

**PHYSIOLOGICAL AND MOLECULAR DISSECTION OF SALINITY TOLERANCE IN
ARABIDOPSIS AND MAIZE AND NITROGEN UPTAKE IN WHEAT**

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Physiological and Molecular Dissection of Salinity Tolerance in Arabidopsis and Maize and Nitrogen Uptake in Wheat

Suman Lamichhane

Abstract (Academic)

The PROTEOLYSIS 6 (PRT6) branch of the N-end rule pathway is a well-characterized negative regulator of flooding and low oxygen tolerance in plants. This study investigated the role of this pathway in adaptation to salinity stress in Arabidopsis and maize via physiological and molecular characterization of Arabidopsis *prt6-1* and maize *prt6* MU insertion mutants, respectively. Our study demonstrated that the loss of function mutation of *prt6* in Arabidopsis activated hormonal and transcriptional responses associated with adaptation to salinity stress, enhancing high salt tolerance at seed germination, seedling, and adult plant stages. Our data also indicated that salinity tolerance conferred by the *prt6* mutation is attributed to increased mRNA abundance of key transcriptional factors in ABA-dependent (AREB/ABFs) and independent (DREBs) pathways, together with the dominant expression of downstream dehydrins. Furthermore, this study revealed that the *prt6* mutation enhances ethylene and brassinosteroid responses, resulting in restricted Na⁺ accumulation in roots and shoots as well as increased expression of dehydrin genes such as *RD29A* and *RD29B*. Maize *prt6* mutant plants, contrary to our observation in Arabidopsis, showed lower seed germination, primary root elongation, and shoot biomass growth along with increased malondialdehyde (MDA) accumulation under high salt. Moreover, maize *prt6* mutants exhibited reduced grain yield and yield-related components under high salt. These results indicate that *PRT6* functions as a negative regulator for salinity tolerance in Arabidopsis, whereas this gene plays a

positive role in salinity tolerance in maize. In wheat, we compared two genotypes with contrasting nitrogen-use-efficiency (NUE), VA08MAS-369 and VA07W-415, to dissect physiological and molecular mechanisms underlying NUE regulation. Our agronomic data revealed that line 369 maintained yield and yield-related parameters and exhibited greater NUE indexes relative to line 415 under N deficient conditions. Furthermore, our analyses suggested that the significantly higher nitrogen use efficiency (NUE) in line 369 could be attributed to the greater N uptake efficiency in this genotype. In fact, line 369 was able to maintain the development of root systems under N limitation. Consistently, genes encoding high-affinity nitrate transporters such as *TaNRT2.1* and *TaNRT2.2* were expressed more abundantly in the roots of line 369 than line 415 at limited N. Overall, the results of this study characterized physiological and molecular phenotypes associated with high N uptake efficiency in line 369. This is useful information for the development of new wheat accessions with improved NUE.

Physiological and Molecular Dissection of Salinity Tolerance in Arabidopsis and Maize and Nitrogen Uptake in Wheat

Suman Lamichhane

Abstract (General Audience)

In coastal areas, sea-level rise increases the chances of saltwater intrusion into cultivable lands, making a hostile environment for crop growth and production by imposing flooding and salinity stresses simultaneously. Identification of central regulators that regulate the adaptation to both flooding and salinity is a critical step for the development of new crop genotypes with enhanced tolerance to these stresses. Previous studies have characterized the function of the *PROTEOLYSIS 6 (PRT6)* gene in adaptation to flooding stress in plants. This study assessed whether this gene is involved in adaptation to salinity stress in Arabidopsis and maize by evaluating the growth and survival of their respective *prt6* mutants under high salt. Consistent with the flooding tolerance data, our study showed that the *PRT6* gene also functions as a negative regulator of salinity stress tolerance in Arabidopsis. The *prt6* mutation in Arabidopsis activated the key transcriptional and hormone response pathways associated with adaptation to both salinity/osmotic stress and sodium toxicity, expressed as enhanced tolerance to excess salt at seed germination, seedling, and adult plant stages. In maize, disruption of the *PRT6* gene decreased seed germination, primary root elongation, and shoot biomass growth under high salt, which is opposite to our observations in Arabidopsis. Additionally, the maize mutant plants encountered more oxidative stress, as demonstrated by the higher accumulation of malondialdehyde (MDA) under high salt. Moreover, maize *prt6* mutants exhibited reduced grain yield under high salt. Overall, these results indicate

that disruption of the *PRT6* gene confers increased tolerance to high salt in *Arabidopsis*, whereas it conversely reduced salinity tolerance in maize. In wheat, we compared two genotypes with distinct nitrogen use efficiency (NUE), VA08MAS-369 and VA07W-415, to determine critical traits involved in NUE regulation. Our study showed that grain yield and yield-related parameters were significantly higher in line 369 than line 415 under low N. Moreover, high NUE in line 369 was attributed to efficient N uptake in this genotype under limited N. Our root architecture analysis demonstrated that line 369 was able to maintain root depth, volume, and thickness even under N limitation. Consistently, line 369 highly induced expression of genes associated with nitrogen transport at low N. Altogether, this study identified key traits involved in high NUE in wheat, facilitating the breeding of new wheat genotypes with enhanced NUE.

Dedicated to all the farmers around the world

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ATTRIBUTIONS

CHAPTER I: The Central Negative Regulator of Flooding Tolerance, the PROTEOLYSIS 6 Branch of the N-end Rule Pathway Adversely Modulates Salinity Tolerance in Arabidopsis

Takeshi Fukao is an Assistant Professor of Crop Stress Physiology and Genomics in the School of Plant and Environmental Sciences at Virginia Tech. He is the principal investigator of this project.

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CHAPTER II: The Role of an N-end Rule Pathway E3 Ligase, *PROTEOLYSIS6*, in Salinity Tolerance in Maize

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CHAPTER III: Time-course Analysis of Physiological and Molecular Traits Associated with Efficient Nitrogen Uptake and Transport in Wheat

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CHAPTER I

The Central Negative Regulator of Flooding Tolerance, the PROTEOLYSIS 6 Branch of the N-end Rule Pathway, Adversely Modulates Salinity Tolerance in Arabidopsis

ABSTRACT

Seawater intrusion in coastal regions and waterlogging in salinized lands are serious constraints that reduce crop productivity and quality under changing climates. Under these conditions, plants encounter flooding and salinity concurrently or sequentially. Identification and characterization of genes and pathways associated with both flooding and salinity adaptation are critical steps for the simultaneous improvement of plant tolerance to these stresses. The PROTEOLYSIS 6 (PRT6) branch of the N-end rule pathway is a well-characterized process that negatively regulates flooding tolerance in plants. Here, we determined the role of the PRT6/N-end rule pathway in salinity tolerance through the physiological and molecular characterization of the Arabidopsis *prt6* mutant. We demonstrated that the *prt6* mutation enhances biomass growth and plant viability under sublethal and lethal salinity, respectively, at the germination, seedling, and adult plant stages. Maintained chlorophyll content and root growth under high salt in the *prt6* mutant were linked with the restricted accumulation of Na⁺ in shoots and roots of the mutant genotype. The *prt6* mutation also stimulated mRNA accumulation of key transcription factors in ABA-dependent and independent pathways of osmotic/salinity tolerance, accompanied by the prominent expression of their downstream genes such as dehydrins. Furthermore, the *prt6* mutant displayed increased sensitivity to ethylene and brassinosteroids, which suppress root Na⁺ uptake and transport and promote expression of dehydrin genes under salinity. Altogether, the *prt6* mutation contributed to proper modulation of transcriptional, hormonal and physiological adaptations to excess salt. This

study provides genetic evidence that both salinity and flooding tolerance is coordinated through a common regulatory pathway in *Arabidopsis*.

Keywords

Arabidopsis thaliana, salinity, PRT6, N-end rule, ethylene, brassinosteroids

INTRODUCTION

Most commercially important plants are susceptible to high salt. Therefore, soil salinization is a serious constraint that threatens food security worldwide. Currently, more than 6% of the total land surface and 20% of the total irrigated areas are affected by excess salt, a condition that is increasingly widespread (Munns and Tester 2008). To meet growing demands for plant-based products at the global level, it is imperative to improve salinity tolerance in major crops.

Plants encounter multiple abiotic stresses simultaneously or sequentially in an agricultural or natural environment. In the context of salinity stress, plants can be exposed to excess salt and water in consequence of flooding in salinized areas and seawater intrusion in coastal regions. Importantly, much of the world's saline land is subjected to flooding because of shallow water tables and low infiltration of surface water (Barrett-Lennard 2003). It has been predicted that sea-level rise and elevated frequency of storm surge will increase seawater inundation in low-lying grasslands and croplands, leading to serious agricultural losses in coastal zones (Chen et al. 2011, Hoggart et al. 2014, Hanley et al. 2019). For these reasons, there is an urgent need for the development of new crop varieties with enhanced tolerance to both salinity and flooding.

Identification and functional characterization of genes and pathways associated with adaptation to both salinity and flooding are critical steps for the simultaneous improvement of

plant tolerance to these stresses. In this study, we evaluated whether a well-characterized signaling pathway involved in flooding tolerance, the PROTEOLYSIS 6 (PRT6) branch of the N-end rule pathway, regulates adaptation to high salt. The N-end rule pathway is an enzymatic cascade that determines the half-life of protein substrates depending on the identity of their N-terminal residues (Varshavsky 2011, Dissmeyer 2019). In the PRT6 branch of the N-end rule pathway, PRT6, an E3 ubiquitin ligase, is the last enzyme that recognizes the specific N-terminal residues of its substrates, which are subsequently ubiquitinated and transported into the 26S proteasome for degradation (Gibbs et al. 2014a).

Diverse biological roles of the PRT6/N-end rule pathway have been revealed through genetic analysis of loss-of-function mutants of *PRT6* and other enzymes of this pathway. Such roles include the regulation of seed dormancy and germination, seedling establishment, leaf and root development, photomorphogenesis, leaf senescence, and disease resistance as well as flooding / low oxygen tolerance (Yoshida et al. 2002, Graciet et al. 2009, Holman et al. 2009, Gibbs et al. 2011, Licausi et al. 2011, Gibbs et al. 2014b, Weits et al. 2014, Abbas et al. 2015, Riber et al. 2015, Gravot et al. 2016, Zhang et al. 2018, Vicente et al. 2019). Genetic evidence obtained from these studies suggests that proper modulation of regulatory protein levels *via* the PRT6/N-end rule coordinates these traits. Nevertheless, only a limited number of the PRT6/N-end rule substrates have been identified; the most characterized ones are the group VII Ethylene Response Factor (ERF-VII) transcription factors.

The Arabidopsis genome encodes five *ERF-VII* genes, all of which play a pivotal role in the expression of core hypoxia-responsive genes and tolerance to flooding and low oxygen (Bailey-Serres et al. 2012, Tamang and Fukao 2015). The half-life of these transcription factor proteins is controlled by sequential reactions in the PRT6/N-end rule pathway (Appendix I:

Supplementary Figure 1). All ERF-VIIs contain a highly conserved N-terminal motif initiating with methionine (Met) and cysteine (Cys) (Gibbs et al. 2011, Licausi et al. 2011). First, the N-terminal Met is cleaved by methionine aminopeptidase. Next, the exposed Cys is oxidized by plant cysteine oxidases in an oxygen and nitric oxide-dependent manner (Gibbs et al. 2014b, Weits et al. 2014). The oxidized Cys is then arginylated by arginyl-tRNA transferases. Finally, the N-terminally modified ERF-VII proteins are ubiquitinated by PRT6, which are targeted to proteasomal degradation (Gibbs et al. 2014a). Due to the oxygen requirement for PCOs, low oxygen and flooding allow ERF-VII proteins to escape from this proteolysis pathway, thereby triggering the expression of hypoxia-responsive genes. Consistently, disruption of the PRT6/N-end rule pathway in *ate1ate2* and *prt6* mutants promotes transcript accumulation of core hypoxia-responsive genes and confers tolerance to oxygen deprivation and submergence (Gibbs et al. 2011, Weits et al. 2014). It is predicted that ERF-VIIs are not only PRT6/N-end rule substrates because phenotypes of PRT6/N-end rule mutants are highly pleiotropic, and Arabidopsis contains over 200 proteins initiating Met and Cys at the N-terminus (Gibbs et al. 2016). In fact, a polycomb repressive complex 2 subunit, VERNALIZATION 2 (VRN2), was recently identified as a new N-end rule target in Arabidopsis, which is accumulated under submergence and associated with hypoxia and waterlogging tolerance (Gibbs et al. 2018).

In the present study, we assessed the role of the PRT6/N-end rule pathway, a key regulatory process of flooding tolerance, in adaptation to salinity stress in Arabidopsis through physiological and molecular characterization of the *prt6-1* mutant. Recently, it was reported that the *prt6* mutation enhances seedling survival under high salt (Vicente et al. 2017). However, the influence of the mutated gene in salt tolerance at other developmental stages and its adaptation mechanisms remain obscure. Here, we investigated the contribution of the *prt6* mutation to salinity tolerance

and relevant traits in *Arabidopsis* at various growth stages. Detailed time-course and dose-response analyses uncovered a negative role of the PRT6/N-end rule pathway in transcriptional, hormonal, and physiological adaptations to excess salt. This study provides genetic evidence that both flooding and salinity tolerance is coordinated through a common regulatory pathway in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana* L.) Columbia-0 [wild-type (WT)] and its T-DNA insertion mutant, *prt6-1*, were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University. The *prt6-1* knockout mutant has been described previously (Garzon et al. 2007, Graciet et al. 2009, Holman et al. 2009, Gibbs et al. 2011). These genotypes were propagated simultaneously under the same growth conditions (23 °C, 50% relative humidity, 16 h day/8 h dark, 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Seeds were sterilized with 2% (w/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 12 min and then rinsed thoroughly using deionized water. The sterilized seeds were immersed in water at 4 °C for 4 d for synchronized germination.

Salinity stress and hormone treatments

All stress and hormone treatments were replicated in at least three independent biological experiments under the growth conditions mentioned above. For germination tests, seeds were incubated on half-strength MS media containing 0, 50, 100 or 150 mM NaCl for up to 5 d. Seedlings with more than 1 mm roots were counted as germinated. For root growth photos, 4-d-

old seedlings were transferred on half-strength MS media containing 0 or 150 mM NaCl and grown upside down for 4 d. For root growth measurements, the root tip of the main root in each seedling was marked after transplanting of 4-d-old seedlings onto salt-containing media (0-150 mM NaCl). Following 4 d of vertical incubation, the length of root growth was measured. For seedling viability tests, 4-d-old seedling grown under non-stress conditions were transferred into half-strength MS media containing 0 or 200 mM NaCl and incubated for 4 d. For adult plant viability tests, 4-d-old seedlings were grown in soil-containing pots for 21 d. These pots were placed in a tray containing 0 or 200 mM NaCl for 12 d.

Hormone treatments were performed by transferring 10-d-old seedlings on half-strength MS media supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC; 0, 1 or 100 μ M) or 24-epibrassinolide (eBL; 0, 0.01 or 1 μ M in 0.02% (v/v) ethanol) and incubating them for 6 h.

Chlorophyll and anthocyanin assays

Chlorophyll and anthocyanin contents were determined using the methods of Porra (2002) and Rabino and Mancinelli (1986), respectively. Chlorophyll was extracted from 50 mg of homogenized tissues in 3 ml of 100% methanol on ice. Following centrifugation at 4 °C for 20 min at 21,000 g, the absorbance of the supernatant was measured at 652.0 and 665.2 nm with a UV-Vis spectrophotometer. Anthocyanin was extracted from 200 mg of homogenized tissues by shaking the tissues in acidic methanol extraction buffer [1% (v/v) HCl] for 16 h at 4 °C. After extraction, 0.4 mL of water and 0.4 mL of chloroform were added. Following centrifugation at 4 °C for 2 min at 21,000 g, the absorbance of the supernatant was measured at 530 and 657 nm.

Na⁺ and K⁺ content analysis

Sodium and potassium ion concentrations were quantified using the method of Rus et al. (2001). Four-day-old seedlings were grown in pots containing Turface MVP (Turface Athletics, Buffalo Grove, IL, USA) for 21 d. These pots were placed in a tray containing 0 or 200 mM NaCl, and shoot and root tissues were harvested on the specified days. After drying at 65 °C for 2 d, tissues (100 mg) were homogenized in 20 mL of 0.1 N HNO₃ for 30 min. Following filtration with quantitative analysis-grade filter paper, the extract was subjected to Na⁺ and K⁺ analysis using an inductively coupled plasma atomic emission spectrometer (ICP-AES).

Hormone sensitivity evaluation

For ethylene response analysis, seeds were incubated on half-strength MS media containing 0, 1, 10 or 100 µM ACC in the dark for 5 d. For brassinosteroid response analysis, seeds were grown on half-strength MS media containing 0, 0.01, 0.1 or 1 µM eBL in the light (120 µmol m⁻² s⁻¹) for 5 d. After incubation, hypocotyl and root length was measured using Image J software (Schneider et al. 2012).

Quantitative RT-PCR analysis

Total RNA was extracted from 100 mg of seedling tissue using RNeasy Plant Mini kit (Qiagen, Hilden, Germany). Genomic DNA was eliminated by the on-column digestion method described by the manufacturer's protocol. cDNA was synthesized from 2 µg of total RNA as described by Fukao et al. (2006). Real-time RT-PCR was conducted in a 15 µL reaction using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in the CFX Connect real-time PCR

detection system (Bio-Rad). Amplification specificity was validated by melt-curve analysis at the end of each PCR experiment. Relative transcript abundance was determined using the comparative cycle threshold method (Livak and Schmittgen 2001). Primer sequences used for this analysis are listed in Supplementary Table S1. *UBQ10* (AT4G05320) and *18s RNA* (AT2G01010) genes were used as internal controls. qRT-PCR was repeated three times using tissues generated from three independent plant culture under identical growth conditions.

Statistical analyses

Statistical analyses were performed using JMP Pro 14 (SAS Institute) and MS-Excel 2019 (Microsoft).

RESULTS

The *prt6* mutation enhances salinity tolerance at various developmental stages

To determine the role of the *prt6* mutant in salinity tolerance at different growth stages, we performed various stress tolerance evaluations. First, we assessed seed germination performance under moderate (sub-lethal) salinity (≤ 150 mM NaCl) (Figure 1A). Salinity stress reduced seed germination of both wild-type (WT) and mutant genotypes in a dose-dependent manner, but *prt6* seeds germinated more vigorously at 100 and 150 mM NaCl. Consistently, the time-course observation demonstrated that *prt6* seeds germinated more actively than WT on day 3, 4, and 5 (Figure 1B).

