consistently, seedlings with enhanced expression of this ncRNA exhibited reduced transpirational water loss and were more drought-tolerant than the wild type. Future analyses of the mechanism for its role in drought tolerance may help us to understand how plant drought tolerance could be further regulated by this novel ncRNA.
ACKNOWLEDGEMENTS

First I want to offer my sincerest gratitude to my supervisor, Dr. Liming Xiong for his useful comments, patience and support through the learning process of this master thesis. Furthermore, I would like to thank Dongjin Kim for introducing me to the topic as well for the support on the way. Also, I like to thank Zhenyu Wang for his help and assistance throughout my laboratory work. Finally, I would like to thank my parents for supporting me throughout my studies and my husband who recently joined me in my life journey but had a remarkable influence on my performance and determination to finish my masters, both by keeping me harmonious and helping me putting pieces together. I will be grateful forever for your love.
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LIST OF ABBREVIATIONS

• ABA, abscisic acid
• bZIP, basic leucine zipper
• ABRE, ABA-responsive element
• ROS, reactive oxygen species
• dsRNA, double-stranded RNA
• ncRNA, non-coding RNAs
• IncRNAs, long non-coding RNAs
• lincRNAs, long intergenic noncoding RNAs
• miRNA, microRNA
• siRNA, small interfering RNA
• ta-siRNAs, trans-acting siRNAs
• TGS, transcriptional gene silencing
• PTGS, post-transcriptional gene silencing
• NFY, nuclear factor Y
• ORF, open reading frame
• IPS1, Induced by phosphate starvation
• NAT, natural antisense transcript
• fSATs, fully overlapping sense–antisense transcripts
• TAIR, Arabidopsis Information Resource
• Col-0, ecotype Columbia
• ABRC, Arabidopsis Biological Resource Centre
• LB, left border
• LP, left primer
• RP, right primer
• PCR, polymerase chain reaction
• M-MLV, Moloney murine leukemia virus
• RT, reverse transcription
• MS, Murashige and Skoog media
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CHAPTER 1

Section 1.1

INTRODUCTION

Drought, high soil salinity, nutrient deprivation, and high or low temperature are the major environmental stresses that plants frequently encounter in nature and that influence their survival, growth and productivity [1]. To tolerate the cellular damage caused by abiotic stresses, plants have evolved diverse but well coordinated mechanisms operating at the transcriptional (induction or suppression of genes), post-transcriptional (transcript processing, modification, and stability) and post-translational (protein modification, stability and degradation) levels [2]. Gene expression studies have demonstrated that there is an altered expression of numerous stress-related genes during drought stress. This results in accumulation of stress-responsive proteins and metabolites such as proline and antioxidants [3, 4]. Moreover, drought stress activates the phytohormone abscisic acid (ABA) biosynthesis genes that dramatically increase the ABA level [5]. ABA activates ABA-responsive element binding proteins/factors (AREB/ABF) transcription factors belonging to the basic leucine zipper (bZIP) family. These transcription factors in turn bind to the ABA-responsive element (ABRE) in the promoter region of many stress-responsive genes and trigger their expression. Accordingly, overexpression of AREB/ABF transcription factors such as AREB1/ABF2 or AREB2/ABF3 resulted in decreased water loss and enhanced drought tolerance [6].

In addition to these protein-encoded genes, there are non-protein encoding genes that may also be involved in plant stress response and stress tolerance. Recent studies revealed that non-coding RNAs (ncRNA) acquire a much larger portion of eukaryote transcriptomes than previously believed [1, 2]. These ncRNAs include small RNAs and long non-coding RNAs (lncRNAs). Small ncRNAs are 18-30 nt single-stranded RNAs of a distinct category of regulatory RNAs found in diverse organisms including plants [7]. Among them, microRNA (miRNA) and small interfering RNA (siRNA) are conserved riboregulators in plants that are distinguished
based on their modes of biogenesis and functions. siRNAs are generated from perfectly double-stranded RNAs, whereas miRNA precursors have a partially double-stranded stem-loop structure. The identification of endogenous small RNAs and several other ncRNAs as stress-responsive regulators in model plants by causing either transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) has contributed to better understanding of the underlying mechanisms of small RNAs in biotic and abiotic stress responses [8, 9].