Next, we determined the ability of the *prt6* mutant in root elongation under moderate salinity at the seedling stage. In root-bending assays, WT and *prt6* seedlings displayed similar root

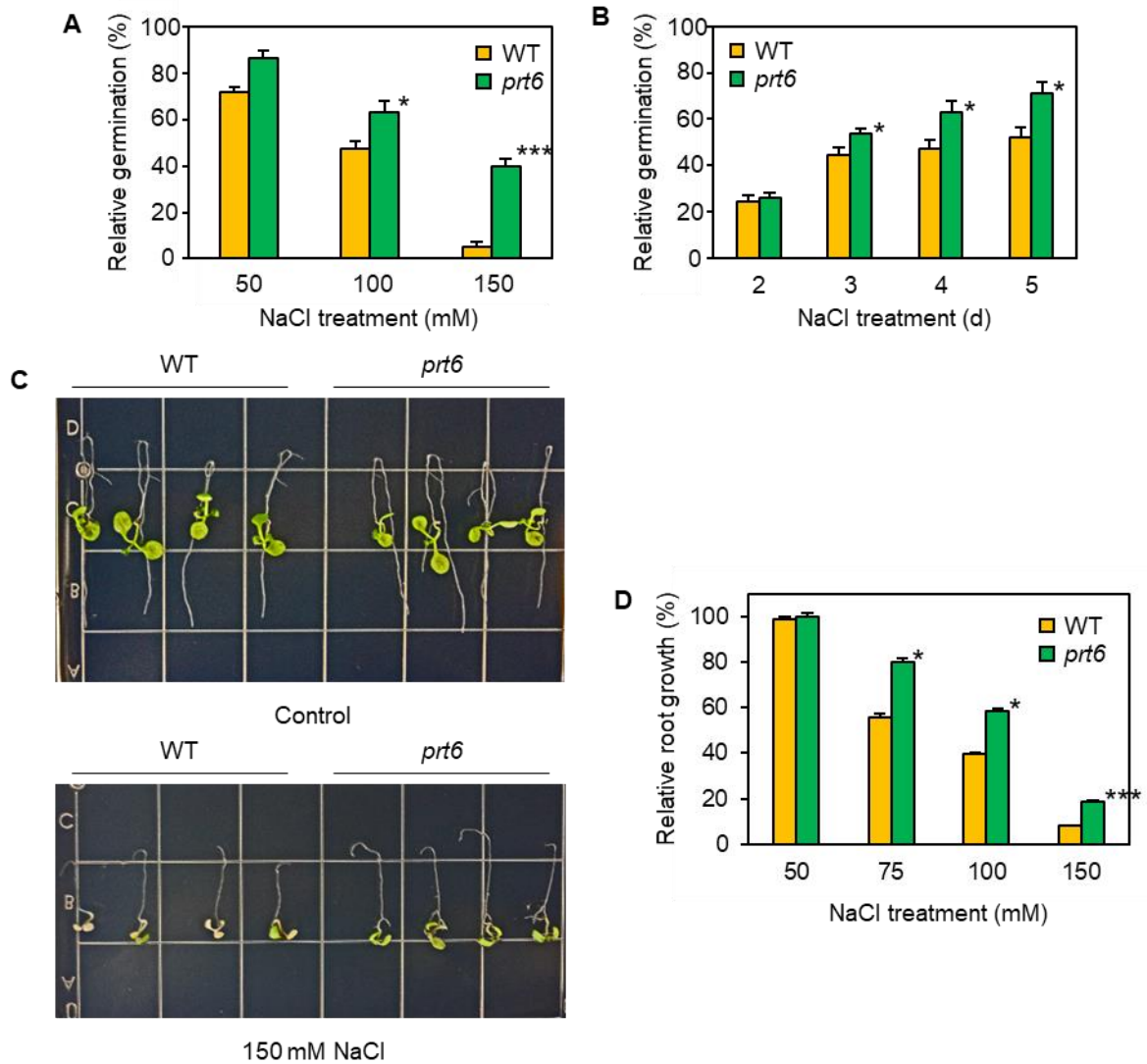


Figure 1. Loss-of-function mutation of *prt6* enhanced tolerance to moderate (sublethal) salinity at the seed germination and seedling stages. (A) Dose-response of wild-type (WT) and *prt6* seeds to NaCl during seed germination. Seeds were incubated under sublethal levels of NaCl (≤ 150 mM) for 4 d. (B) Time-course observation of seed germination in WT and *prt6* seeds under sublethal salinity. Seeds were incubated under 100 mM NaCl for up to 5 d. (C) Photos of WT and *prt6* seedlings that were grown upside down under non-stress or moderate salinity. Four-day-old seedlings grown under non-stress conditions were transferred on half-strength MS plates containing 0 or 150 mM NaCl and incubated upside down for 4 d. (D) Dose-response of WT and *prt6* root growth to moderate salinity. Four-day-old seedlings were grown vertically on half-strength MS plates containing a range of NaCl concentrations for 4 d. Relative germination and root growth in (A), (B), and (D) were calculated by comparison to non-stressed WT or *prt6*. Data represent means $\pm$ SE [$n = 40 \times 3$ replicates in (A, B); $n = 24$ in (D)]. Asterisks indicate significant difference between WT and *prt6* (* $P < 0.05$; *** $P < 0.001$).

elongation under control conditions, whereas roots of *prt6* seedlings grew more vigorously than WT under salinity (Figure 1C). Quantitative assays of root elongation further supported that the *prt6* mutation contributes to maintained root growth under moderate salinity (75-150 mM NaCl) (Figure 1D).

We also evaluated whether the *prt6* mutation affects seedling viability under high (lethal) salinity. WT and *prt6* seedlings showed identical growth under control conditions (Figure 2A). When exposed to high salinity (200 mM NaCl), more than 90% of WT seedling died, but the majority (>60%) of *prt6* seedlings were viable (Figure 2A, B). Correspondingly, seedling biomass

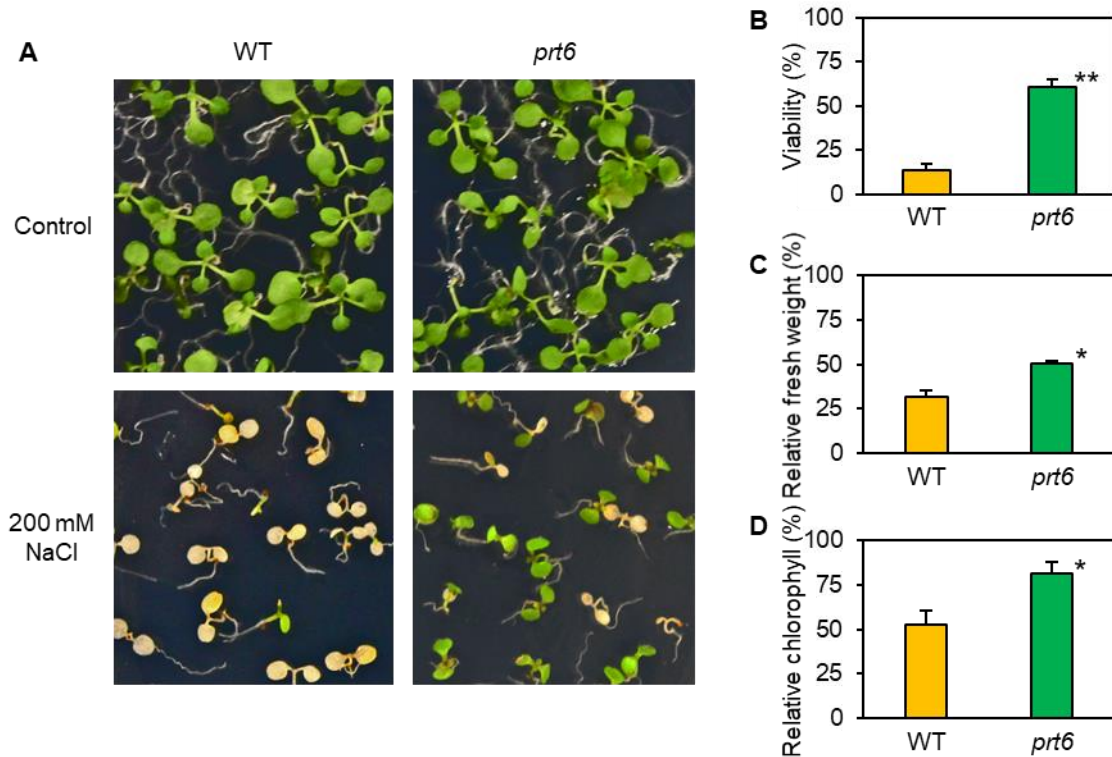


Fig. 2. The *prt6* mutation increased seedling viability under high salinity. (A) Photos of wild-type (WT) and *prt6* mutant seedlings exposed to non-stress or high salinity conditions. Four-day-old seedlings were transferred onto half-strength MS media containing 0 or 200 mM NaCl and grown for 4 d. Seedling viability (B), relative fresh weight (C), and relative chlorophyll content (D) of WT and *prt6* seedlings under high salinity. Seedlings exposed to 0 or 200 mM NaCl as described in (A) were used for data collection in (B-D). Relative fresh weight and chlorophyll were calculated by comparison to non-stressed WT or *prt6*. Data represent means $\pm$ SE [$n = 35 \times 3$ replicates in (B); $n = 3$ in (C, D)]. Asterisks indicate significant difference between WT and *prt6* (* $P < 0.05$; ** $P < 0.01$).

and leaf chlorophyll content were significantly higher in the *prt6* mutant than WT under high salinity (Figure 2C, D).

Finally, we analyzed adult-plant tolerance to high salinity. Under control conditions, *prt6* plants displayed early senescence, relative to WT (Figure 3A). However, *prt6* plants had more green leaves than WT under high salt. Consistent with the phenotypic observation, aboveground biomass and chlorophyll content were significantly higher in the *prt6* mutant under salt stress (Figure 3C, D). In contrast with the chlorophyll data, anthocyanin was highly accumulated in WT plants (Figure 3D), indicating that WT suffered from more severe salinity damage. Taken together,

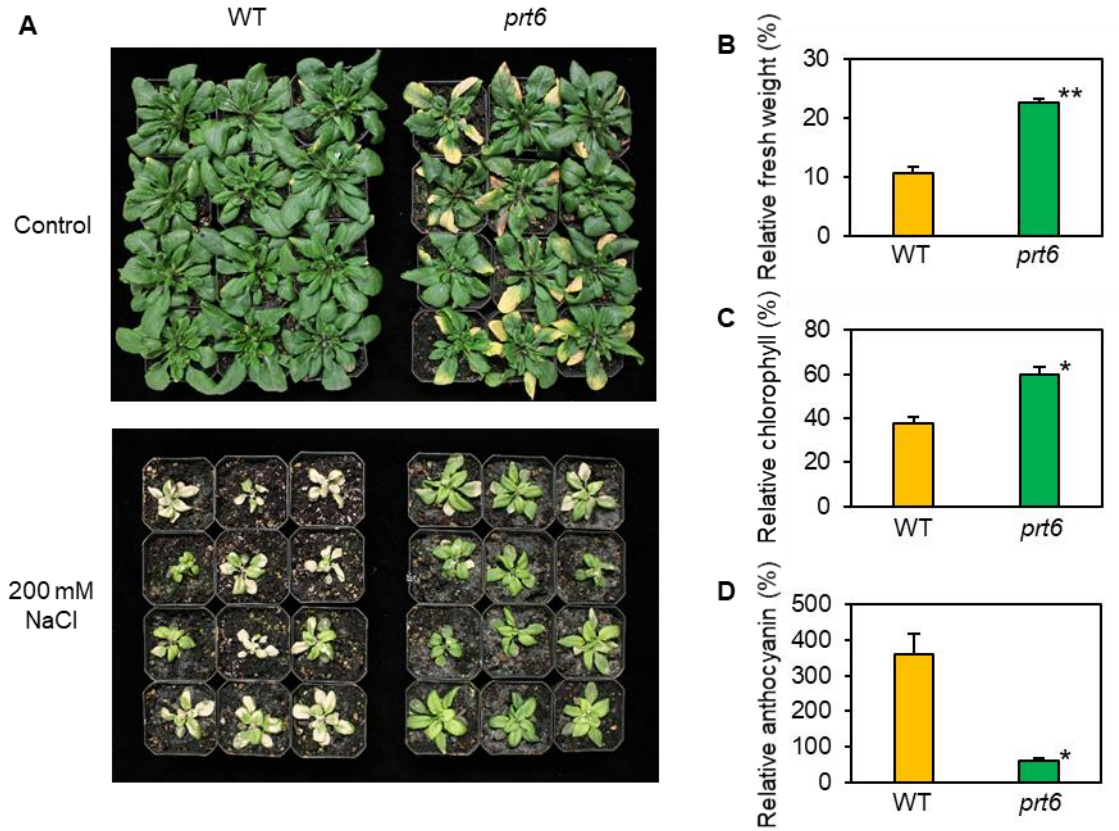


Fig. 3. The *prt6* mutation increased adult-plant tolerance to high salinity. (A) Photos of wild-type (WT) and *prt6* plants exposed to non-stress or high salinity. Plants were grown in pots under regular growth conditions for 21 d and then irrigated with fertilized water containing 0 or 200 mM NaCl for 12 d. Relative fresh weight (B), chlorophyll (C), and anthocyanin (D) of WT and *prt6* plants exposed to salinity. These relative values were calculated by comparison to non-stressed WT or *prt6*. Data represent means $\pm$ SE [$n = 12$ in (B); $n = 3$ in (C, D)]. Asterisks indicate significant difference between WT and *prt6* (* $P < 0.05$; ** $P < 0.01$).

the qualitative and quantitative data presented here demonstrated that loss-of-function mutation of *prt6* increases salinity tolerance at the seed germination, seedling, and adult-plant stages.

The *prt6* mutant plants show restricted accumulation of Na⁺ in roots and shoots.

Salinity-mediated reductions in chlorophyll content and root growth were less severe in the *prt6* mutant than in the WT. We hypothesized that these results are attributed to a restricted accumulation of Na⁺ in the mutant line. To test this, we monitored the abundance of Na⁺ in root and shoot tissues of plants exposed to salinity stress. This analysis revealed that the *prt6* mutant contained a smaller amount of Na⁺ in both roots and shoots (Figure 4). In shoots, the effect of this mutant allele was still significant on day 5, while this effect was not observed in roots at that time. These results suggest that the *prt6* mutation contributes to the suppression of root-to-shoot Na⁺ transport as well as Na⁺ uptake into roots.

We also quantified the level of K⁺ in roots and shoots (Figure 4). The influence of the mutant allele in K⁺ accumulation was minimal or not observed in these tissues.

The *prt6* mutation augments responsiveness to ethylene and brassinosteroids.

Ethylene and brassinosteroids (BR) are positive regulators for salinity tolerance in plants (Archard et al. 2006, Cao et al. 2007, Vriet et al. 2012, Peng et al. 2014). To discern the capability of the *prt6* mutant in regulating sensitivity to these hormones, we performed hormone response assays. When incubated under constant darkness, *prt6* mutant seedlings displayed moderate triple response phenotypes even without 1-aminocyclopropane-1-carboxylic acid (ACC), an immediate precursor of ethylene (Figure 5A). When ACC was supplied, more severe restriction of hypocotyl

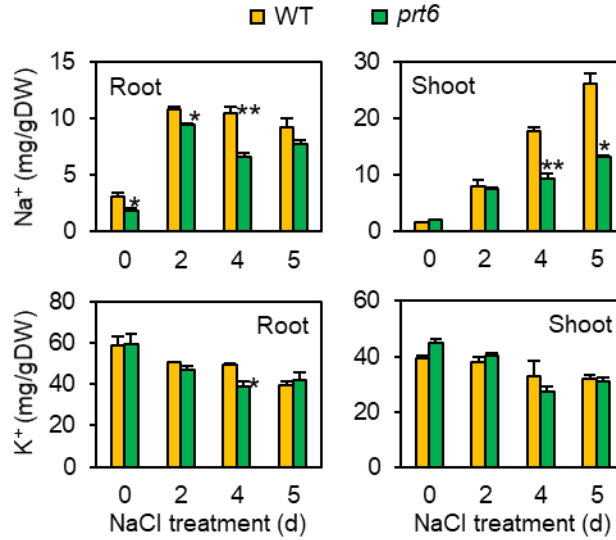


Figure 4. The *prt6* mutation restricted accumulation of Na⁺ in shoots and roots of Arabidopsis plants under salinity. Wild-type (WT) and *prt6* plants were grown in Turface under regular growth conditions for 21 d and then irrigated with fertilized water containing 150 mM NaCl for up to 5 d. Shoot and root samples were harvested at the specified time points, washed thoroughly, and subjected to Na⁺ and K⁺ assays. Data represent means $\pm$ SE (n = 3). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; ** P < 0.01).

elongation and enhancement of apical hook formation were observed in the mutant seedlings. Dose-response analysis further supported that ACC-mediated reductions in hypocotyl growth were greater in the *prt6* mutant at all ACC concentrations tested (Figure 5B). We also evaluated the effect of the mutant allele on mRNA accumulation of ethylene-responsive genes. All the four representative marker genes, *PDF1.2*, *ACO2*, *ERF1*, and *RAP2.3 (EBP)* (Solano et al. 1998, Deslauriers et al. 2015), were highly induced in response to ACC in the *prt6* mutant, compared to WT (Figure 5C). These results indicate that the *prt6* mutation leads to increased responsiveness to ethylene at the molecular and physiological levels.

We also examined the impact of the *prt6* mutation on BR sensitivity. Both WT and *prt6* seedlings grew similarly under mock (0.02% ethanol) conditions (Figure 6A). Application of 24-

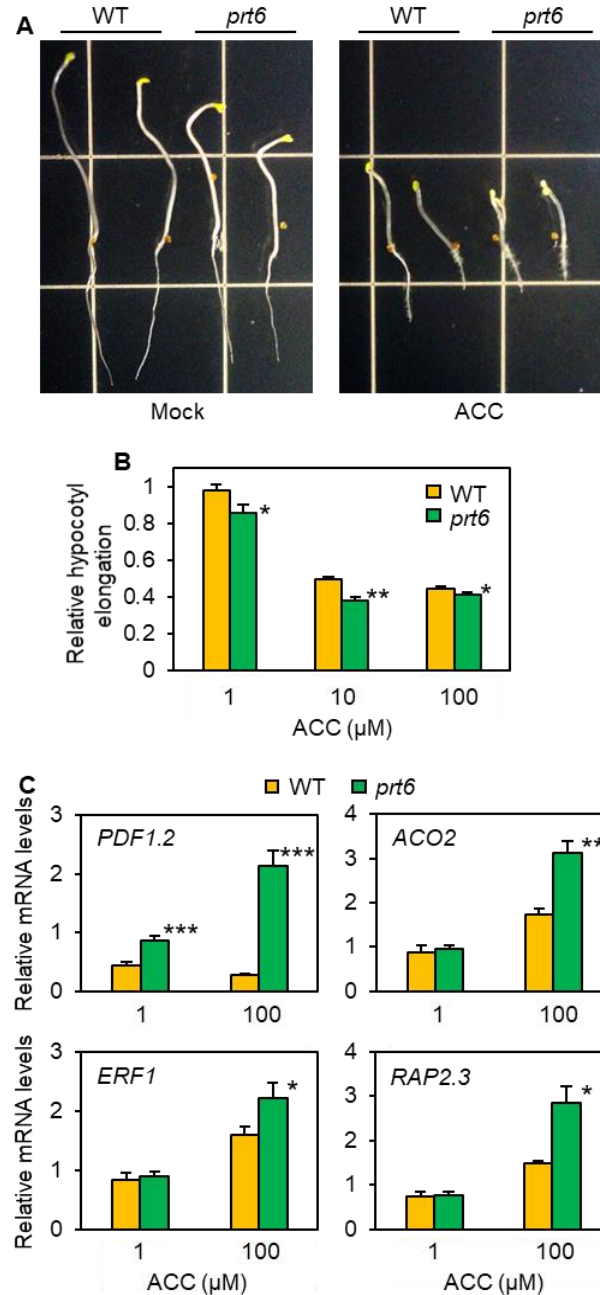


Figure 5. The *prt6* mutation enhanced ethylene responsiveness. (A) Photos of wild-type (WT) and *prt6* seedlings that were grown on half-strength MS media containing 0 (mock) or 10 μ M ACC for 5 d in the dark. (B) Relative hypocotyl elongation of WT and *prt6* seedlings treated with ACC. Seeds were incubated on half-strength MS plates containing a range of ACC concentrations for 5 d in the dark. Hypocotyl length was measured using Image J software. Data represent means $\pm$ SE (n = 24). (C) Relative mRNA levels of ethylene-responsive genes. Ten-d-old seedlings were treated with 1 or 100 μ M ACC for 6 h. Relative hypocotyl elongation and mRNA levels were calculated by comparison to the corresponding genotype (WT or *prt6*) under mock (0 mM ACC) conditions. Data represent means $\pm$ SE (n = 3). Asterisks indicate significant difference between WT and *prt6* (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$).

epibrassinolide (eBL), a bioactive BR, considerably limited root growth in the two genotypes, with a more severe reduction in the mutant seedlings. Dose-dependent analysis verified that root elongation was more strictly inhibited by 0.1 and 1 μ M eBL in the *prt6* mutant than WT. BR responsiveness in WT and the *prt6* mutant was further evaluated by expression analysis of BR-responsive genes. All the four marker genes, *BAS1*, *PAE8*, *XTH33* (*BRU9*), and *Saur_Ac1* (Goda et al. 2002, Fan et al. 2012), were expressed more abundantly in the mutant line than WT when treated with 1 μ M eBL (Figure 6C). Altogether, these data demonstrated that the *prt6* mutation contributes to amplified sensitivity to BR.

The *prt6* mutation activates ABA-dependent and independent pathways involved in salinity / osmotic stress tolerance.

It has been widely accepted that adaptive responses to drought and salinity are regulated through ABA-dependent and independent pathways at the transcriptional level (Zhu et al. 2002, Shinozaki and Yamaguchi-Shinozaki 2007). To determine if the *prt6* mutation alters mRNA accumulation of transcription factors involved in ABA-dependent and independent pathways, the transcript levels of representative genes were monitored by qRT-PCR (Figure 7A). Of the four *AREB/ABFs* in ABA-dependent pathways, *ABF1*, *ABF3*, and *AREB2* mRNAs were more abundantly accumulated in the *prt6* mutant than WT in at least one time point under salinity stress. Similarly, *DREB2A* and *DREB2B*, representative transcription factors in ABA-independent pathways, were more highly induced by salinity stress in the mutant line relative to WT in at least one time point. In contrast with these results, the level of *DREB2A* transcript was higher in WT than the mutant line on day 0, although the difference between the two genotypes was minimal. We also investigated the expression levels of dehydrin genes such as *RAB18*, *RD29A*, and *RD29B*, direct

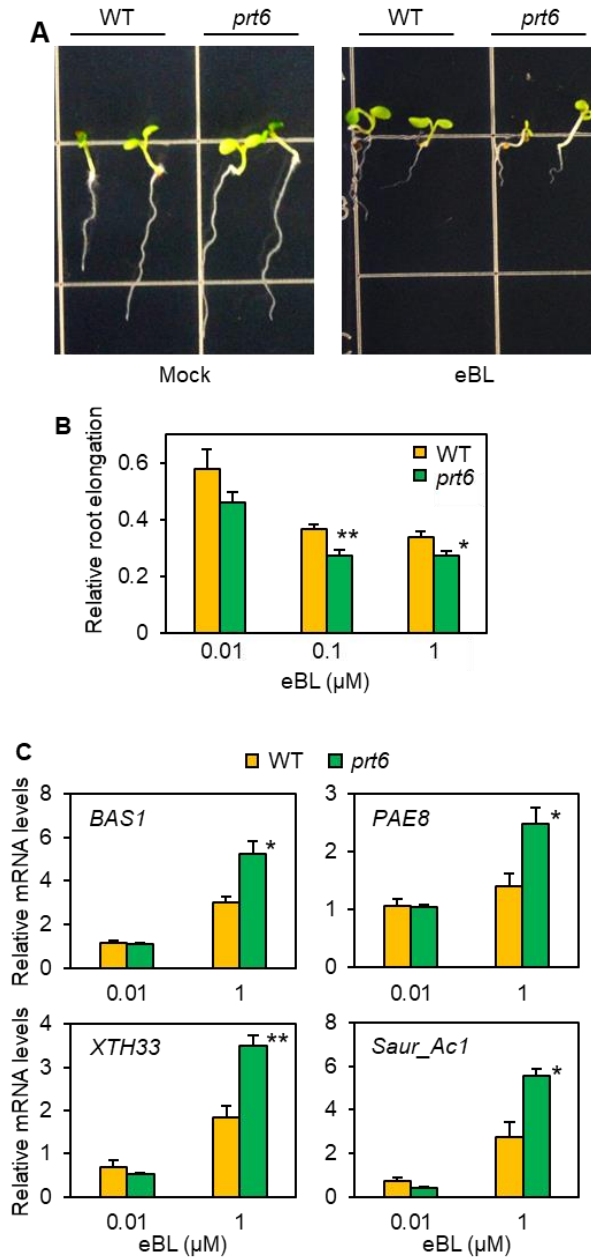


Figure 6. The *prt6* mutation augmented brassinosteroid responsiveness. (A) Photos of WT (wild-type) and *prt6* seedlings that were grown under mock (0.02% ethanol) or 24-epibrassinolide (eBL; 1 μM in 0.02% ethanol) for 5 d. (B) Relative root elongation of WT and *prt6* seedlings treated with eBL. Seeds were incubated on half-strength MS plates containing a range of eBL concentrations for 5 d. Root length was measured using Image J software. Data represent means $\pm$ SE (n = 18). (C) Relative mRNA levels of brassinosteroid-responsive genes. Ten-d-old seedlings were treated with eBL (0.01 or 1 μM in 0.02% ethanol) for 6 h. Relative root elongation and mRNA levels were calculated by comparison to the corresponding genotype (WT or *prt6*) under mock (0.02% ethanol) conditions. Data represent means $\pm$ SE (n = 3). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; ** P < 0.01).

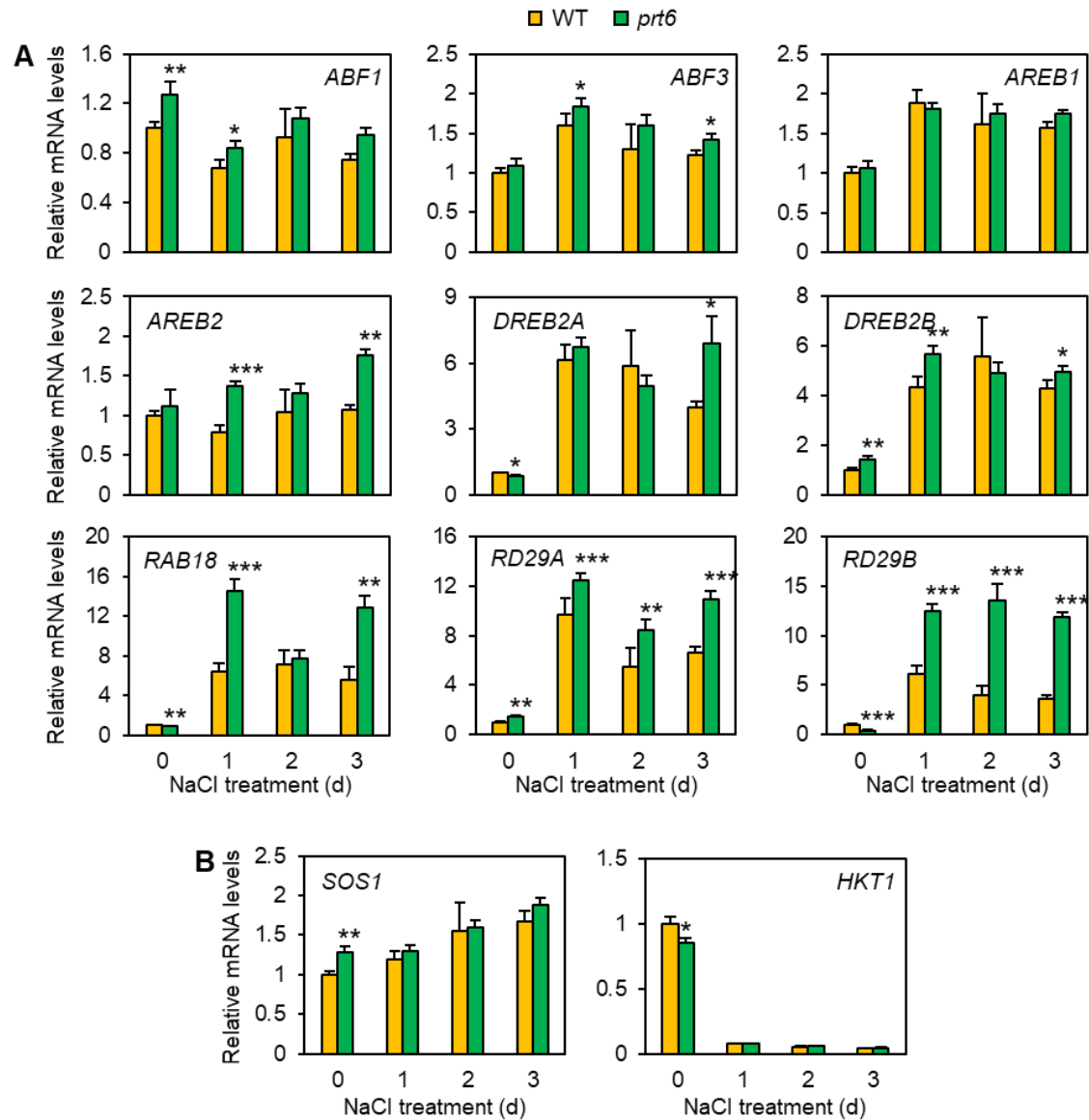


Figure 7. The *prt6* mutation induced mRNA accumulation of genes associated with salinity tolerance. (A) Transcription factors involved in ABA-dependent (*AREB/ABFs*) and independent (*DREB2s*) pathways of salinity/osmotic stress tolerance and their downstream genes, dehydrins. (B) Sodium transporter genes. Ten-d-old seedlings were treated with 150 mM NaCl for up to 3 d and subjected to qRT-PCR analysis. Data represent means $\pm$ SE (n = 3). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; ** P < 0.01, *** P < 0.001).

targets of *AREB/ABFs* and *DREB2s* (Sakuma et al. 2006, Yoshida et al. 2015). All of these genes were highly induced in response to salinity, with greater expression in the *prt6* mutant during

salinity stress (Figure 7A). These results are in accordance with the expression patterns of their upstream transcription factors.

The *prt6* mutant displayed restricted accumulation of Na⁺ in root and shoot tissues (Figure 4). This data raised the question of whether the *prt6* mutation affects the expression of genes associated with Na⁺ transport. To answer this question, we monitored the mRNA accumulation of *SOS1* and *NKT1* by qRT-PCR (Figure 7B). The level of *SOS1* transcript was more abundant in the mutant than WT on day 0, but no significant difference was observed in the two genotypes during salinity stress. The mRNA level of *HKT1* was higher in WT than the mutant line on day 0, but its expression was drastically declined by salinity stress, with no significant difference between the two lines. These results suggest that low accumulation of Na⁺ in mutant roots and shoots is not controlled through transcriptional regulation of these Na⁺ transporters.

The *prt6* mutation induces *ERFVII*s at the mRNA accumulation level under salinity stress.

PRT6 encodes the last enzyme in the PRT6/N-end rule pathway that regulates the turnover of ERF-VII proteins in Arabidopsis (Bailey-Serres et al. 2012). Therefore, a knockout mutation of *prt6* increases the accumulation of ERF-VII proteins (Gibbs et al. 2011, Licausi et al. 2011, Gibbs et al. 2014b). To determine the influence of the *prt6* mutation on the expression of *ERF-VII*s, we performed qRT-PCR analysis. Of the five *ERF-VII* genes, *HRE1* and *HRE2* are inducible under oxygen deprivation (Licausi et al. 2010, Hess et al. 2011, Papdi et al. 2015). Similar to the low oxygen response, these genes were upregulated by salinity stress in WT (Figure 8). In the *prt6* mutant, *HRE1* and *HRE2* were constitutively expressed, and their mRNA level was significantly higher in the mutant than WT under non-stress (day 0) and salinity conditions (days 1 and 3). *RAP2.2*, *RAP2.3*, and *RAP2.12* are not low oxygen-responsive (Licausi et al. 2010, Papdi et al.

2015). Likewise, the transcript abundance of the three *RAP2* genes was not clearly altered under salinity stress in the two genotypes, with higher mRNA accumulation of these genes in the *prt6* mutant. *ALCOHOL DEHYDROGENASE 1* (*ADH1*) is a downstream gene of most ERF-VIIs; overexpression of *HRE1*, *RAP2.2*, *RAP2.3*, or *RAP2.12* upregulates the expression of *ADH1* even under non-stress conditions (Licausi et al. 2010, Papdi et al. 2015). Consistent with the *ERF-VII* gene expression data presented here, *ADH1* transcript was highly accumulated in the *prt6* mutant under non-stress and salinity conditions.

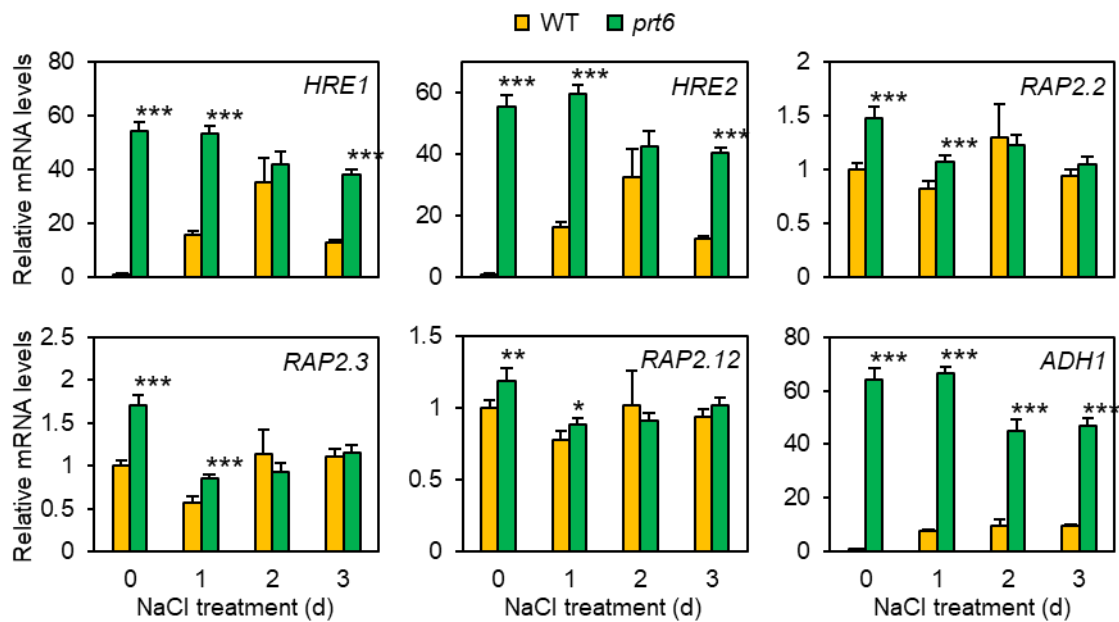


Figure 8. The *prt6* mutation promoted mRNA accumulation of *ERF-VII* genes. Ten-d-old seedlings were treated with 150 mM NaCl for up to 3 d and subjected to qRT-PCR analysis. Data represent means $\pm$ SE (n = 3). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; ** P < 0.01, *** P < 0.001).

DISCUSSION

Salinity and flooding are closely related abiotic stresses which can occur sequentially or concurrently (Barrett-Lennard 2003, Hanley et al. 2019). Therefore, the simultaneous

improvement of both salinity and flooding tolerance is a desirable trait in crop plants. The PRT6/N-end rule pathway is the key signaling process that adversely regulates tolerance to flooding and low oxygen (Bailey-Serres et al. 2012, Gibbs et al. 2014a). In the present study, we demonstrated that this pathway also plays a negative role in salinity tolerance at the seed germination to adult plant stages through detailed time-course and dose-response observations of the *Arabidopsis prt6* mutant and wild-type plants (Figure 1-3). Functional characterization of the mutated *prt6* uncovered the transcriptional and hormonal pathways coordinated by the PRT6/N-end rule under high salt.

Ethylene is a crucial hormone that regulates plant responses and adaptation to salinity (Archard et al. 2006, Cao et al. 2007, Peng et al. 2014). For example, exogenous application of an ethylene precursor, ACC, suppressed the accumulation of Na⁺ in *Arabidopsis* roots and reduced membrane damage under excess salt (Li et al. 2014). In the *eto1* mutant that constitutively overproduces ethylene, the Na⁺ concentrations in both stellar cells and xylem sap were reduced under salinity, leading to enhanced plant survival and leaf chlorophyll content (Jiang et al. 2013). These results indicate that ethylene plays a pivotal role in limiting Na⁺ uptake in roots and transport to shoots under high salt. Besides Na⁺ influx/transport regulation, mutant and transgenic studies revealed that ethylene signaling is necessary for the salt-induced accumulation of dehydrin mRNAs such as *RD29A*, *RD29B*, and *COR15A* in *Arabidopsis* (Wang et al. 2007, Zhang et al. 2011). In the present study, we demonstrated that the *prt6* mutation reduces Na⁺ levels in both roots and shoots (Figure 4) and increases mRNA accumulation of dehydrins including *RD29A* and *RD29B* under salinity compared to WT (Figure 7). It is likely that these adaptive responses enhanced by the *prt6* mutation result from increased sensitivity to ethylene in the mutant (Figure 5).

BR is another positive regulator for salinity tolerance (Li et al. 2007, Cui et al. 2012, Vriet et al. 2012). Treatment of barley and canola plants with eBL reduced Na⁺ levels in roots and shoots, contributing to maintenance of growth under salinity stress (Liu et al. 2014, Azhar et al. 2017). Application of eBL also promoted mRNA accumulation of *RD29A* in *Arabidopsis* under excess salt (Kagale et al. 2007), whereas mRNA accumulation of this gene was restricted in a brassinosteroid deficient mutant, *det2* (Zeng et al. 2009). These results indicate that Na⁺ influx/transport and dehydrin gene expression under salinity stress were coordinated by BR as well as ethylene. It is expected that increased sensitivity to BR in the *prt6* mutant (Figure 6) contributed to the restricted accumulation of Na⁺ in roots and shoots and stimulated expression of dehydrin genes under high salt (Figure 4, 7). The PRT6/N-end rule pathway is responsible for proteasomal degradation of ERF-VII and VRN2 proteins (Gibbs et al. 2015, Gibbs et al. 2018), but this pathway may also regulate targeted proteolysis of other proteins. Further investigation is required to uncover how disruption of the PRT6/N-end rule pathway enhances plant response to ethylene and BR through stabilization of ERF-VII, VRN2, and other unidentified proteins.

Previous studies showed that mutations in *prt6* increase ABA sensitivity during germination and seedling establishment in *Arabidopsis* (Holman et al. 2009, Zhang et al. 2018). Transient expression and chromatin immunoprecipitation (ChIP) analyses demonstrated that RAP2-type ERF-VIIs directly interact with the promoter region of *ABI5*, a major downstream transcription factor in the ABA signaling pathway, activating its expression (Gibbs et al. 2014b). Additionally, GUS staining assays showed that *promABI5::GUS* activity is induced in *prt6* mutant seeds, but not in wild-type seeds. These data indicate that ABA hypersensitivity in *prt6* mutants is caused by enhanced expression of *ABI5* via stabilization of its transcriptional regulators, RAP2-type ERF-VIIs. Our study revealed that the *prt6* mutation increased mRNA accumulation of three

AREB/ARF genes under excess salt (Figure 7). Similar to ABI5, AREB/ABFs serve as downstream transcription factors in the ABA signaling pathway. AREB/ABFs primarily regulate expression of ABA-responsive genes associated with stress tolerance (Yoshida et al. 2014), whereas ABI5 coordinates expression of ABA-responsive genes related to seed germination and dormancy (Skubacz et al. 2016). It is anticipated that increased mRNA accumulation of AREB/ABFs in the *prt6* mutant may be attributed to ERF-VII-mediated activation of their respective promoters.

Although many stress-responsive genes are upregulated by ABA, these genes are also induced in an ABA-independent manner (Yoshida et al. 2014). DREB2A and DREB2B are major transcription factors responsible for ABA-independent gene expression. Our study found that the *prt6* mutation increases mRNA accumulation of *DREB2A* and *DREB2B* under high salt, accompanied by high expression of key ABA-dependent transcription factors including *ABF1*, *ABF3*, and *AREB2* (Figure 7). A previous study demonstrated that the expression of *DREB2A* is regulated by direct binding of ABF3, AREB1, and AREB2 to its promoter region under osmotic stress (Kim et al. 2011). It is likely that elevated accumulation of *DREB2A* mRNA in the *prt6* mutant results from transcriptional activation mediated by ABF3 and AREB2.

The PRT6/N-end rule pathway regulates the turnover of all five ERF-VII proteins (Gibbs et al. 2011, Licausi et al. 2011, Gibbs et al. 2014b). Thus, disruption of *prt6*, which encodes an essential enzyme of the proteolysis pathway, leads to overaccumulation of all five ERF-VII proteins. However, the impact of the *prt6* mutation on mRNA accumulation of ERF-VII genes remains unknown. This study revealed that the *prt6* mutation increases mRNA levels of all ERF-VII genes under salinity, along with the dramatic induction of *ADH* mRNA, a downstream target of ERF-VII transcription factors (Figure 8). The expression patterns of HRE-type and RAP2-type

ERF-VIIs were apparently distinct, reflecting the notion that these two types of ERF-VIIs function differently in the regulation of hypoxia-responsive gene expression and seed germination (Gibbs et al. 2014b, Bui et al. 2015, Gasch et al. 2016). The mechanisms underlying overaccumulation of ERF-VII mRNAs in the *prt6* mutant are unknown, but this process must assist the further synthesis of ERF-VII proteins. Transgenic and mutant studies demonstrated that RAP2-type ERF-VIIs and HRE2 are positive regulators of osmotic and salinity stress tolerance in *Arabidopsis* (Park et al. 2011, Papdi et al. 2015). Therefore, overaccumulation of ERF-VII mRNAs and proteins in the *prt6* mutant must contribute to enhanced tolerance to excess salt.