Early indication for the functions of ncRNAs in plant stress responses appeared from in silico analysis of miRNA and their complementary genes, and cloning of miRNAs from stress-treated Arabidopsis plants, which detected new miRNAs that had not been previously cloned from plants grown in unstressed conditions [10]. Environmental stress causes plants to over- or under-express certain miRNAs or to synthesize new miRNAs to cope with stressful conditions such as drought, cold, salinity, and nutrient deficiency [11]. Micro-RNA expression profiling under drought stress has been carried out in Arabidopsis and several drought-responsive miRNAs were identified. These include, for example, miR168, miR167, miR165, miR171, miR396, miR319, miR159, miR393, miR394, miR156, miR158, and miR169 [12].

The regulation of plant drought stress tolerance by miRNA was demonstrated with the work on miR169 and Nuclear Factory Y (NFY) transcription factor genes in Arabidopsis. A study showed that NFYA5 and several other members of Arabidopsis NFYA family are recognised by the miR169 family of miRNA. Plants exposed to drought stress showed enhanced expression of NFYA5 combined with dramatic decrease in the expression level of miR169a and miR169c. Furthermore, mutant plants (nfya5 knockout and miR169 knockin) were sensitive to drought while NFYA5 knockin transgenic plants were drought-tolerant. The NFYA5 gene was expressed in guard cells as well as other cell types. The suggested function of NFYA5 in guard cells is to control stomatal aperture, while NFYA5 in other cell types possibly contributes to the expression of stress-related genes [6].

The recent application of the genome profiling technology on rice exposed to drought stress at different developmental stages revealed that drought-responsive miRNAs belonged to 30 different miRNA families. Eight families were highly up regulated
(miR474, miR395, miR845, miR854, miR851, miR901, miR1125 and miR903) and 11 were down regulated (miR172, miR170, miR397, miR529, miR408, miR1030, miR896, miR1050 miR1035, miR1126 and miR1088) during the stress. The likely role of these miRNAs in drought tolerance was further suggested by studying regulation of predicted and confirmed complementary target genes to these miRNAs. Plants are exposed to different types of abiotic stresses that could lead to similar adaptation responses. Likewise, different types of stresses have shown to stimulate the synthesis of similar miRNAs, indicating that pathways that are triggered in response to various abiotic stress responses are commonly shared [13].

Although previous studies of stress gene regulation in plants focused on mRNA and miRNA, recently there has been a significant surge of interest to understand another subclass of ncRNAs known as long non-coding RNA (IncRNA). These RNAs are transcribed from introns of protein-coding genes, the opposite DNA strand of coding or non-coding genes, or intergenic regions. This is a large and heterogeneous population of transcripts that vary in length from 200 nt to >100 kb and generally does not contain any long open reading frame (ORF) (e.g., no ORF coding more than 70 amino acids). Regardless of the limited protein coding potential of these genes, it has been established that their RNA moiety plays a critical role in their function, as they seem to interact with proteins to modulate transcription, translation or RNA stability [2, 14]. Some IncRNA may also work together with miRNA to control gene expression. For example, one IncRNA, induced by phosphate starvation1 (IPS1), has a short 23-nt motif highly complementary to miR-399, which is stimulated in response to phosphate starvation. IPS1 base-pairing with miR-399 is interrupted by a mismatched loop at the expected miRNA cleavage site causing IPS1 to be non-cleavable and instead allows IPS1 to inhibit the ability of miR-399 to interact with its mRNA thus increasing the expression of the target genes [15].

Recent advancement in technology such as Tilling array and Next Generation Sequencing has greatly facilitated the identification of novel transcripts including putative IncRNAs in plants and other organisms. However, unlike small RNAs, few studies have reported on the stress-responsive IncRNAs in plants. In 2009, Ben Amor et al. performed a genome-wide bioinformatic analysis of a full-length Arabidopsis cDNA library and identified 76 Arabidopsis ncRNAs including fourteen
ncRNAs that are antisense to protein-coding transcripts suggesting their potential cis-regulatory roles. Analyses of these new ncRNAs revealed that 34 ncRNAs potentially are precursors to small RNAs since numerous 24-nt siRNA matched to various ncRNAs. Additionally, expression analyses of the identified 76 ncRNAs detected a novel ncRNA that accumulates in a dcl1 mutant but does not appear to form trans-acting siRNA or miRNA. Meanwhile, it was found that abiotic stresses altered the expression of 22 ncRNAs. Two ncRNAs were overexpressed in Arabidopsis plants for functional studies and their regulatory roles in root growth during salt stress and leaf differentiation were revealed, respectively. The work demonstrated that at least a subset of these newly identified ncRNAs function in the regulation of differentiation, development and stress adaptation [2].