We propose a model for salinity-tolerance mechanisms that are negatively regulated by the PRT6/N-end rule pathway (Figure 9). When exposed to high salt, plants are damaged mainly due to salt-induced osmotic stress and sodium toxicity. The present study demonstrated that *PRT6* restricts signaling processes associated with adaptation to both osmotic stress and sodium toxicity.

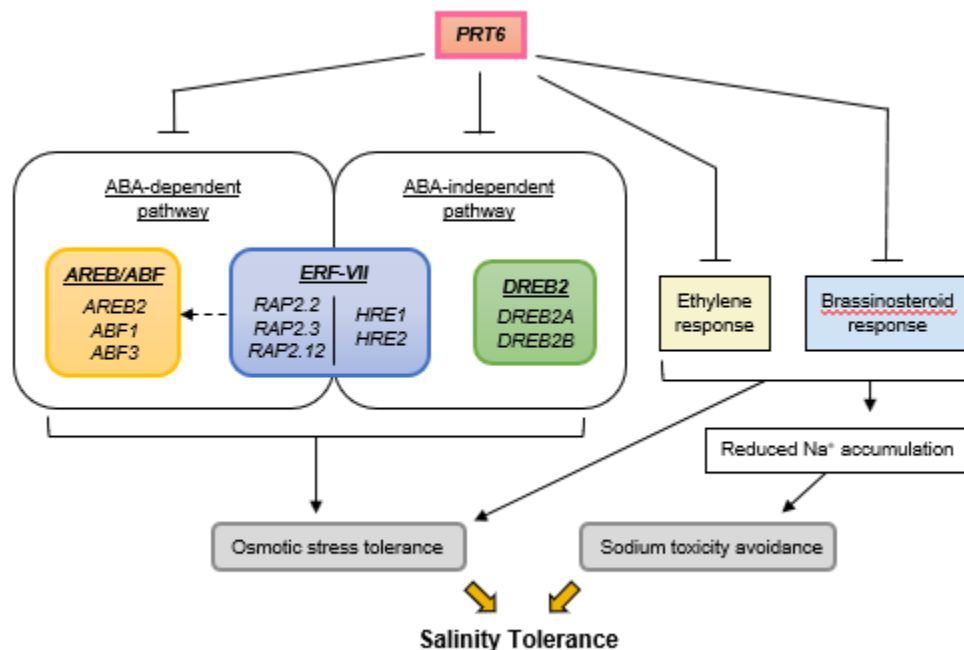


Figure 9. Model for salinity-tolerance mechanisms that are negatively regulated by the PRT6/N-end rule pathway. A dashed line indicates a hypothetical relationship.

For example, *PRT6* downregulates mRNA accumulation of *AREB/ABFs* and *DREB2s*, well-characterized transcription factors involved in the ABA-dependent and independent pathways of osmotic stress tolerance, respectively. *PRT6* also dampens responsiveness to ethylene and BR, key hormones that enhance expression of stress-responsive genes such as dehydrins and restrain root Na^+ uptake and root-to-shoot Na^+ transport. In this model, we propose that *RAP2*-type *ERF-VII*s serve as members of the ABA-dependent pathway, whereas *HRE*-type *ERF-VII*s function in the ABA-independent pathway due to the following reasons: 1) All *RAP2*-type *ERF-VII* genes are ABA-responsive (Papdi et al. 2015). 2) The three *RAP2*-type *ERF-VII*s directly regulate expression of *ABI5*, a downstream transcription factors in the ABA signaling pathway (Gibbs et al. 2014b). 3) Inducible expression of each of the three *RAP2*-type *ERF-VII*s increases sensitivity to ABA (Papdi et al. 2015). 4) Both *HRE*-type *ERF-VII* genes are not ABA-inducible (Yang et al. 2011, Papdi et al. 2015). 5) *HRE2* activates the expression of reporter genes by direct binding to the promoter's DRE/CRT motif, a *cis*-acting element in the ABA-independent pathway (Narusaka et al. 2003, Lee et al. 2015). These data demonstrate that *RAP2* and *HRE*-type *ERF-VII*s belong to the ABA-dependent and independent pathways of osmotic/salinity stress tolerance, respectively. Based on the evidence (2), it is predicted that *RAP2*-type *ERF-VII*s directly modulate the expression of *AREB/ABFs*, other downstream transcription factors in the ABA signaling pathway. Taken together, these results have revealed that the *PRT6*/N-end rule pathway acts as a negative regulator of salinity tolerance in *Arabidopsis* through modulation of transcriptional and hormonal responses to the stress. Many commercially important plants can encounter salinity and flooding sequentially or simultaneously, reducing crop yield and quality under changing climates. Guided manipulation of the *PRT6*/N-end rule pathway can be a promising approach to generate new crop varieties with improved tolerance to both salinity and flooding.

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CHAPTER II

The Role of an N-end Rule Pathway E3 Ligase, *PROTEOLYSIS 6*, in Salinity Tolerance in Maize

ABSTRACT

Currently, more than 800 million hectares of land is affected by excess salt, and a considerable proportion of fertile land is turning into saline as a consequence of global climate changes and human-mediated activities such as repeated use of salt-containing groundwater as a source of irrigation. Maize is a salinity-sensitive crop, whose production is threatened by soil salinization worldwide. In Chapter I, we demonstrated that an E3 ligase of the N-end rule pathway, *PROTEOLYSIS6 (PRT6)*, acts as a negative regulator of salinity tolerance in Arabidopsis. In this chapter, we evaluated the role of *PRT6* in tolerance to high salt in maize. Our analyses showed that the *prt6* mutation reduced seed germination, primary root elongation, and shoot biomass growth in maize under high salinity. In addition, salinity-induced malondialdehyde accumulation was significantly higher in the *prt6* mutant than wild-type, indicating that the mutant line encountered more severe oxidative damage. Reduced tolerance to salinity stress in the *prt6* mutant was also observed at the reproductive stage. High salt significantly delayed anthesis and silking days and reduced grain yield in the *prt6* mutant. Altogether, this study revealed the pivotal role of *PRT6* in tolerance to high salt in maize, opposite to our observations in Arabidopsis. Further investigation is required to dissect the molecular mechanisms underlying the distinct function of this E3 ligase in salinity tolerance between maize and Arabidopsis.

Keywords: Salinity stress, *PRT6*, N-end rule pathway. *Zea mays*

INTRODUCTION

Maize (*Zea mays* L.) is an important cereal crop widely grown throughout the world under a wide spectrum of soil and climatic conditions. It is a major staple crop and has the highest production of all the cereal crops, surpassing rice and wheat, with 1.1 billion tons being produced in 2017 worldwide (FAOSTAT, 2017). The United States is a major maize producer and the world's largest exporter. Of the total global maize production in 2017, 32.7% were produced in the United States and approximately 17% of the production was exported to other countries (USDA-ERS, 2019).

Over the past few decades, maize yield and planted acreage have been constantly increasing (Haarhoff and Swanepoel 2018). However, a steady increase in maize production may not meet the global demand for this crop in the future due to increasing rates of its consumption for food, feed, and biofuel production (Ashraf et al. 2018). Importantly, abiotic stresses including salinity have seriously threatened global maize production (Zhang et al. 2019). Maize is categorized as a salinity-susceptible species (Fortmeier and Schubert 1995, Farooq et al. 2015). Therefore, maize growth and productivity are reduced in coastal areas, barriers island, and areas along brackish tidal rivers and estuaries where the salt level is elevated. Soil salinity is also a major issue in irrigated maize production in inland areas because repeated irrigation of the salt-containing groundwater increases the level of salt in the soil. Furthermore, salt tolerance in maize may become more important in the future when irrigation is more widely used as a consequence of climate change. Considering a global demand for maize and increasing soil salinization problems, it is crucial to advance the present knowledge of salinity tolerance mechanisms in maize, facilitating the development of salt-tolerant varieties with higher productivity.

When exposed to high salt, plants experience osmotic stress at the initial phase and then encounter ionic toxicity at the later phase. This biphasic model of salinity response has already been confirmed in maize by Fortmeier and Schubert (1995). In salt-sensitive crops, leaf expansion, new leaf emergence, and photosynthesis are all reduced at the initial phase of salinity response. To minimize the impact of salinity stress on shoot growth, plants accumulate various osmolytes such as proline, soluble sugars, and organic acids, contributing to the readjustment of cellular osmotic potential (Roy et al. 2014). High salt also activates antioxidant systems, promoting the detoxification of reactive oxygen species (ROS) overaccumulated under the stress (Szabados and Savoure 2010, Wang et al. 2019). The second, ion toxicity, phase starts when ions such as Na^+ are further accumulated to toxic concentrations in the plant cells. Ionic toxicity accelerates a severe reduction in photosynthetic capacity, premature leaf senescence, and even plant death (Munns and Tester 2008, Roy et al. 2014).

In Chapter I, we demonstrated that the *PRT6* acts as a negative regulator for salinity tolerance in Arabidopsis. Besides what is known for Arabidopsis, the molecular function of *PRT6* to salinity responses is not yet understood. In this chapter, we evaluated the role of *PRT6* in tolerance to high salt in maize. Using the *MU* transposon insertion mutant of *prt6* and its background genotype, we evaluated how the *prt6* mutation influences adaptability to salinity at various developmental stages. Taking advantage of the fact that maize is a crop plant, we also assessed the effect of the *prt6* mutation on crop yield under high salt, which was not evaluated in Arabidopsis. This study demonstrated that *PRT6*, a negative regulator for salinity tolerance in Arabidopsis, functions as a positive regulator in maize, providing new insight into the functional role of this signaling component in salinity tolerance regulation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

A *Mu* transposon insertion mutant in *prt6* and its wild-type (WT) were obtained from Dr. Alice Barkan at University of Oregon (May et al., 2003). Both genotypes were subjected to semi-quantitative RT-PCR to confirm the presence and absence of *prt6* expression. Seeds were sterilized with 2% (v/v) sodium hypochlorite and 0.1 % (v/v) Tween-20 for 12 minutes and then rinsed thoroughly using water. Sterilized seeds were used for various experiments as described below.

Germinability evaluation under salinity

Seeds were placed on wet paper towels containing 0, 100, and 150 mM NaCl and incubated at 23 °C under constant light ($120 \mu\text{mol m}^{-2} \text{s}^{-1}$). The number of germinated seeds was recorded 4, 5, and 6 days after sowing. Seedlings with more than 5 mm root were counted as germinated.

Shoot and root growth analysis under salinity

Germinated WT and *prt6* seeds (radicle > 5mm) grown under non-stress conditions were transferred onto damp paper towels containing 0, 100, and 150 mM NaCl and grown for 5 days as described above. Shoot and root elongation of each seedling was obtained by subtracting the initial from the final length.

Shoot growth evaluation

Seeds were incubated on wet paper towels for 5 days as described above. The germinated seedlings were transferred into soil-containing pots (14x14x16 cm) and grown until the 3-leaf stage. Pots were then treated with 0 or 200 mM NaCl for up to 2 weeks. At the end of the treatments, shoot tissues were harvested, and their dry weight was recorded.

Leaf relative water content analysis

Leaf relative water content (RWC) was measured in the uppermost leaves of the salt-treated plants. Each leaf blade was detached from a plant, and the fresh weight (FW) was immediately measured. After the measurement, leaf blades were floated on water for 24 h to rehydrate. Leaves were reweighed to measure turgid weight (TW). Finally, leaf blades were dried at 65 °C for 3 d, and the dry weight (DW) was measured. Leaf RWC was determined using the following equation: $RWC (\%) = (FW - DW) / (TW - DW) \times 100$.

Na⁺ and K⁺ content analysis

Dried leaf tissues (100 mg) was homogenized in 20 mL of 0.1 N HNO₃ for 30 min (Rus et al. 2001). Following filtration using quantitative analysis-grade filter paper, the extract was subjected to Na⁺ and K⁺ analysis using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES).

Carbohydrate assay

Fifty milligrams of frozen leaf tissue were homogenized in 1 ml of cold water on ice. Following centrifugation at 4 °C for 10 min at 21,000 g, the supernatant was collected in a new tube. This

process was repeated twice, and the three extracts were combined. Total soluble carbohydrate content was assayed by the anthrone method as described by Fukao et al. (2006) with glucose as a standard. The carbohydrate extract (100 μ L) was mixed with 1 mL of 0.14% (w/v) anthrone solution in 100 % H_2SO_4 and incubated at 100 $^\circ\text{C}$ for 20 min. The absorbances of the solution were measured at 620 nm using a spectrophotometer.

Starch content was quantified from the pellet obtained after the soluble sugar extraction process as described by Fukao et al. (2012). The pellet was washed with water, resuspended in 1 mL of water containing 10 units of heat-resistant alpha-amylase and incubated at 95 $^\circ\text{C}$ for 15 min. After cooling, the suspension was adjusted to pH 4.8 with 25 mM sodium citrate (pH 4.8), and 5 units of amyloglucosidase were added. After incubation at 55 $^\circ\text{C}$ for 30 min, the reaction mixture was centrifuged for 30 min at 21,000 g. The glucose content in the supernatant was quantified by the anthrone method as described above.

Proline assay

Proline content was quantified using a colorimetric assay described by Abraham et al. (2010). Frozen tissue (50 mg) was homogenized in 350 μ L of 3% salicylic acid on ice. Following centrifugation at room temperature for 5 minutes at 21,000 g, 200 μ L of the supernatant was mixed with 200 μ L of glacial acetic acid and 200 μ L of acidic ninhydrin. After incubation at 96 $^\circ\text{C}$ for 60 minutes, the reactions were terminated on ice and 1 mL of toluene was added to the reaction mixture. Following 20-second vigorous mixing, the solution was kept at room temperature for 5 minutes to allow the separation of the organic and water phases. The absorbance of the top chromophore was measured at 520 nm using toluene as the reference.

Lipid peroxidation analysis

Malondialdehyde (MDA) – the end product of lipid peroxidation, was quantified using the thiobarbituric acid assay (Hodges et al. 1999). Frozen tissue (50 mg) was homogenized in 1 mL of 80% (v/v) ethanol on ice. After centrifugation at 4 °C for 20 min at 21,000 g, the supernatant (0.5 mL) was mixed with 0.5 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric acid. The mixture was then incubated at 95 °C for 30 min and cooled immediately on ice. Following centrifugation at 4 °C for 10 min at 10,000 g, absorbance at 532 and 600 nm were measured.

Yield component analysis

A total of 96 plants (2 genotypes x 3 treatments x 16 plants) were laid out in a completely randomized design. Each plant was grown in a pot (9.08 L) containing ProMix Brk20 with mycorrhizae under greenhouse conditions (28 °C day, 23 °C night) at Virginia Tech. Both genotypes were grown under non-stress conditions until the V9 stage. Plants were then treated with either 0, 33, or 66 mM NaCl until harvest. Yield components, including grain yield per plant, the number of seeds per plant, and 1000-grain weight were measured.

Statistical analyses

Statistical analyses were performed using JMP Pro 14 (SAS Institute) and MS-Excel 2019 (Microsoft).

RESULTS

In the previous chapter, we demonstrated that a loss of function mutant of *PRT6* enhanced salinity tolerance at various developmental stages in *Arabidopsis*, indicating that *PRT6* is a negative regulator for adaptation to high salt. To determine whether *PRT6* plays a negative role in salinity tolerance in a commercially important crop as well, we performed comparative analysis of a maize *prt6* mutant and its wild-type (WT) under high salt. Semi-quantitative RT-PCR analysis confirmed that *PRT6* is not expressed in the mutant line, whereas mRNA accumulation of this gene was clearly visualized in the WT (Figure 1).

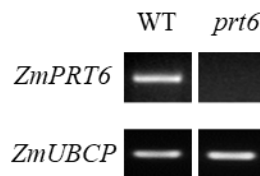


Figure 1. Semi-quantitative RT-PCR analysis of maize *PRT6* (*ZmPRT6*) gene in wild-type (WT) and a *prt6* knockout mutant lines. *ZmUBCP* gene was used as a loading control.

Effect of the *prt6* mutation on salinity tolerance at the seed germination and seedling stages

To evaluate the role of the *prt6* mutation in salinity tolerance in maize, we first conducted stress evaluation tests at the seed germination and seedling stages. It is well known that seed germination, a critical stage initiating a plant life cycle, is severely affected by salinity stress (Debez et al. 2018, He et al. 2019). Vigorous germination under high salt is the first and major acclimation response of plants to salinity stress. We monitored germination of the mutant and WT seeds under sub-lethal salinity conditions. High NaCl reduced seed germination in both genotypes, but the *prt6* mutant exhibited lower seed germination than WT on days 4 and 5 (Figure 2A). Consistently, primary root elongation was significantly reduced in the *prt6* mutant compared to WT at 100 mM and 150

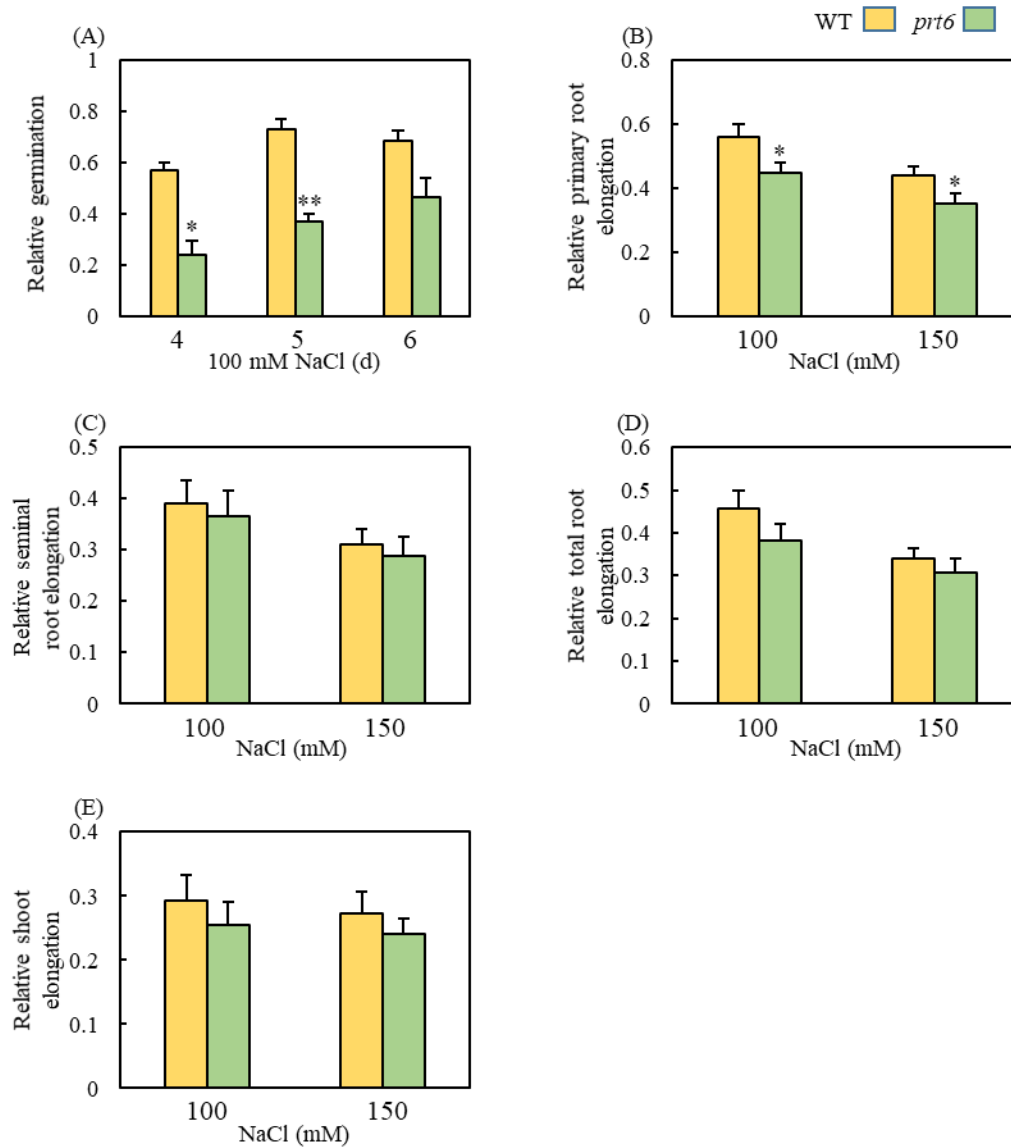


Figure 2. The *prt6* mutation reduced salt tolerance at the seed germination and seedling stages. (A) Relative germination of WT and *prt6* seeds grown under 100 mM NaCl. Seeds were incubated under 100 mM NaCl for 4, 5, and 6 days. (B-E) Germinated seedlings grown under non-stress conditions were transferred onto wet paper towels containing 100 or 150 mM NaCl and incubated for 5 days. Relative elongation of primary root (B), seminal root (C), total root (D), and shoot (E) of WT and *prt6* seedlings were analyzed. Relative values were calculated by comparison to the corresponding genotype (WT or *prt6*) under non-stressed (0 mM NaCl) conditions. Data represent means $\pm$ SE (n = 32). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; *** P < 0.001).

mM NaCl (Figure 2B). These results indicate that *PRT6* is a positive regulator for salt tolerance at the seed germination and seedling stages in maize, which is contrary to our findings in *Arabidopsis*. We also evaluated more detailed traits associated with root morphology such as seminal root, total root, and shoot elongation (Figure 2C, 2D, 2E). These values were not significantly distinct between the *prt6* mutant and WT under salinity stress, suggesting that the *prt6* mutation is mainly functional in primary root elongation.

Impact of the *prt6* mutation on salinity tolerance at the vegetative stage

We extended our stress tolerance evaluation to the vegetative stage. Both *prt6* and WT plants were treated with 0 or 200 mM NaCl solution for two weeks (Figure 3A). Salinity stress reduced the dry weight of shoot tissue in both genotypes, but *prt6* mutants demonstrated significant higher reduction than the WT (Figure 3B). Accordingly, the relative water content of the uppermost leaves was lower in the *prt6* mutant than WT under high salt (Figure 3C). We also determined the level of Na⁺ in shoot tissue. High salt dramatically increased the accumulation of Na⁺ in WT and mutant lines, but no significant difference was detected between the two genotypes (Figure 3D). Altogether, these results indicate that *PRT6* plays a positive role in maize tolerance to salinity at the vegetative stage as well as seed germination and seedlings stages.

Influence of the *prt6* mutation in accumulation of metabolites associated with salinity adaptation

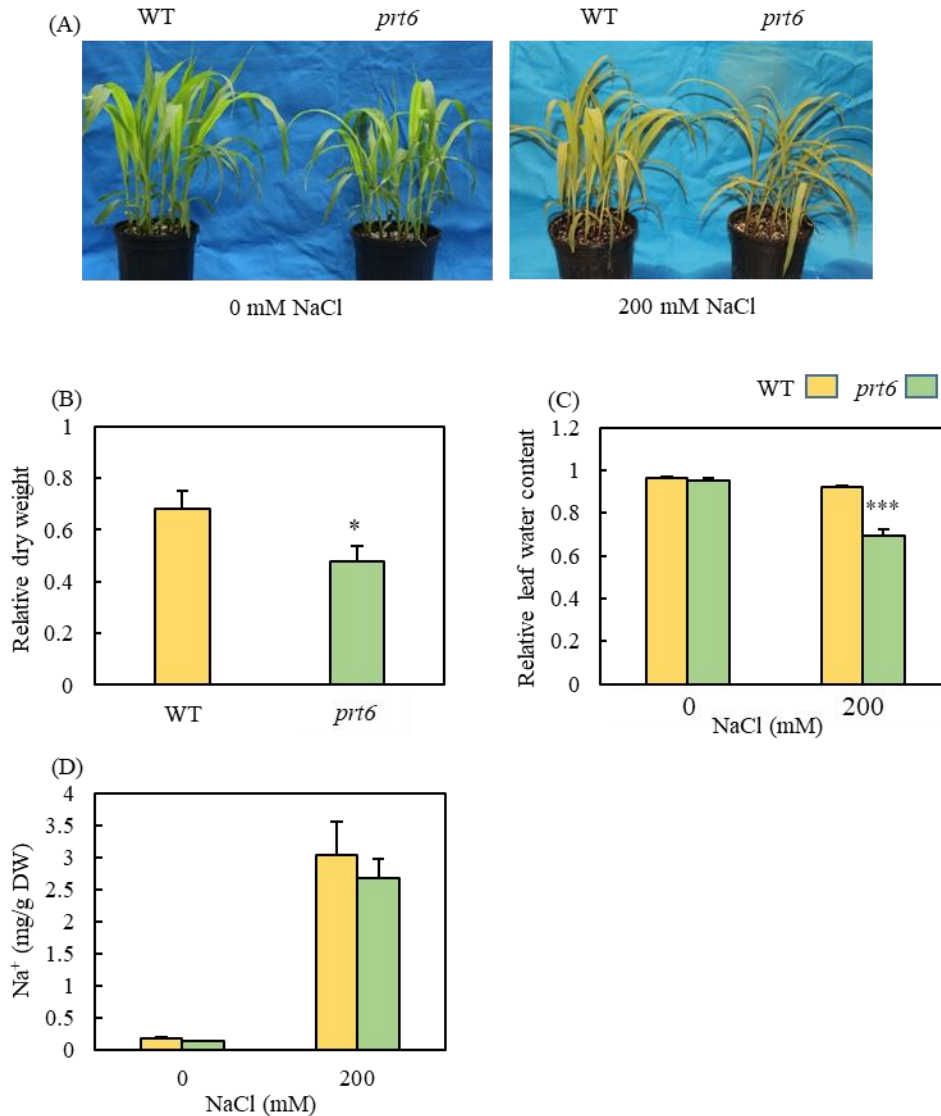


Figure 3. The *prt6* mutation reduced salt tolerance at the early vegetative stage. (A) Photos of WT and *prt6* seeds grown under 0 or 200 mM NaCl. Plants at the 3-leaf stage were treated with 0 or 200 mM NaCl for 2 weeks. Relative dry weight (B), relative water content (C), and Na⁺ content (D) were quantified in shoot tissues of the two genotypes. Relative values were calculated by comparison to the corresponding genotype (WT or *prt6*) under non-stressed (0 mM NaCl) conditions. Data represent means $\pm$ SE (n = 10). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; *** P < 0.001).

We also analyzed the levels of metabolites associated with salinity tolerance in the *prt6* mutant and WT. Malondialdehyde (MDA), an end product of lipid peroxidation, is an indicator of oxidative damage caused by abiotic stresses including salinity stress (Velikova et al. 2008, Blande

et al. 2014). Under non-stress conditions, MDA accumulated to similar levels in both genotypes. Under salinity stress, however, the MDA level was significantly higher in the *prt6* mutant than WT, indicating that the *prt6* mutant was exposed to more severe oxidative damage. Increased accumulation of cellular osmolytes such as proline and soluble sugars is a well-characterized adaptive response to salinity stress (Diaz et al. 2010, Kong et al. 2011). Under non-stress conditions, the proline content was similar in the two genotypes. Salinity stress drastically elevated the abundance of proline in both genotypes, but its accumulation was significantly repressed in the *prt6* mutant.

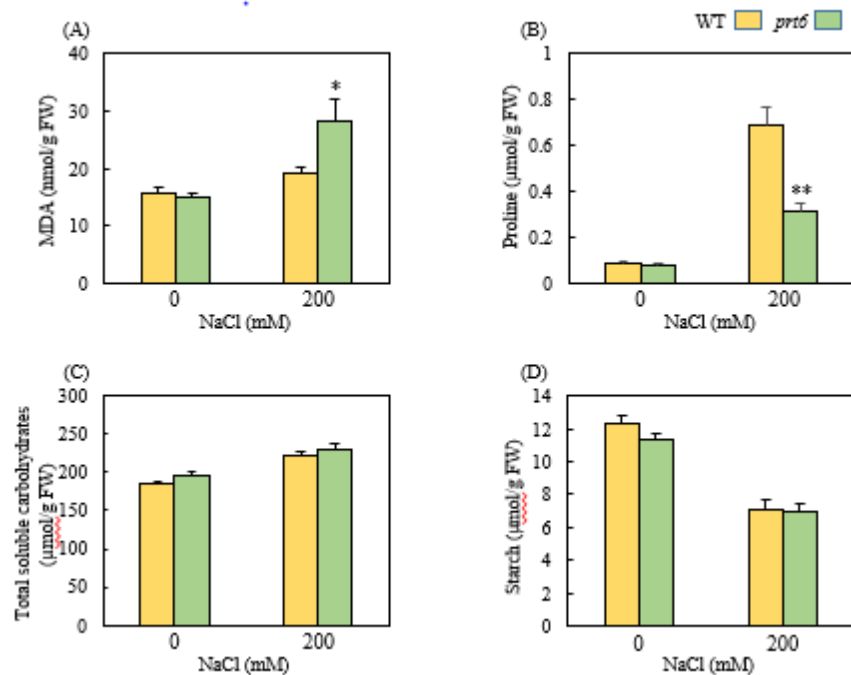


Figure 4. The effect of the *prt6* mutation on the accumulation of metabolites associated with salinity adaptation. Plants at the 3-leaf stage were treated with 0 or 200 mM NaCl for 2 weeks, and leaves were subjected to metabolite assays. MDA (A), proline (B), soluble sugar (C), and starch (D) contents were measured. Data represent means $\pm$ SE (n = 6). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; *** P < 0.001).

We also analyzed the levels of total soluble carbohydrates and starch. Salt stress slightly increased the abundance of total soluble carbohydrates, an adaptive response to osmotic/salinity stress (Figure 4C). However, the effect of the *prt6* mutation was not observed on the content of this metabolite. Consistent with soluble carbohydrate accumulation under salinity stress, the starch content decreased in response to this stress in both genotypes, but the influence of the *prt6* mutation was not detected (Figure 4D).

Effect of the *prt6* mutation on flowering time under salinity

To determine the impact of the *prt6* mutation on salinity tolerance at the reproductive stage, we evaluated flowering time in the *prt6* mutant and WT under high salt. Flowering time is a key attribute influencing grain yield and quality in maize (Song et al. 2017). Plants usually display a delayed flowering phenotype under high salinity conditions (Achard et al. 2006). In this study,

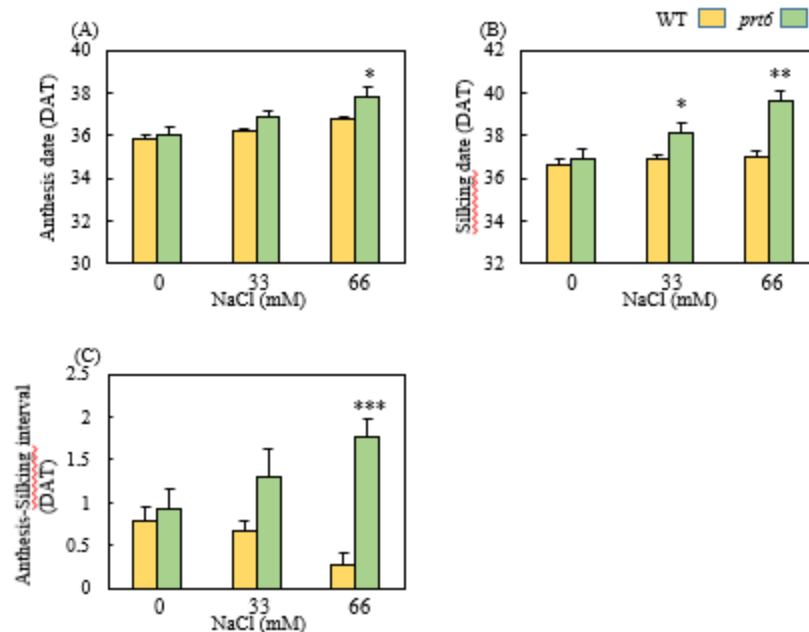


Figure 5. The *prt6* mutation delayed anthesis and silking and increased the anthesis-silking interval under salinity. Plants at the V9 stage were treated with 0, 33, or 66 mM NaCl until harvest. Anthesis date (A), silking date (B), and anthesis-silking interval (C) of WT or *prt6* plants were recorded. DAT indicates days after transplanting. Data represent means $\pm$ SE (n = 13). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; *** P < 0.001).

salinity stress delayed both anthesis and silking dates in both genotypes, but these phenotypes were more significantly affected in the mutant than the WT (Figure 5A and 5B). The anthesis-silking interval (ASI) is a critical trait for successful fertilization in cross-pollinated species such as maize (Singh et al. 2012). Our study showed that salinity stress increased ASI in the two genotypes, but the stress effect was more significant in the *prt6* mutant (Figure 5C).

Effect of the *prt6* mutation on grain yield and yield components under high salt

Salinity stress brings about a significant impact on kernel set and development in maize (Henry et al. 2015). Kernel abortion and poor kernel setting are the major causes of reduced grain weight and grain number under salt stress (Farooq et al. 2015, Henry et al. 2015). Grain weight and grain number are the important parameters that determine the final grain yield of maize. To evaluate the

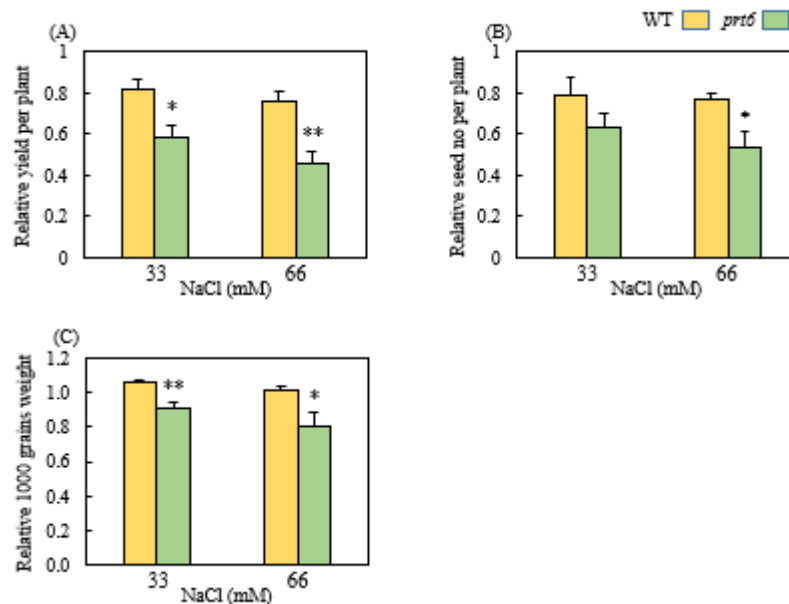


Figure 6. The *prt6* mutation decreased grain yield and related parameters under salinity. Plants at the V9 stage were treated with 0, 33, or 66 mM NaCl until harvest in a greenhouse. Relative yield per plant (A), relative no of seeds per plant (B), and relative 1000-grain weight (C) of WT or *prt6* plants were analyzed. Relative values were calculated by comparison to the corresponding genotype (WT or *prt6*) under non-stressed. Data represent means $\pm$ SE (n = 13). Asterisks indicate significant difference between WT and *prt6* (* P < 0.05; *** P < 0.001).

effect of the *prt6* mutation on grain yield and yield components under salinity, we treated the mutant and WT plants with moderate salt stress at the V9 to harvest stages. Our study showed that relative grain yield was more significantly reduced in the *prt6* mutant than WT under salinity (Figure 6A). Consistently, relative seeds number per plant and 1000-grain weight were lower in the mutant under the stress (Figure 6B and 6C). Because grain yield is the product of the total number of grains and grain weight, it is obvious that lower grain yield was caused by the reduced yield parameters in the *prt6* mutant.

DISCUSSION

In the present study, we characterized the role of an N-end rule pathway (NERP) E3 ligase, *PROTEOLYSIS 6 (PRT6)*, in salinity tolerance in maize. In Chapter I, we revealed that *PRT6* plays a negative role in adaptation to high salt in Arabidopsis. However, our morphological and physiological analyses demonstrated that *PRT6* gene functions as a positive regulator of salinity tolerance in maize.

Our analysis showed that a loss of function mutant, *prt6*, attenuated seed germination and seedling root growth under high salt (Figure 2A and 2B). Similarly, salinity stress reduced vegetative growth in the mutant plants more severely (Figure 3B). These observations are opposite to those in Arabidopsis shown in Chapter I. Maintaining vigorous root growth to ensure continued water uptake is an adaptation trait of a plant to salinity stress (Snapp and Shennan 1992, Shelden et al. 2019). A significant reduction in primary root elongation in the *prt6* seedlings reflected more severe water loss in the mutant leaves under high salt (Figure 3C).

High salt induces over-accumulation of reactive oxygen species (ROS) (Bose et al. 2014). The accumulated ROS reacts with different cellular components, causing oxidative damage and even cell death (Mittler 2017). Our study revealed that MDA accumulation was higher in the *prt6* mutant than WT under high salt (Figure 4A). MDA is an indicator of lipid peroxidation caused by oxidative stress. Thus, this result indicates that the *prt6* mutant was more severely damaged by excessive ROS under salinity. It is known that proline contributes to scavenging reactive oxygen species (ROS) and modulating redox homeostasis (Szabados and Savoure 2010). This study showed that salinity stress stimulated the accumulation of proline in both genotypes, but the level of proline was significantly lower in the *prt6* mutant than WT (Figure 4B), linked with more severe oxidative damage in the mutant under high salt.

Besides ROS detoxification, proline also functions as an osmolyte that adjusts cellular osmotic potential (Diaz et al. 2010, Szabados and Savoure 2010). Accumulation of osmolytes such as proline and soluble carbohydrates is an adaptive response to water loss induced under high salt. This study evaluated the effect of the *prt6* mutation on the accumulation of soluble sugars under salinity, but the genotypic effect was not detected (Figure 4C). It appears that the *prt6* mutation negatively impacts only proline accumulation under salinity stress.

Maize plants use more water during flowering than any other developmental stages (Udom and Kamalu 2019). Therefore, low water availability around the time of anthesis and silk emergence can be disastrous for maize grain production because of its severe impact on seed set up and development (Denmead and Shaw 1960, Barker et al. 2005, Henry et al. 2015). This study showed that salinity delayed both anthesis and silking days and increased anthesis-silking interval (ASI) more significantly in the *prt6* mutant (Figure 5). ASI is a critical trait for successful fertilization in cross-pollination species including maize, but the increased ASI observed in salt-

treated *prt6* mutant (~ 1 day) may not be sufficient to explain reduced grain yield in this genotype (Figure 6).

In summary, the result presented here demonstrated that maize *PRT6* serves as a positive regulator for salinity tolerance, which is contrary to the role of the Arabidopsis *PRT6* gene in stress adaptation. *PRT6* is an E3 ligase of the N-end rule pathway, which regulates the turnover of particular target proteins. The distinct function of *PRT6* in salinity tolerance between maize and Arabidopsis may suggest that the N-end rule pathway in the two species controls the fate of different target proteins. Identification and functional characterization of novel *PRT6*'s targets in maize and Arabidopsis will provide useful information to elucidate the distinct regulatory mechanisms underlying *PRT6*-mediated salinity tolerance/susceptibility in the two species.

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CHAPTER III

Time-course Analysis of Physiological and Molecular Traits Associated with Efficient Nitrogen Uptake and Transport in Wheat

ABSTRACT

Application of nitrogen fertilizers is pivotal for high grain yield and desired grain protein content in wheat. However, only 30-50% of the applied N is actually absorbed by the plants and the remaining N is lost to water bodies and atmosphere, leading to water and air pollution. A comprehensive understanding of physiological and molecular mechanisms that underpin efficient nitrogen uptake and transport is a crucial step for the development of new wheat varieties with increased nitrogen use efficiency (NUE) while maintaining grain yield. In this study, comparative analysis was conducted between two soft red winter wheat genotypes, VA08MAS-369 and VA07W-415, to characterize the agronomic, physiological, and molecular traits related to efficient nitrogen uptake and transport in wheat. Under ample N supply, both genotypes displayed similar yield, yield parameters, and NUE indexes. At low N supply, however, line 369 maintained yield and yield-related parameters and exhibited higher NUE indexes. Our study also revealed that high NUE in line 369 was attributed to high nitrogen uptake efficiency (NUpE) under limited N. Plant ability to absorb nitrogen is largely influenced by root morphology and functions. We demonstrated that line 369 maintained root growth parameters such as maximum root length, total root volume and total surface area under low N, whereas these parameters were significantly lower in line 415. Additionally, genes encoding high-affinity nitrate transporters such as *TaNRT2.1* and *TaNRT2.2* were expressed more abundantly in roots of line 369 than line 415 at limited N. Altogether, our results suggested that line 369 is more adapted to limited N conditions due to

maintained root development and increased function of nitrogen uptake. Improvement of these traits using line 369 could be incorporated into a wheat breeding program to develop varieties with more efficient N uptake.