One group of the lncRNA, long intergenic noncoding RNAs (lincRNAs) are poorly characterized and regulation of their biogenesis remains unclear in plants. Liu et al. analyzed 200 transcriptome libraries of Arabidopsis thaliana and discovered 13,230 intergenic transcripts of which 6480 can be classified as lincRNAs. RNA sequencing analysis revealed that 2708 lincRNAs were expressed, and transcriptome profiling showed that the majority of these lincRNAs are expressed at a modest level between those of mRNAs and miRNAs precursors. Moreover, a group of lincRNA genes showed organ-specific expression, while others were responsive to biotic and/or abiotic stresses [15]. One important subclass of lncRNA is natural antisense transcript (NAT) which is lncRNA generated from the opposite strand of coding or non-coding genes. The latest study on lncRNA (published on March 2014) carried out by Wang et al., aimed to identify NATs and investigate their response to light in Arabidopsis. In the study, a total of 37,238 NAT pairs and 70% of annotated protein coding transcripts to be associated with antisense transcripts in Arabidopsis were identified. Using a custom-designed ATH NAT array, the expression profiles of sense–antisense pairs under dark and light conditions was analyzed. The spatial and developmental-specific light effects on 626 concordant and 766 discordant NAT pairs were observed. This study illustrated a strong correlation between light-responsive NAT pairs and histone acetylation [16]. In another study using Arabidopsis tiling arrays for whole transcriptome studies under drought, cold, high-salinity and ABA treatment conditions, Matsui et al. identified 7000 novel transcriptional units. These include a population of stress-responsive antisense transcripts that are longer than
500 nt and do not have sequence complementary with protein-coding genes. About 80% of them belong to pairs of the fully overlapping sense–antisense transcripts (fSATs). Interestingly, a significant linear correlation between the expression ratios (treated/untreated) of the sense and antisense RNAs was observed in the fSATs. *RD29A* and *CYP707A1* antisense RNA sequences that are drought- and ABA-inducible showed sequence complementary to that of the sense mRNAs, suggesting that expression of the antisense transcripts depends on that of the sense mRNAs [1].

In the current study, we identified a novel ncRNA in *Arabidopsis thaliana* named *NCRD1* (non-coding RNA for drought 1). This gene is annotated in the Arabidopsis Information Resource (TAIR) version 9 as “other RNA”. TAIR annotated 350 transcripts as the “other RNAs,” many of which are derived from intergenic regions. Similar to the group of 76 non-protein-coding RNAs, the 350 “other RNAs” group consists of NATs, potential lincRNAs and small RNA-related transcripts, as well as several transcripts of high protein-coding potential [15]. We obtained an insertional mutant *ncrd1* (referred to as *ncrd1* hereafter for convenience) with increased expression of this ncRNA and analysed the phenotypes. We found that the *ncrd1* plants were more drought-tolerant than the wild type, indicating a role of this novel non-coding RNA in regulating plant drought stress tolerance.

Section 1.2

MATERIALS AND METHODS

1.2.1 Plant materials and growth conditions

*Arabidopsis thaliana* wild type (ecotype Columbia, Col-0) and a homozygous T-DNA insertion mutant *ncrd1* (SAIL_813_G12) were used in this study. Seeds were surface-sterilized with 50% bleach for five minutes followed by three rinses with sterile water and incubated at 4°C for 2 days. Sterilized seeds were then transferred into Petri dishes containing ½ Murashige and Skoog (MS) salts, 0.6% sucrose and 1.2% agar and incubated at 22°C for germination. After seven days, seedlings were
used for stress tolerance assays on plates or transferred to soil (Metro-Mix 360, Sun Gro Horticulture, USA) for growth at 22°C under a 16-h light/8-h dark regime.

1.2.2 Plant genotyping
The T-DNA insertion mutant ncrd1 (SAIL_813_G12) was obtained from the Arabidopsis Biological Resource Centre (ABRC). Homozygous mutant line was selected by PCR-based genotyping using a T-DNA left border-specific primer (LB) and gene-specific primers (LP and RP). The primer sequences are as follow: LB: 5’-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3’, LP: 5’-TCGAGTACCAACCATTTCCTG-3’ and RP: 5’-TGGCTAAAGGGTTGGGTTTAG-3’

1.2.3 Phenotype analysis
To investigate the tolerance of ncrd1 seedlings to various stresses, seedlings were grown in MS-sucrose agar medium for four days and were then transferred to MS agar plates supplemented with different concentrations of mannitol, NaCl or ABA. For root elongation assays, the plates were placed vertically. After one week, seedlings were photographed with a digital camera and the length of the primary root was determined.

1.2.4 Seed germination assay
To evaluate seed germination response to stress or ABA, surface-sterilized seeds of the wild type (Col-0) and mutant (ncrd1) were stratified for three days at 4°C and then planted on the same MS-sucrose agar medium containing various concentrations of ABA, NaCl, mannitol or sorbitol and were then incubated at 22°C with a 16-h-light/8-h-dark cycle. Germination is defined as the obvious appearance of the radicle through the seed coat and it was scored daily for five days and the germination rate was calculated as a percentage of the total number of seeds plated. There were three replicates for each treatment.

1.2.5 Drought stress treatment and measurement of transpiration rate
To investigate the sensitivity of plants to drought stress, Arabidopsis seeds planted onto MS-sucrose medium were allowed to germinate and grow for one week in a growth chamber before the seedlings were transferred to soil and allowed to grow
with sufficient water in a growth room at 22°C under a 16-h light/8-h dark regime for four weeks. Subsequently, irrigation was stopped for two weeks and surviving plants were counted three days after re-watering. To measure the transpiration rate, detached leaves from 4-week-old soil-grown plants of similar developmental stages were placed with abaxial side up on a piece of weighting paper and weighed immediately at designated time points at room temperature. Water loss was calculated by subtracting measured weight at different time intervals from the initial fresh weight and expressed as a percentage of the initial weight. All tests were repeated at least three times.

1.2.6 Analysis of gene expression
The ncrd1 and Col-0 seedlings were grown on the same MS agar plate for one week before being treated with ABA, Salt (NaCl) and drought. ABA treatment was carried out by spraying the plants with 50 µM ABA solution and incubated for three hours. For salt-stress treatment, plants were immersed in 130 mM NaCl solution for three hours. For drought-stress treatment, seedlings were spread out on a Petri dish and weighted. Afterward, the plates were left exposed to air and plant weight was measured frequently till around 6% of its weight was lost. Upon ending of the stress treatment, seedlings were immediately frozen in liquid nitrogen prior to RNA extraction and expression analysis.

1.2.7 PCR amplification
The standard reaction for the polymerase chain reaction (PCR) was prepared using the following: 34 µl of 10 x buffer, 10.2 µl Mg²⁺, 0.85 µl Taq DNA polymerase, 1 µl template and 17 µl dNTP in 50 µl volume. PCR thermal cycling conditions were: 4 min at 94°C; 30 cycles of 30s at 94°C, 30 s at 55-62°C, and 0.5-1.5 min at 72°C; and finally 7 min at 72°C.

1.2.8 Reverse-Transcription PCR (RT-PCR) Amplification
The expression of the target ncRNA in the mutant (ncrd1) was examined by semi-quantitative RT-PCR. To extract RNA, 200 mg of frozen tissues were finely ground using microbeads in liquid nitrogen. Total RNA was isolated using Trizol solution (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was generated using 5 mg of DNase-treated total RNA and the Moloney murine leukemia
virus (M-MLV) reverse transcriptase (Promega, USA). The housekeeping gene ACTIN2 was used as an internal control. The following cycling conditions were used for the PCR reactions: 5 min at 94°C; and 25 or 27 cycles of 30s at 94°C, 30s at 52°C, and 20s at 72°C. PCR primers used to amplify the NCRD1 cDNAs were: F: 5'-ATGAGCAGGGAGTGTGTTCCC-3' and R: 5'-CTACCGGAGCGTAGACTGCA-3'. The RT-PCR product was analyzed by gel electrophoresis.