Keywords: *Triticum aestivum*, NUE, root architecture, N transporters

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a major staple crop, which provides nutrition to more than one-third of the world population. In 2014, wheat was cultivated on approximately 220 million hectares of land across the world (FAOSTAT 2014), making the crop more widely grown than any other cereals (Tamang et al. 2017). The application of nitrogen (N) fertilizers is essential for high grain yield and appropriate grain protein content in wheat. Out of total N applied globally, 18% is used for wheat, making it the largest N applied for any crops (Ladha et al. 2015). However, only 30-50% of N supplied is taken up by cereal including wheat (Delogu et al. 1998, Kant et al. 2011, Li et al. 2017, Tamang et al. 2017). The non-absorbed N is lost to water bodies and atmosphere through leaching, surface runoff, and denitrification, resulting in soil, water, and air pollution (Wang et al. 2014). Improvement of wheat ability to absorb N can contribute to mitigating this environmental issue and reducing the production cost for this major crop.

Plants, as sessile organisms, have evolved complex N uptake systems to support their life under fluctuating environmental conditions. Regulation of N transporter activities and modulation of root morphology and architecture in response to changing environments are well-documented mechanisms for efficient N uptake in plants (Xu et al. 2012). Plants acquire N from the soil through their roots mainly in the form of nitrate and ammonium. A set of plasma-membrane localized

transporters are responsible for the uptake of nitrate and ammonium into the root cells. Nitrate uptake in plants is mediated by two families of transporters; NITRATE TRANSPORTER 1 / PEPTIDE TRANSPORTER family (NPF) (formerly known as NRT1 family) and NITRATE TRANSPORTER 2 (NRT2) family (O'Brien et al. 2016, Wang et al. 2018). NRT2 transporters belong to the high-affinity nitrate uptake system and are highly expressed under nitrate limiting conditions (Wittgenstein et al. 2014, Tegeder and Masclaux-Daubresse 2018). In Arabidopsis, seven NRT2s have been identified to date (Tsay et al. 2007). Of these genes, *AtNRT2.1*, *AtNRT2.2*, *AtNRT2.4*, and *AtNRT2.5* are responsible for the uptake of approximately 95% of the total nitrate under limited N conditions (Li et al. 2007, Lezhneva et al. 2014). In rice, four NRT2 genes have been identified so far (Cai et al. 2008, Fan et al. 2015, Fan et al. 2017). In partnership with *OsNAR2.1*, high-affinity nitrate transporters, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3*, are actively involved in root nitrate influx (Yan et al. 2011). On the other hand, members of the NPF gene family have a low-affinity for nitrate and participate in the uptake and transport of various molecules including nitrate, peptides, and amino acids (Tegeder and Masclaux-Daubresse 2018). Unlike NRT2s, most of the NPF members are not responsive to low nitrogen (Wittgenstein et al. 2014). An exception is *NPF6.3* (*NRT1.1*), which has dual-affinity transport and sensing functions (Tsay et al. 1993, O'Brien et al. 2016). Although a large number of NPFs (Arabidopsis >51 NPFs, rice >80 NPFs) have been identified, only limited members have been functionally characterized to date (Li et al. 2017). Another major N source, ammonium, is absorbed by ammonium transporters (AMT) in plants. Six members of AMT have been identified in Arabidopsis, four of which (*AMT1:1*, *AMT1:2*, *AMT1:3*, and *AMT1:5*) are involved in the uptake of approximately 90% of ammonium (Loque et al. 2006,

Yuan et al. 2007). In rice, 10 AMT genes have been found, and *OsAMT1;1*, *OsAMT1;2*, and *OsAMT1;3* play a major role in ammonium uptake in roots (Li et al. 2016).

Besides N transporter activities, modification of root morphology in response to N supply is another documented mechanism for efficient N uptake in plants. N deficient conditions trigger a set of morphological and physiological responses in roots, called foraging (O'Brien et al. 2016). Enhanced root growth and increased absorptive surface of the root system, thereby directing roots towards the nutrient-rich part of the soil, is one of the classical responses of a plant to N limitation (Kiba and Krapp 2016). Previous studies have reported that higher root growth under N deficient conditions correlates with shoot growth, grain yield, and yield components in rice and maize (Jian-Bo et al. 2010, Peng et al. 2010, Qi et al. 2019), indicating an interdependent relationship between shoots and roots under limited N conditions. Therefore, breeding crop varieties for enhanced root system architecture is essential for improving N acquisition efficiency under limited N supply.

Plant ability to absorb N is regulated by complex genetic, morphological, and environmental factors. An advanced understanding of the regulatory mechanisms associated with nitrogen uptake and transport will facilitate the development of new crop varieties with enhanced NUE. However, past studies have been mostly on *Arabidopsis* and rice, and the underlying regulatory mechanisms are still elusive for other cereal crops including wheat. In this chapter, we explored morphological and physiological responses of soft red winter (SRW) wheat to different N supply, identifying key traits associated with efficient nitrogen uptake and transport.

MATERIALS AND METHODS

Plant Materials and Greenhouse Growth Conditions

In this study, we used two soft-red-winter wheat accessions, VA08MAS-369 and VA07W-415. Plants were grown in pots in a greenhouse at Virginia Tech under two nitrogen regimes (normal vs. low N) in a completely randomized design (336 plants; 21 plants x 2 nitrogen treatments x 2 genotypes x 4 time-points). First, seeds were grown in cell inserts (72 cells/flat) containing Metro mix 360 potting soil under natural light at 22 °C day/13 °C night. At the 3-leaf-stage, seedlings were transferred into a vernalization chamber and incubated at 9 °C under 8 h light (80 mmol/m²/s) and 16 h dark for 9 weeks. Vernalized plants were then transplanted into pots (2.4 L, one plant per pot) containing a mixture of 50% (v/v) Metro Mix 360 and 50% (v/v) sand and grown in the greenhouse under 16 h light (22 °C) and 8 h (13 °C) dark. All plants were supplied with a half-strength Hoagland solution without N (50 mL/plant) plus ammonium nitrate (5 mg N/plant) twice a week until the maximum tillering stage. After this developmental stage, 5 mg N/plant (normal N) or 1 mg N/plant (low N) was provided twice a week until harvest. Leaves, stems, and spikes (if available) were harvested at maximum tillering, jointing, anthesis, and harvest stages. For yield component analysis, 96 plants (6 plants x 2 nitrogen treatments x 2 genotypes x 4 replications) were used. For biomass and N contents analyses, 240 plants (5 plants x 2 nitrogen treatments x 2 genotypes x 3 time-points x 4 replications) were used.

Biomass and N Measurements

Harvested leaves, stems, and spikes (if available) were dried in an oven at 65 °C for 3 d, and their dry weights were measured. The concentration of N in dried leaves, stems, and spikes were

measured by combustion analysis using Vario MAX cube CNS (Elementar, Frankfurt, Germany) following the protocol developed by Kim et al. (2011).

NUE Index Analysis

Grain yield was calculated as grain weight (g) per plant. Nitrogen use efficiency (NUE) indexes were computed as follows. NUE for Yield (NUEY) = Grain yield (g) / N supplied (g); NUE for Protein (NUEP) = Grain protein content (g) / N supplied (g); N-uptake efficiency (NUE) = Total biomass N content (g) / N supplied (g); N-utilization efficiency (NUE) = Grain yield (g) / Above-ground biomass N content (g) (Moll et al. 1982, Good et al. 2004).

Hydroponic Culture Conditions

Plants were grown in a hydroponic system in a growth chamber under 16 h light (300 $\mu\text{mol}/\text{m}^2/\text{s}$) /8 h dark at 20 °C and 50% relative humidity. As a nutrient solution, a half-strength Hoagland's solution without N was used. As a nitrogen source, ammonium nitrate (1 mM NH_4NO_3 for normal N and 0.2 mM NH_4NO_3 for low N) was added to the solution. Four-day-old germinated seedlings were precultured in a hydroponic system for 10 days under normal N. Plants were then grown either under normal or low N for additional 8 days. Both root and shoot tissues were harvested at 0, 2, and 8 days after distinct N treatments. The collected tissues were frozen in liquid nitrogen, homogenized, and stored at -80 °C until use.

Root Morphology Analysis

Four-day-old seedlings were pre-cultured using a “cigar rolled method” (Watt et al. 2013) for 3 days under normal N condition. Then, the plants were incubated in the same system under normal or low N for additional 6 days. Normal and low N solutions used for this analysis were identical to those used for the hydroponic culture. Root morphology parameters were quantified using WinRhizo 2017a (Regent Instrument Inc. Canada).

Carbohydrate Compound Analysis

Carbohydrate and starch contents were quantified as described in Chapter II.

Nitrate, Ammonium, Amino acids, and Protein Assays

Quantification of nitrate and ammonium was performed according to the protocol described by van Veen et al. (2013). Tissues (75 mg) were homogenized in 450 μL of 0.83 N perchloric acid on ice. After centrifugation, 300 μL of the supernatant was mixed with 75 μL of 1 M bicine (pH 8.3) and 42 μL of 4 M KOH. The sample tubes were centrifuged and the neutralized supernatant was used for nitrate and ammonium assays. For nitrate assay, 10 μL of the extract was mixed with 40 μL of 5% (w/v) salicylic acid in 100% H_2SO_4 and incubated at 25 $^\circ\text{C}$ for 20 min. After incubation, 950 μL of 2 M NaOH was added to the solution (50 μL) and A_{410} of the mixture was determined with a spectrophotometer. For ammonium, 25 μL of the extract was mixed with 375 μL of 8.8% (w/v) salicylic acid, 10 M NaOH, 21.5 mM EDTA, and 6.7 mM sodium nitroferricyanide (III) dehydrate. The mixture was added to 625 μL of 70 mM NaH_2PO_4 (pH 12.0) and 45 mM sodium

dichloroisocyanurate, and then incubated at 25 °C for 2 h. After incubation, A_{660} of the solution was measured with a spectrophotometer.

Total amino acid was assayed from a neutralized extract obtained from nitrate and ammonium assays. The extract (80 μ L) was mixed with 50 μ L of 0.2 mM sodium cyanide resolved in 8 mM glacial acetic acid and 50 μ L of ninhydrin resolved in 100% 2-methoxyethanol. Following incubation at 100 °C for 15 min, 1 mL of 50% isopropanol was added immediately to the mixture. After cooling down the mixture to room temperature, the absorbance at 570 (A_{570}) was measured using a spectrophotometer. Ninhydrin reacts with both amino acids and ammonium. Therefore, to correct the measurements, a standard curve for ammonium was generated following the ninhydrin method. The ammonium absorbance was then computed using this ninhydrin standard curve and the ammonium concentration data obtained from the same extract. The actual amino acid absorbance was finally calculated by subtracting generated ammonium absorbance from total amino acid sample absorbance.

Total protein was quantified from 50 mg tissues in 400 μ L of an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) IGEPAL CA-360, and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. Protein concentrations of the samples were determined by Coomassie Plus protein assay reagent (Life Technology). Bovine serum albumin (BSA) was used as a standard.

Gene Expression Analyses

RNA extraction and qRT-PCR analyses were performed according to the protocols described in Chapter I.

Statistical analyses

Statistical analyses were performed using JMP Pro 14 (SAS Institute) and MS-Excel 2019 (Microsoft).

RESULTS

Effect of differential N application on yield and yield components

In this study, we grew two soft-red winter wheat accessions, VAMAS-369 (line 369) and VA07W-415 (line 415), in a greenhouse under normal or low N conditions to investigate the effect of N supply on yield and yield parameters (Figure 1). The two genotypes displayed similar grain weight per plant (yield) under normal N supply. Low N reduced grain yield in both wheat accessions, but line 369 maintained higher yield relative to line 415. Spike number per plant, grain number per spike, and grain number per plant are the major attributes determining grain yield in wheat. At N deficient conditions, line 369 exhibited significantly larger values with respect to these parameters. Furthermore, this analysis showed that 1000-grain weights, another attribute determining final grain weight, were not significantly distinct between the two genotypes under normal or low N. Altogether, these data indicated that higher grain yield in line 369 under limited N was attributable to maintained spike number per plant, grain number per spike, and grain number per plant.

Effect of differential N application on Nitrogen Use Efficiency (NUE) indexes

Nitrogen use efficiency for yield (NUEY) in cereal crops can be simply defined as the yield of crops per unit N supply. Similarly, NUE for protein (NUEP) is the result of total grain protein

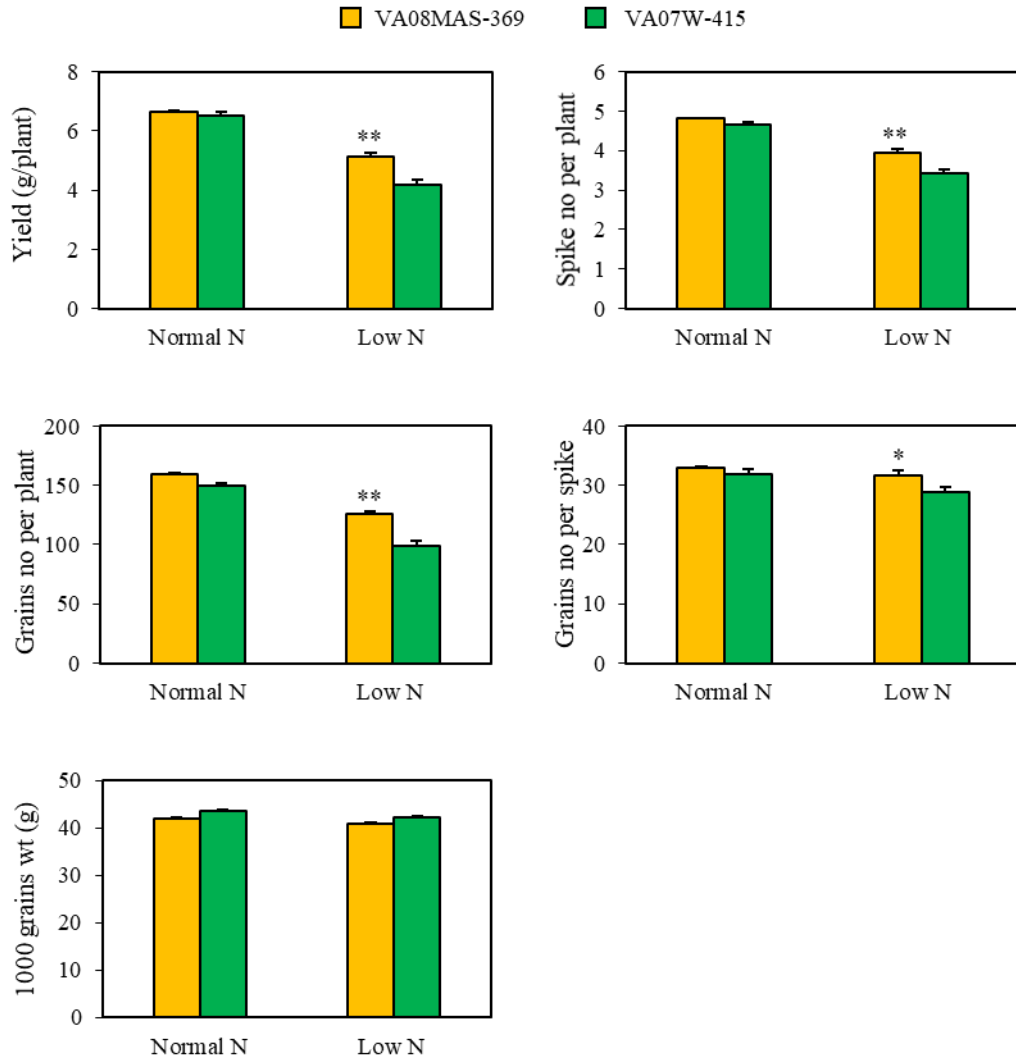


Figure 1. Effect of nitrogen application on yield and related components in two wheat accessions, VA08MAS-369 and VA07W-415. Data represents means $\pm$ SE (n = 4). *P < 0.05, **P < 0.01.

divided by total N supplied. NUEY can be separated into two components, Nitrogen uptake efficiency (NUpE) and Nitrogen utilization efficiency (NUtE) (Moll et al. 1982). NUpE is the efficiency of a plant to absorb available nitrogen from the soil and is calculated by dividing above ground biomass N content by the total N supplied. On the other hand, NUtE describes how efficiently absorbed N is utilized for final grain production. The NUtE is obtained by dividing

grain yield by the total N content. In this study, we investigated the effect of differential N applications on these NUE indexes (Figure 2). Both NUEY and NUEP were similar between the two accessions under normal N conditions. However, line 369 showed significantly higher NUEY and NUEP at low N supply. Likewise, no genotypic difference was observed between the two accessions for NUtE and NUpE under normal N input. Under N deficient condition, line 369 exhibited significantly higher NUpE compared to line 415. However, no statistical difference was detected between the two lines for NUtE under limited N conditions. Altogether, these results indicated that significantly higher NUEY in line 369 under limited N resulted from the efficient nitrogen uptake capacity in the genotype.

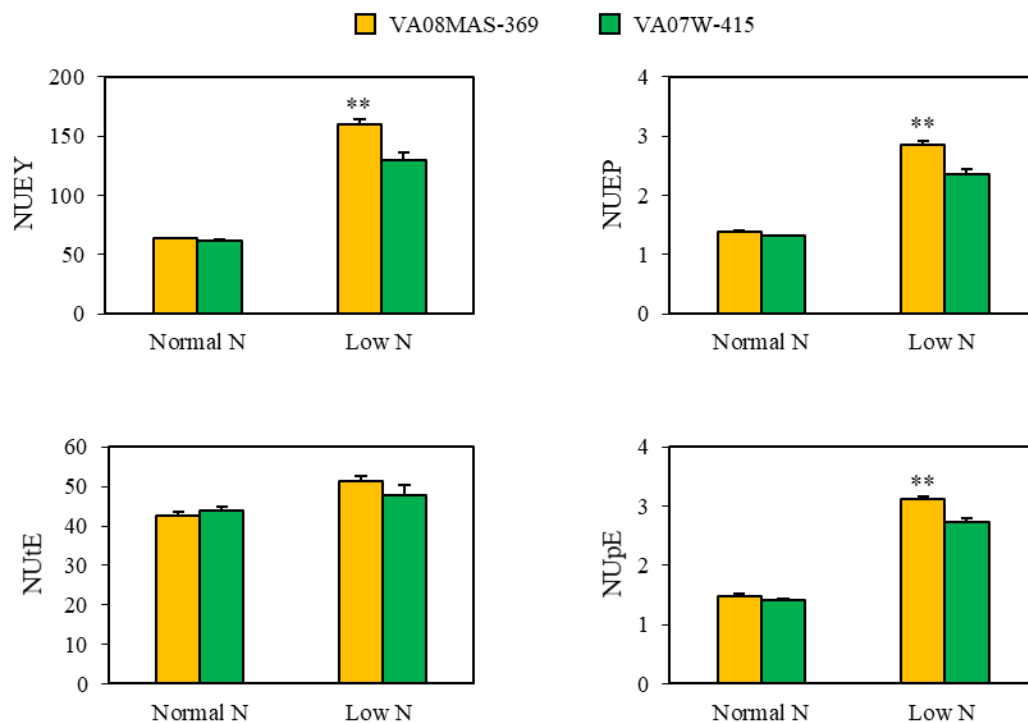


Figure 2. Nitrogen Use Efficiency (NUE) indexes of two wheat accessions, VA08MAS-369 and VA07W-415, grown under normal and low N conditions. NUEY, Nitrogen Use Efficiency for Yield = Grain yield / Total N supplied. NUEP, Nitrogen Use Efficiency for Protein = Grain protein content / Total N supplied. NUtE, Nitrogen Utilization Efficiency = Grain yield / Aboveground N content. NUpE, N Uptake Efficiency = Aboveground biomass N content / Total N supplied. Data represents means $\pm$ SE (n = 4). *P < 0.05, **P < 0.01.