1.2.9 Real-Time PCR analysis
To evaluate the expression level of the NCRD1 gene, quantitative real-time PCR analysis was performed. RNA extraction and reverse transcription (RT) were performed as described above for RT-PCR. PCR amplification was performed with gene-specific primers as follows: F: 5'-AAACAAGGGAGTGTGTTCCC-3' and R: 5'-CTACCGGAGCGTAGACTGCA-3'. Amplification of the cDNA was carried out using the Real Master Mix kit (SYBR Green) employing the following protocol: denaturation 95°C/10 min, 45 cycles of 95°C/10 s and 60°C/1 min. The reference gene ACTIN2 was used as an internal control and for data normalization.
CHAPTER 2

Section 2.1

RESULTS

2.1.1 Isolation of the _ncrd1_ mutant

To identify non-coding RNA potentially involved in plant stress response and tolerance, we conducted a large-scale stress-treated transcriptome analysis in Arabidopsis. Briefly, Arabidopsis seedlings were treated with salt, ABA, or drought stress and the transcriptomes were sequenced using the Illumina High-seq 2000 platform. The transcriptomes were then analysed and potential ncRNA that are regulated by stresses were identified (our unpublished data). One of the candidates, At1g21529, which we subsequently named as _NCRD1_, was chosen for further study.

The genomic DNA of this gene is 755 bp long located on Chromosome 1 and was annotated in TAIR (version 9) as encoded “other RNA”. The other RNAs are a subset of RNAs (around 350 transcripts) transcribed from intergenic regions comprising lincRNAs, NATs, small RNA-related transcripts, and transcripts of high protein-coding potential [15]. This RNA molecule does not have an open reading frame and seems not to encode any peptide and thus is a non-coding RNA.

To understand whether this ncRNA has any role in plant stress response, we obtained a putative T-DNA insertion mutant, SAIL_813_G12, from the Arabidopsis Biological Resource Center (ABRC). We first conducted genotyping to confirm the insertion and also to isolate the homozygous line. The predicted T-DNA insertion in this line occurred between two genes (promoter of At1g21528 and 3’URT of At1g21529) (Figure 1A). Genotyping using the LP and RP primer pair could amplify the predicted fragment from the wild type but not the mutant. In contrast, using the T-DNA border sequence primer LB together with genomic DNA primer RP could amplify a single fragment from the mutant. This confirms the T-DNA insertion and we were able to obtain homozygous insertion of the T-DNA (Figure 1B).
To determine whether the insertion had any impact on the expression of either gene, we conducted qRT-PCR analyses of the expression of both genes in the mutant. While the insertion did not have any obvious impact on the expression of At1g21528, it dramatically increased the expression of At1g21529. qRT-PCR assay revealed that the expression level of At1g21529 in the mutant was nearly 40 times higher than that in the wild type seedlings (Figure 1C). Thus, the mutant overexpresses the NCRD1 gene. This homozygous T-DNA insertion mutant was used in all subsequent experiments.

Figure 1: The T-DNA insertion in the ncrd1 mutant enhances the expression of the NCRD1 gene. (A) Position of the T-DNA insertion. The T-DNA was located between two genes (promoter of At1g21528 and 3' URT of At1g21529). The position and direction (5' to 3') of the gene-specific PCR primers (RP and LP) used for PCR amplification and genotyping are shown by arrows. (B) PCR analysis of homozygous
mutant (ncrd1) and wild-type (Col-0) plants. (C) The At1g21529 transcript level was quantified by qRT-PCR relative to ACTIN2, which was used as an internal control. Asterisks indicate significant differences from the corresponding control value determined by Student’s t test (* P= 0.01).

2.1.2 Morphology of the ncrd1 mutant
Arabidopsis rosette leaf consists of a proximal petiole and a distal blade. Wild type (Col-0) Arabidopsis leaves are characterized by an ovate blade attached to the main plant stem by a thin petiole. In contrast to Col-0, leaves of four-week-old ncrd1 seedlings are slightly smaller particularly under short-day growth conditions. Furthermore, ncrd1 mutant leaves have a rounded blade shape and the mutant flowered later than the wild type (Figure 2). Nonetheless, there was no other obvious difference between ncrd1 and the wild type in phenotypic characteristics.
2.1.3 The germination of *ncrd1* seeds is hypersensitive to ABA and osmotic stress

The transition from a dormant seed to a young seedling (i.e., seed germination) is a critical stage of a plant's life that is particularly sensitive to environmental stress. To investigate the potential role of NCRD1 in plant stress response, we first checked the response of seed germination of *ncrd1* to ABA. Seeds of the wild type and *ncrd1* mutant were sown on media containing various concentrations of ABA and seed germination (obvious radicle emergence) was scored beginning from the 3 d after imbibition and continued for one week. While in the absence of ABA, there was no significant difference in seed germination rate of different genotypes (Figure 3A). *ncrd1* seeds were more sensitive than the wild type to the inhibition of seed germination by ABA. As shown in Figure 3B, in the presence of 0.5 µM ABA, *ncrd1* exhibited significantly reduced seed germination percentage than the wild type. At Day 7, wild type seeds reached 100% germination while mutant seeds only had 51% germination.