Time-course observation of biomass and N content changes under low and high N

To determine the developmental stages when efficient N uptake occurs, we observed biomass and N content changes in leaves, stems, spike, and whole plant at different growth stages under normal and low N (Figure 3). At regular N input, leaf, and whole plant biomass was greater in line 415 at the maximum tillering and anthesis stages, respectively, but no genotypic difference was observed in any tissues at the harvest stage. Under low N, the biomass of all tissues analyzed was larger in line 369 than 415 at the harvest stage. These data indicated that line 369 has the ability to maintain biomass growth of leaves and stems as well as heads (grains) under limited N, especially after anthesis. This observation was consistent with the N accumulation patterns over the course of N treatments. Under low N, the N contents in stems, heads, and whole plants were significantly higher in line 369 at harvest. In leaves, the N content was lower in line 369 than line 415, presumably due to more efficient N transport in the high NUE accession, line 369. Overall, these results suggested that higher NUPE in line 369 under low N was caused by larger absorption at the post-anthesis stage in this genotype.

Changes in root morphological parameters in response to different N application

The root is the primary organ to receive nutrients from the soil and therefore essential for the efficient uptake of soil nutrients (Samejima et al. 2005, Bai et al. 2013). To elucidate the mechanisms by which line 369 takes up N efficiently from the soil, we investigated root architecture parameters in both lines under normal and low N conditions using a cigar rolled method (Figure 4) (Watt et al. 2013). Our analysis revealed that root morphological parameters such as maximum root length, average root diameter, total root surface area, and total root volume

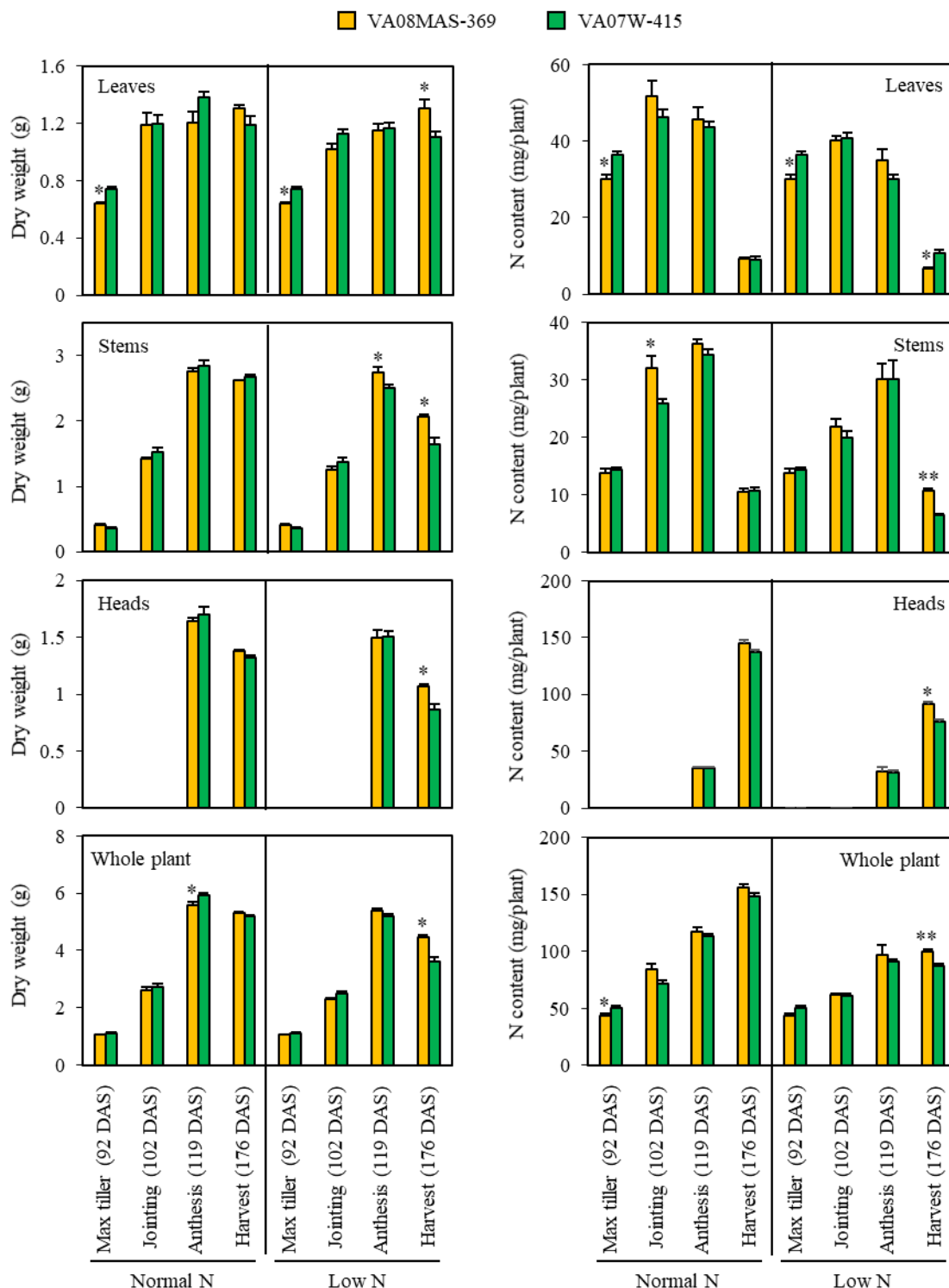


Figure 3. Biomass and N content changes in leaves, stems, and heads of plants grown under normal and low N during vegetative and reproductive stages. Plants were grown in pots under normal or low N in a greenhouse. Leaves, stems, and heads (if available) were harvested at the specified developmental stages and exposed to biomass and N content analysis. Data represents means $\pm$ SE ($n = 4$). * $P < 0.05$, ** $P < 0.01$.

were reduced by low N supply in line 415. However, in line 369, these root parameters were maintained or even increased under N deficient conditions. These data demonstrated that line 369 has the capability to maintain root growth under N limitation, contributing to high NUpE in this genotype.

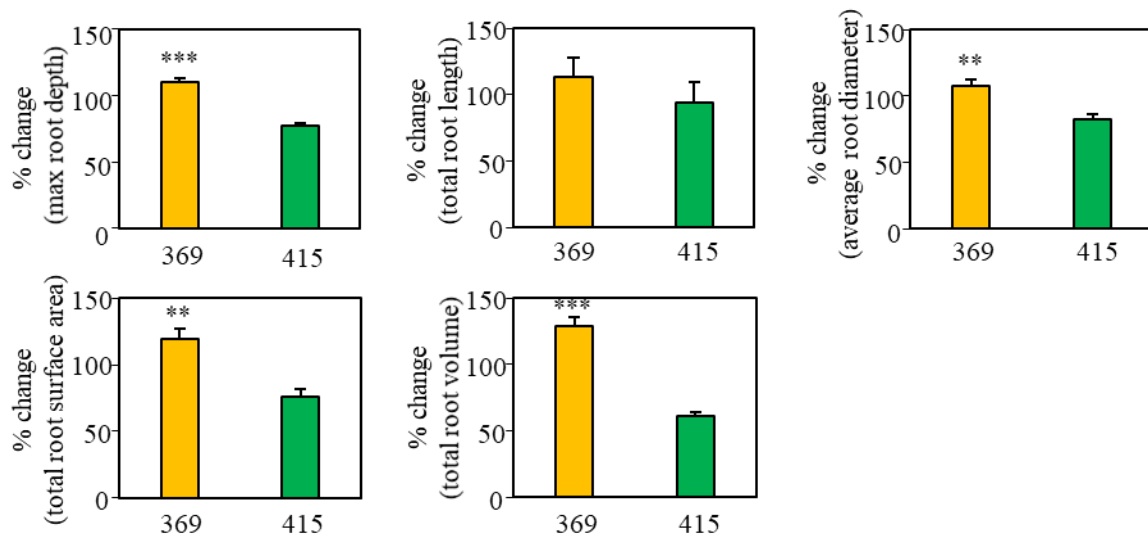


Figure 4. Root morphology of VA08MAS-369 and VA07W-415 grown under normal and low N. Four-day-old seedlings were pre-cultured using the cigar role method for 3 d under normal N. Then, seedlings were incubated in the same growth system under normal or low N for additional 6 d. Root morphology parameters were quantified using WinRhizo 2017a. Data represents means $\pm$ SE (n = 12). 369; VA08MAS-369. 415; VA07W-415. **P < 0.01, ***P < 0.001.

Effect of N application on biomass, C and N contents in a hydroponic system

Besides root architecture, the ability of roots to take up N is a critical factor determining NUpE in plants. In this study, we decided to monitor mRNA accumulation of nitrate and ammonium transporters in the two genotypes under regular and low N. This analysis required intact root tissues exposed to distinct N treatments. To harvest the tissue, we cultured wheat plants using a hydroponic system. We then investigated shoot and root biomass growth under normal and low

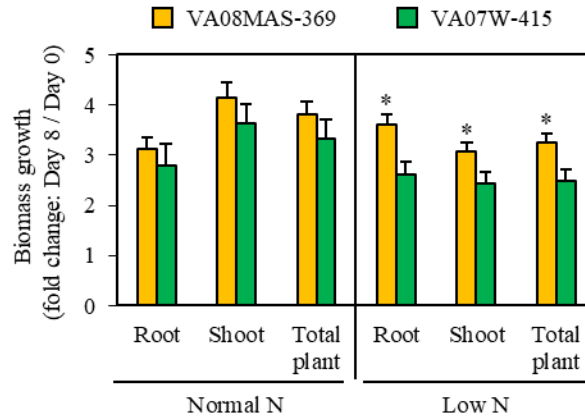


Figure 5. Biomass growth of VA08MAS-369 and VA07W-415 grown under normal and low N in the hydroponic culture system. Four-day-old seedlings were pre-cultured in the hydroponic system for 10 d under normal N. Then, plants were incubated in the same growth system under normal or low N for additional 8 d. Shoot and root tissues were harvested on day 0 (at the end of pre-culture) and 8 (at the end of differential N treatment) for dry weight measurements. Data represents means $\pm$ SE (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001.

nitrogen applications to evaluate whether their growth performance was similar under the hydroponic and greenhouse culture systems. Consistent with the greenhouse observations, root, shoot, and total plant biomass was larger in line 369 than line 415 under limited N in the hydroponic system (Figure 5). We further quantified the accumulation of carbohydrates compounds in the shoots and roots of plants grown under hydroponics system. In shoots, the total soluble carbohydrate and starch contents were greater in line 369 than line 415 at 2 and 8 d after low N treatment (Figure 6). In roots, the total soluble carbohydrate content was higher in line 369 only at 2 d after low N application. These results indicated that line 369 can maintain biomass growth and C accumulation under N limitation.

We also quantified major N components in shoots and roots of plants grown in the hydroponic system (Figure 7). Under regular N, overall accumulation patterns of nitrate, ammonium, amino acids and protein in the two genotypes were similar in shoots and roots. The

nitrate content was higher in line 415, whereas the ammonium and amino acid levels were greater in line 369. The protein content was not significantly different between the two genotypes. Under low N, the shoot nitrate level was lower in line 369 at the final time point, but the root nitrate level was conversely higher in this genotype. The level of ammonium was generally higher in line 369 than line 415 under low N in both shoots and roots although the time of ammonium accumulation was distinct

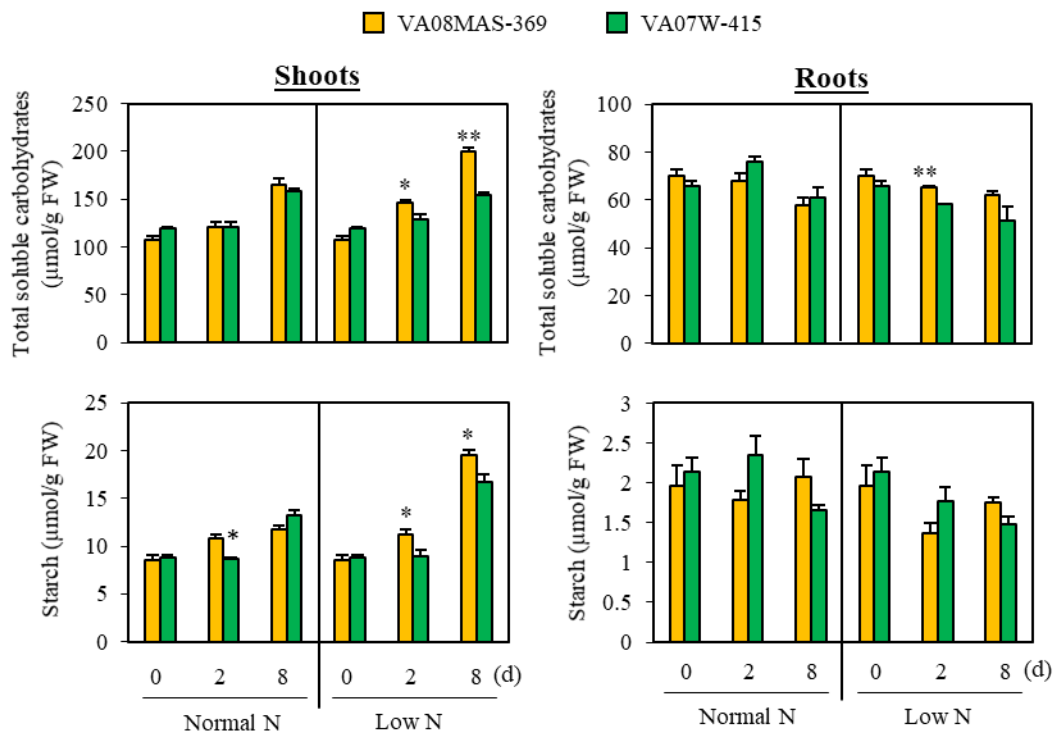


Figure 6. The concentrations of carbohydrate compounds in shoots and roots of VA08MAS-369 and VA07W-415 grown under normal and low N plants in the hydroponic culture system. Four-day-old seedlings were pre-cultured in the hydroponic system for 10 d under normal N. Then, plants were incubated in the same growth system under normal or low N for additional 8 d. Shoot and root tissues were harvested on day 0 (at the end of pre-culture), 2, and 8 for metabolite analysis. Data represents means $\pm$ SE (n = 3). *P < 0.05, **P < 0.01.

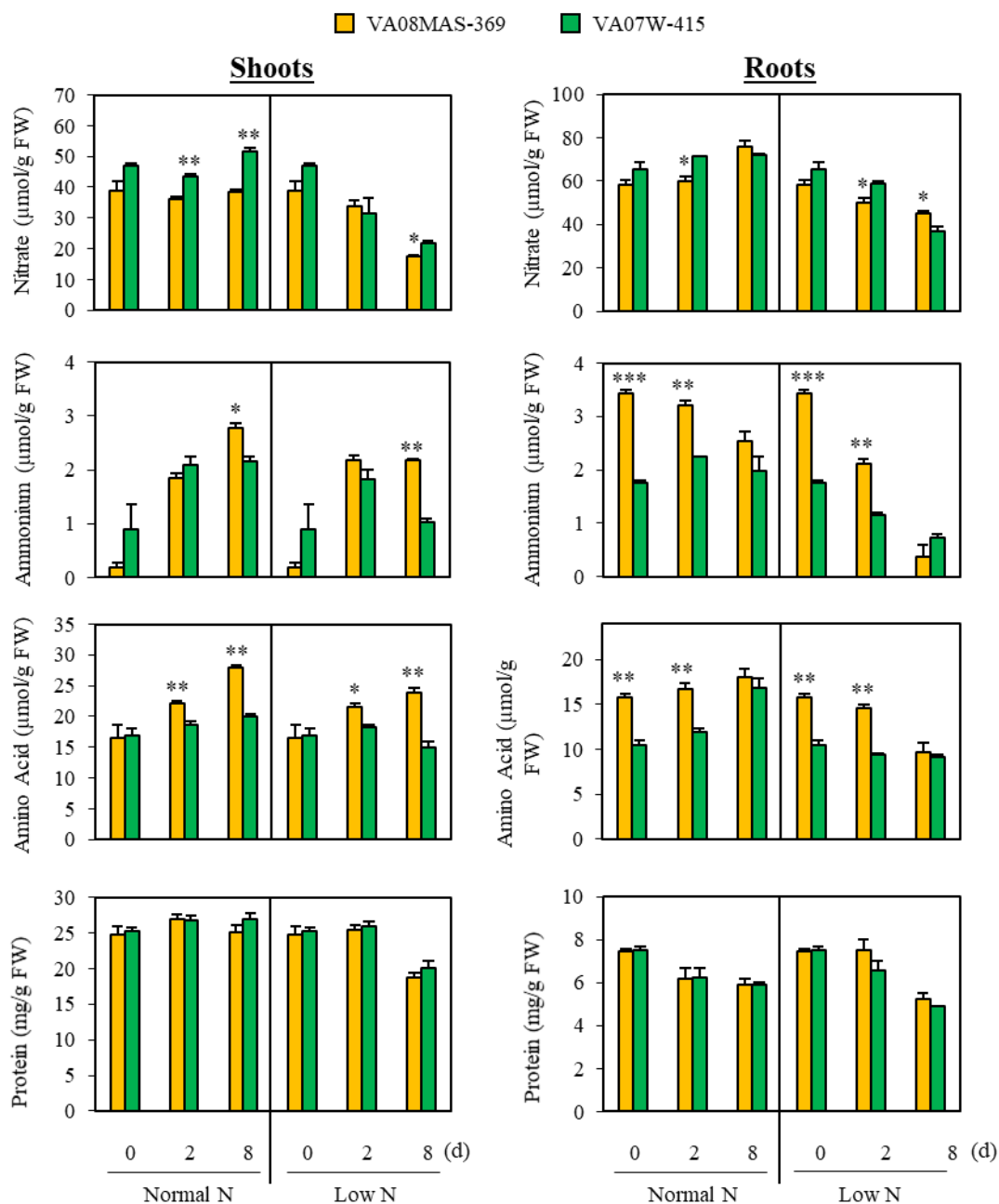


Figure 7. The concentrations of nitrogen compounds in shoots and roots of VA08MAS-369 and VA07W-415 grown under normal and low N in the hydroponic culture system. Four-day-old seedlings were pre-cultured in the hydroponic system for 10 d under normal N. Then, plants were incubated in the same growth system under normal or low N for additional 8 d. Shoot and root tissues were harvested on day 0 (at the end of pre-culture), 2, and 8 for metabolites analysis. Data represents means $\pm$ SE (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001.

between the two tissues. A similar trend was observed in the amino acid content. The protein level was not significantly distinct between the two genotypes in shoots and roots under low N.

Transcription accumulation of nitrate and ammonium transporter genes under regular and low N

Plants have developed two nitrate transport systems to support their life under fluctuating N availability. These two transport systems are called the high-affinity transport system (HATS) and low-affinity transport system (LATS) (Bajgain et al. 2018). This study investigated the mRNA accumulation of representative high-affinity (*NRT2*) and low-affinity (*NRT1/NPF*) genes in shoots and roots of wheat plants exposed to normal and low N conditions. Under regular N, the transcript level of *NRT2.1* was higher in line 369 than line 415 on day 0 in both shoots and roots (Figure 8). Under low N, this trend was observed on day 0 only in shoots. On day 2 and 8, the *NRT2.1* mRNA was highly accumulated in line 415. In roots, however, this transcript was greatly accumulated in the counterpart on day 8. Another high-affinity nitrate transporter, *NRT2.2*, was not detected in shoots, presumably due to no or low expression of this gene. In roots, accumulation of this transcript was more abundant in line 369 than 415 under both regular and low N.