In addition to ABA, germination of *ncrd1* seeds was also delayed by salinity and osmotic stresses (generated by adding mannitol and sorbitol). NaCl (200 mM) inhibited germination of *ncrd1* seeds significantly as only 20% of *ncrd1* seeds germinated after one week whereas 80% of wild type seeds germinated under the same conditions (Figure 3C). Mutant seeds grown on mannitol (200mM) containing media displayed slower germination rate and were not fully germinated whereas wild type reached 100% germination on Day 7 (Figure 3D). Similarly, *ncrd1* seeds were more sensitive to various concentrations of sorbitol and showed significant delay of germination compared to those of Col-0 (Figure 3E and data not shown).
Figure 3: Sensitivity of seed germination to ABA and stress treatments. Germination rates of ncrd1 and wild-type seeds in the absence (A) or presence of (B) 0.5 µM ABA, (C) 200 mM NaCl, (D) 200 mM Mannitol or (E) 200 mM Sorbitol. The percentage of germinated seeds was determined daily for 7 days. The values are means ± SE of experiments performed at least in triplicate (n ≥ 100 each).
2.1.4 Abiotic stress responses of the *ncrd1* mutant

To reveal the possible role of *NCRD1* in the regulation of plant responses to various environmental stresses, we performed seedling growth assays under different stress treatments. In addition to inhibiting seed germination, high concentrations of ABA also suppress shoot and root growth. To examine the possible role of *ncrd1* in ABA inhibition of seedling growth, 4-days-old seedlings grown on MS medium were transferred to media containing different concentrations of ABA (0, 10, 20, 50, and 100 μM) and grown for 10 days before root growth was assessed. In the absence of ABA, seedling growth of wild type and *ncrd1* was largely similar (Figure 4A). In the presence of different concentrations of ABA, primary roots of the *ncrd1* plants were shorter than those of the wild type (Figures 4A and 4B).

When treated with NaCl, it was found that wild type and mutant seedlings were equally sensitive to high salt concentrations (Figure 4A). In the presence of hyperosmotic stress generated by using polyols such as mannitol and sorbitol, *ncrd1* seedlings showed no difference in primary root length compared to Col-0. However, we noticed that *ncrd1* seedlings can develop a significant amount of lateral roots within one week under control condition whereas, those treated with mannitol (at 200 mM) did not develop or were delayed in lateral root development, indicating increased sensitivity of lateral root growth to osmotic stress inhibition in the mutant (Figure 4A).
Figure 4: Sensitivity of *ncrd1* seedlings to ABA and osmotic stress. (A) Morphology of seedlings growing on vertical plates for 2 weeks in the presence of 2.5µM ABA, 100 mM NaCl or 200 mM mannitol. (B) Relative primary root growth (root length) of the wild type and *ncrd1* on MS medium supplemented with various concentrations of ABA. Primary root length on MS medium without ABA was taken as 100%.

2.1.5 Drought tolerance of *ncrd1*
The noticeable difference between the *ncrd1* mutant and the wild type in their sensitivity to ABA in seedling growth and seed germination assays motivated us to further investigate whether *ncrd1* is more drought-resistant than the wild type. The *ncrd1* mutant and wild-type plants were grown in pots under water-sufficient conditions. After 4 weeks, the plants were subjected to drought stress by withholding water. After 2 weeks of drought treatment, Col-0 leaves showed clear wilting signs, but *ncrd1* leaves remained turgid (Figure 5A). The number of survived plants 3 days after re-watering in short and long day conditions were recorded (Figure 5B). Because the *ncrd1* mutants were slightly smaller than the wild-type plants, and since leaf surface area affects the rate of transpiration, we thus also directly measured the transpiration rate from detached leaves. The rate of water loss was slower from detached *ncrd1* leaves than from detached wild-type leaves (Figure 5C), which is consistent with the drought-tolerant phenotypes of *ncrd1* plants growing in soil.
Figure 5: *ncrd1* seedlings are drought-tolerant and have reduced transpiration water loss. (A) Drought tolerance of the wild-type and *ncrd1* plants grown under short day (left panels) or long day conditions (right panels). Four-week-old plants grown in soil were subjected to drought stress by stopping irrigation for 2 weeks. (B) Survival rates of plants tested in (A) 3 days after re-watering. Average survival rates (%) were calculated by counting surviving plants in three independent experiments (n = 20 each). (C) Transpiration water loss rates of wild-type and *ncrd1* detached leaves. Leaves at similar developmental stages were excised and weighed over a period of the indicated time. The values are means ±SE of experiments performed in triplicate (n=5 each).

Section 2.2

**DISCUSSION**

Water with its biological roles as transport medium and solvent, as electron donor in the Hill reaction, and as evaporative coolant is often in deficit in plants under various adverse environmental conditions. Although plant species differ in their sensitivity and response to the decrease in water content caused by drought, high salinity, or low temperature, it is believed that all plants have encoded capability for stress perception, signaling, and response. First, majority of cultivated species have wild relatives that display greater tolerance to abiotic stresses. Second, biochemical studies have demonstrated similarities in processes triggered by stress that resulted in accumulation of certain metabolites in vascular and nonvascular plants, fungi, algae and bacteria. These metabolites include nitrogen-containing compounds and hydroxyl compounds. Third, molecular studies have shown that a wide range of species express a common gene pool and similar proteins when stressed. Although functions for many of these gene products have not yet been definitely assigned, it is likely, based on their characteristics, that these proteins play active roles in the response to stress [17].

In 1982, Boyer indicated that abiotic stress may limit crop production by as much as 70% [18]. Learning about the biochemical and molecular mechanisms by which plants tolerate abiotic stresses is essential for genetic engineering to improving crop
performance under stress. A diverse range of genes with different functions controls the responses of plants to water deficit conditions. As water content is reduced in the cell, regulatory processes are initiated that adjust cellular metabolism to the new cellular conditions. Concurrently, growth inhibition and alterations of development may occur. One important consequence of cellular dehydration is the accumulation of the phytohormone ABA, which is a key regulator of stomatal movement and many other stress responses including the activation of ABA-responsive genes. The products of these genes may collectively enhance plant tolerance to drought and other stresses.

In addition to protein-encoding genes, non-protein encoding genes may play important roles in plant growth and development as well as response to environmental stresses. Non-coding RNA-mediated gene regulation is an ancient and conserved mechanism. Although there are extensive studies in various organisms for the functions of ncRNAs, their modes of action still remain obscure except for small RNAs whose biogenesis and functions are now well documented [19, 20]. In this study, we investigated the possible function of a novel gene that encodes a ncRNA, in abiotic stress tolerance particularly drought stress tolerance. We obtained a T-DNA insertionally mutant named ncrd1 that has an enhanced expression of the NCRD1 transcript. The mutant has increased sensitivity to ABA during seed germination and in root growth. Consistent with the increased sensitivity to ABA, ncrd1 seedlings had reduced water loss in detached leaves and also increased drought tolerance. These results suggest that this ncRNA may regulate drought stress responses through an ABA-dependent signalling pathway. We noticed as well increased sensitivity of ncrd1 seedlings to osmotic stress generated by using mannitol. Unlike in control plate, ncrd1 seedlings displayed reduced number of lateral roots under the mannitol treatment (200 mM). This reduced lateral growth under osmotic stress is an adaptive response and correlates with increased tolerance of the entire plant to drought stress as demonstrated in genetic studies [21].

Another process affected by this ncRNA is flowering time. Plant flowering time is crucial for adaptation to the environment, determination of reproductive success, and also has a great impact on crop production. ncrd1 plants flowered later than the wild type plants. Due to time limitation, I was only able to perform an initial investigation
for this gene; however, further physiological and genetic studies including guard cell
ABA responses, transcriptome analyses under various abiotic stresses as well as
complementation assays are required in order to confirm the role of this ncRNA in
drought tolerance and reveal the underlying mechanisms.

Section 2.3

CONCLUSION

In summary, this work characterizes a T-DNA insertion mutant with increased
expression of a novel ncRNA that has not been characterized previously. Compared
with the wild type, the insertional mutant is ABA sensitive during seed germination
and early seedling growth. Seedlings of the ncrd1 mutant were also drought-tolerant
and were able to survive for a longer period of time than wild type under drought
stress conditions. Due to lack of time, I was not able to extensively define and study
the expression profiles of this gene under various abiotic stress conditions, however
this is a groundwork that provokes more investigation to reveal the molecular
mechanisms of its roles in abiotic stress tolerance.
REFERENCES