We also analyzed five low-affinity nitrate transporters, *NPFs* (Figure 9). In shoots, the *NPF2.1* mRNA level was not significantly different between the two genotypes. In roots, this transcript was highly accumulated in line 369 on day 0 and 2 under both regular and low N. The *NPF2.2* mRNA was generally more abundant in line 369 than 415 under regular and low N in shoots and roots, although the level of this mRNA was higher in 415 on day 0 under regular N in shoots. The *NPF6.3* mRNA level in shoots was mostly greater in line 369 than line 415 under

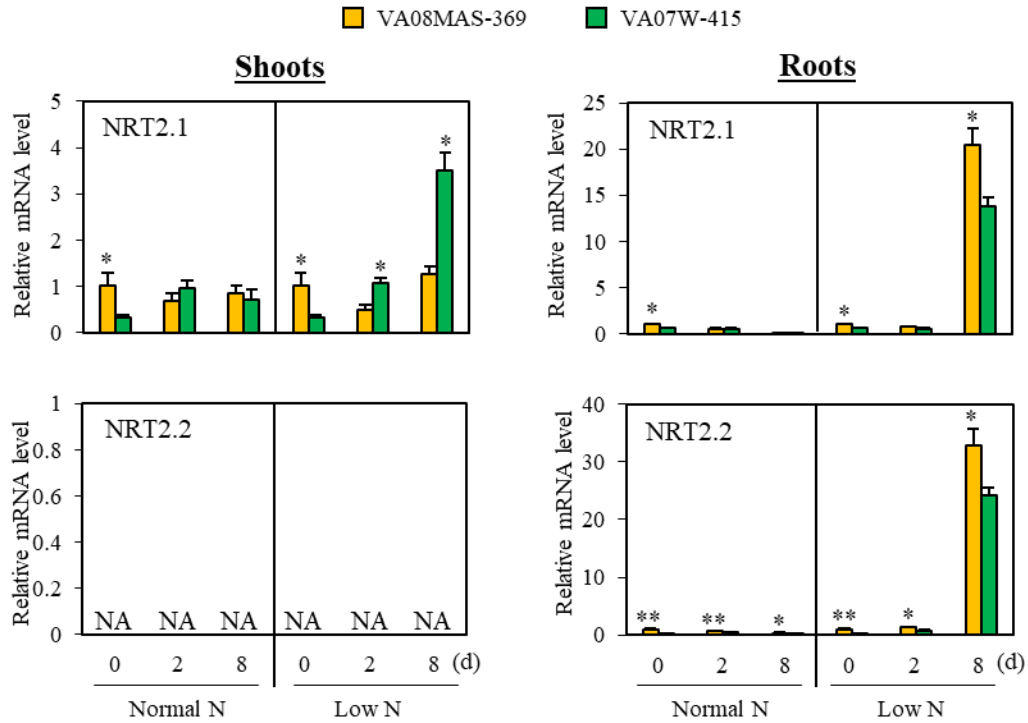


Figure 8. Relative mRNA levels of high-affinity nitrate transporters in shoots and roots of VA08MAS-369 and VA07W-415 grown under normal and low N in the hydroponic culture system. Four-day-old seedlings were pre-cultured in the hydroponic system for 10 d under normal N. Then, plants were incubated in the same growth system under normal or low N for additional 8 d. Shoot and root tissues were harvested on day 0 (at the end of pre-culture), 2, and 8 for gene expression analysis. Data represents means $\pm$ SE (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001.

normal N. At limited N, this transcript in shoots was less abundant in line 369 on day 2, but more abundant in this genotype on day 8. In roots, the *NPF6.3* mRNA accumulated to a greater extent in line 415 than line 369 on day 2 under both normal and low N. The mRNA level of *NPF7.1* in shoots was lower in line 369 than line 415 on day 0 under both regular and low N, although this level was higher in this genotype on day 8 under N limitation. In roots, the *NPF7.1* transcript was highly accumulated in line 369 on day 2 and 8 at low N. In terms of *NPF7.2*, this transcript level was not significantly distinct between the two genotypes during normal or low N treatment in shoots or roots. We further monitored mRNA accumulation of ammonium transporter genes

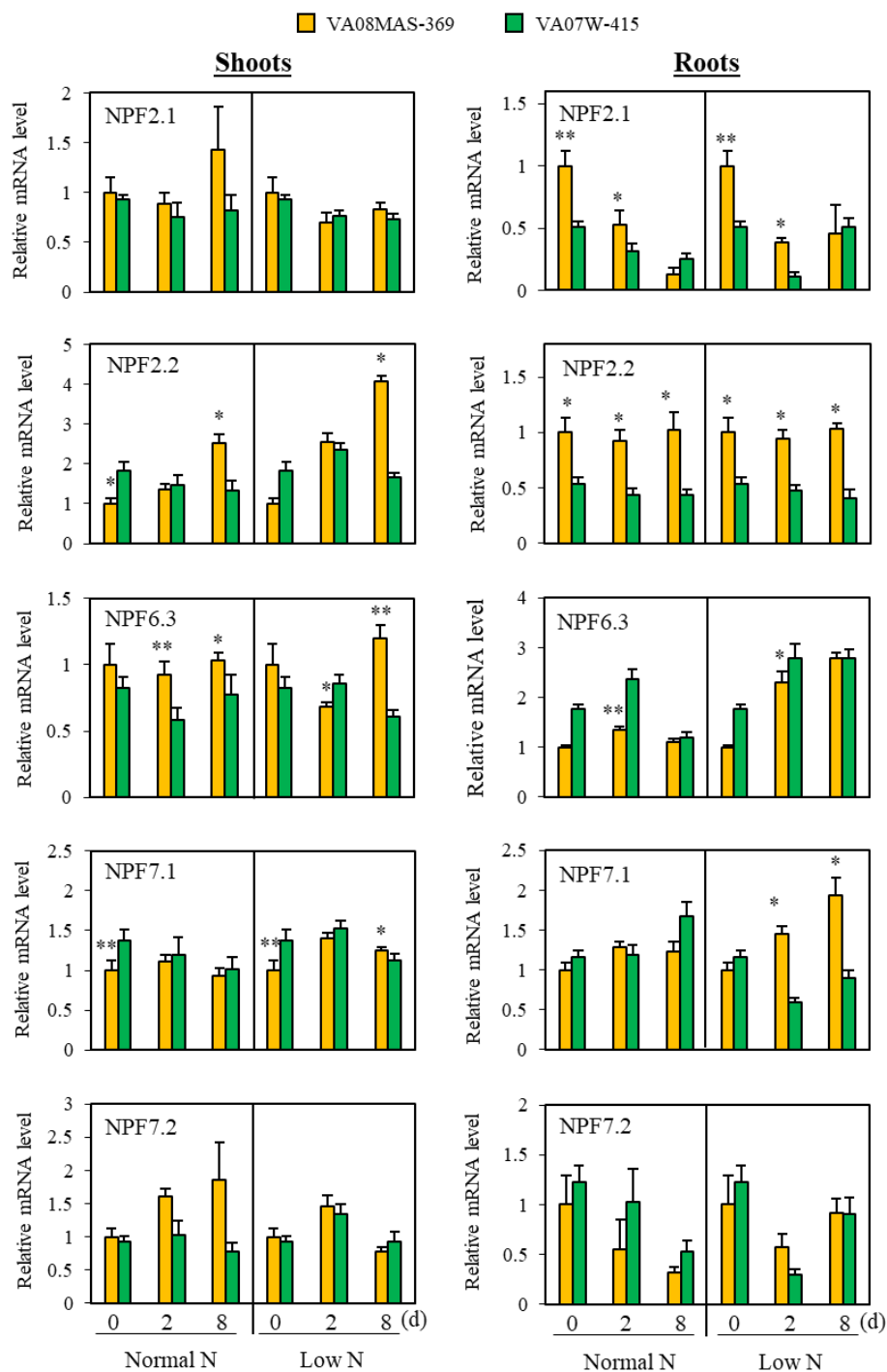


Figure 9. Relative mRNA levels of low-affinity nitrate transporters in shoots and roots of VA08MAS-369 and VA07W-415 grown under normal and low N in the hydroponic culture system. Four-day-old seedlings were pre-cultured in the hydroponic system for 10 d under normal N. Then, plants were incubated in the same growth system under normal or low N for additional 8 d. Shoot and root tissues were harvested on day 0 (at the end of pre-culture), 2, and 8 for gene expression analysis. Data represents means $\pm$ SE (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001

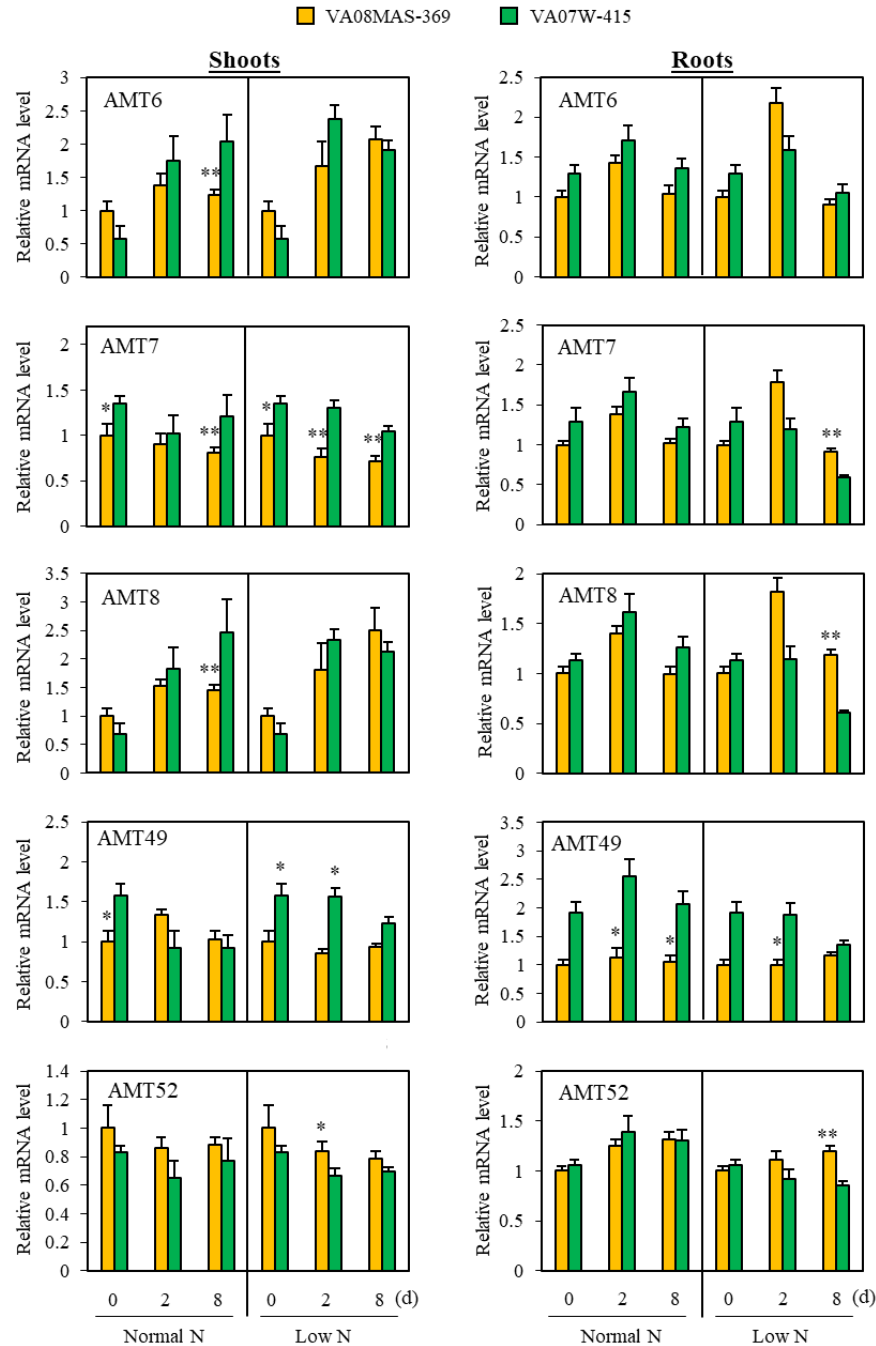


Figure 10. Relative mRNA levels of ammonium transporters in shoots and roots of VA08MAS-369 and VA07W-415 grown under normal or low N in the hydroponic culture system. Four-day-old seedlings were pre-cultured in the hydroponic system for 10 d under normal N. Then, plants were incubated in the same growth system under normal or low N for additional 8 d. Shoot and root tissues were harvested on day 0 (at the end of pre-culture), 2, and 8 for gene expression analysis. Data represents means $\pm$ SE (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001.

(Figure 10). The *AMT6* mRNA was more abundant in shoots of line 415 than line 369 on day 8 under normal N. The level of *AMT7* transcript in shoots was mostly larger in line 415 under both regular and low N, whereas in roots, this mRNA level was greater in line 369 on day 8 at limited N. The abundance of *AMT8* mRNA in shoots was higher in line 415 on day 8 under normal N, but this mRNA was highly accumulated in line 369 on day 8 under low N. Regarding *AMT49*, this transcript was generally more abundantly accumulated in line 415 than line 369 in shoots and roots under both N conditions. The level of *AMT52* mRNA in shoots was higher in line 369 than line 415 under low N on day 2 in shoots and on day 8 in roots.

DISCUSSION

This study first evaluated the yield performance of two SRW wheat genotypes, VA08MAS-369 and VA07W-415, under ample and limited N conditions. Our study demonstrated both genotypes displayed similar grain weight per plant (yield) under normal N supply. However, at low N supply, line 369 exhibited higher grain yield, which was attributed to the superior yield-related parameters such as spike number per plant, grain number per plant, and grain number per spike (Figure 1). A more detailed analysis regarding NUE indexes revealed that higher N uptake efficiency (NUpE) in line 369 was attributable to the high NUE in the genotype (Figure 2). Based on these results, we performed physiological and molecular evaluations to decipher the mechanisms which allow line 369 to absorb N efficiently even at low N supply.

Without a doubt, the plant root system is pivotal for the efficient acquisition of N from the soil. The capability of N acquisition under normal and low N conditions has been shown to correlate with the morphology of root systems (Peng et al. 2010). For example, maize genotypes with few crown root number and a deep root system exhibited significantly higher N uptake from

the soil (Saengwilai et al. 2014). In rice, high NUE was correlated with greater total root length and root surface area (Jian-Bo et al. 2010). In this study, we investigated the effect of limited N on root development in the two wheat genotypes with contrasting NUE. Low N suppressed major parameters associated with root development, such as maximum root depth, average root diameter, total root surface area, and total root volume in line 415, but these parameters were significantly greater in line 369 under N limitation (Figure 4). The ability of line 369 to maintain the development of root systems must contribute to efficient N acquisition at N deficit conditions.

Besides root morphological parameters, the activities of nitrate and ammonium transporters play a crucial role in the acquisition of N from the source (He et al. 2015). Disruption of the high-affinity nitrate transporters *nrt2.1* and *nrt2.2* in *Arabidopsis* reduced the uptake of nitrate in *Arabidopsis* (Cerezo et al. 2001). Similarly, overexpression of an *OsNRT2.3* nitrate transporter in rice improved NUE and grain yield substantially (Fan et al. 2015), indicating that the activity of high-affinity transporters is key for the uptake of nitrate. The present study revealed that the mRNA abundance of the genes encoding high-affinity nitrate transporters, *TaNRT2.1* and *TaNRT2.2*, was significantly higher in the roots of line 369 under N deficient conditions (Figure 8). A previous study demonstrated that the dominant expression of *TaNRT2.1* resulted in a larger influx of nitrate in wheat roots (He et al. 2015), which is also consistent with our observation in line 369 (Figure 7 and 8). In shoots, the expression of *TaNRT2.1* was higher in line 369 than line 415 under low N (Figure 8), suggesting higher root to shoot transport activity in line 415.

Low-affinity NRT1 (NPF) nitrate transporters are also reported to be associated with root nitrate uptake (Li et al. 2017). In this study, we analyzed the expression of representative NPF transporters, *NPF2.1*, *NPF2.2*, *NPF6.3*, *NPF7.1*, and *NPF7.2* in the roots and shoots of both genotypes under normal and low N supply. One of the most interesting results is the inducible

expression of *TaNPF7.1* in roots of line 369 under low N (Figure 9). In line 415, however, *TaNPF7.1* was not induced by low N supply. He et al. (2015) proposed that the higher influx of nitrate in a wheat transgenic line overexpressing the *TaNAC2A* transcription factor might be due to the higher expression of *TaNPF7.1* in roots. Therefore, induced expression of the *NPF7.1* gene in line 369 could be a contributor for higher N uptake in this genotype under limited N. Likewise, we also analyzed the expression of representative ammonium transporters. The expression levels of *TaAMT7* and *TaAMT52* were higher in roots of line 369 on day 8 under limited N (Figure 10). However, the functions and localization of these ammonium transporters have not been characterized in wheat yet.

In this study, we also quantified the amount of carbon and nitrogen compounds in roots and shoots of both genotypes under normal and low N conditions. We observed significantly higher nitrate accumulation in roots of line 369 compared to line 415 on day 8 under low N (Figure 7). This is likely to be related to the higher expression of high-affinity nitrate transporters in roots of line 369 (Figure 8). Interestingly, the accumulation of nitrate was significantly lower in shoots of line 369 than line 415 under limited N. A previous study has demonstrated that root to shoot ratios depend on N availability and higher accumulation of nitrate in shoots inhibited root growth in tobacco (Scheible et al. 1997). Therefore, lower accumulation of nitrate in shoots of line 369 might be a physiological adaptation of the line to maintain higher root biomass under N deficient conditions. Despite the lower shoot nitrate level, line 369 was able to maintain substantially higher levels of ammonium and amino acids under low N, indicating higher N assimilation activity in this genotype. Glutamine synthase (GS) and glutamate dehydrogenase (GDH) are the key genes involved in N assimilation in plants (Kant et al. 2008). Our previous work revealed that expression of *GS1*, *GS2*, *GSr1*, and *GDH1* was higher in line 369 than line 415 in flag leaves at low N input

after anthesis (Alpuerto et al. 2017). This result is consistent with our hypothesis that higher accumulation of ammonium and amino acids in line 369 results from higher N assimilation in this genotype. Further investigation is still necessary to determine how these genes are regulated in young leaves. The amino acid level in plant tissues not only depends upon the availability of nitrogen but also upon the supply of carbon skeletons. It has previously been shown that nitrogen supply affects carbohydrate distribution within the plant (Druege et al. 2004). Higher demand for carbon skeleton for the generation of amino acids drives the accumulation of carbohydrate compounds (Kant et al. 2008). In this study, this increased demand was reflected in the levels of starch and soluble sugars in shoots of line 369 (Figure 7). Alternatively, the significantly higher levels of both starch and soluble sugars in shoots of line 369 at normal and low N might also be an indication of maintained photosynthesis activities in the genotype even under N deficient conditions.

In summary, our physiological and molecular analyses demonstrated that the relatively higher capability of line 369 for nitrogen uptake is attributable to greater performance in terms of root system development and higher expression of genes associated with N uptake and transport. We also observed that line 369 exhibited significantly higher biomass and N content in leaves, stem, and head of the plants grown under low N conditions mainly at the latter time points of the reproductive stage (Figure 3). Additional experiments to investigate N metabolism and the regulation of gene expression at these developmental stages are crucial to advance our understanding of NUE regulation in wheat.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Abiotic stresses such as flooding, drought, salt, nutrient deficiency, heat, and cold are major environmental constraints that reduce plant growth and development, threatening agricultural productivity worldwide. An increase in the frequency of extreme weather events, as the results of global climate changes, will further damage crop yield and quality throughout the world. On the other hand, maintaining food supply to support the ever-increasing population has been a burden for plant scientists. The application of synthetic fertilizers has supported elevated productivity of various crops, but this approach may not be sustainable and environmentally friendly. Therefore, genetic improvement of crop adaptation to abiotic stresses is becoming increasingly important for ensuring food security and maintaining environmentally friendly agriculture.

Plants can encounter multiple abiotic stress simultaneously or sequentially. Sea level rise increases the chances of saltwater intrusion into cultivable lands, making a hostile environment for crop growth and production by imposing flooding and salinity stress concurrently. The PROTEOLYSIS 6 (PRT6) branch of the N-end rule pathway of targeted proteolysis is a well-characterized ‘negative regulator’ for plant tolerance to flooding and low oxygen. In Chapter I, we evaluated whether the PRT6/N-end rule pathway is involved in tolerance to salinity stress in *Arabidopsis* through detailed physiological and molecular characterization of *prt6-1* mutant and wild-type lines. Our study demonstrated that the *prt6* mutation increases salinity tolerance at the seed germination, seedling, and adult plant stages through the activation of hormonal and transcriptional responses associated with salinity adaptation. Tolerance to salinity-induced osmotic stress is regulated via ABA-dependent and ABA-independent pathways. Our study found that the

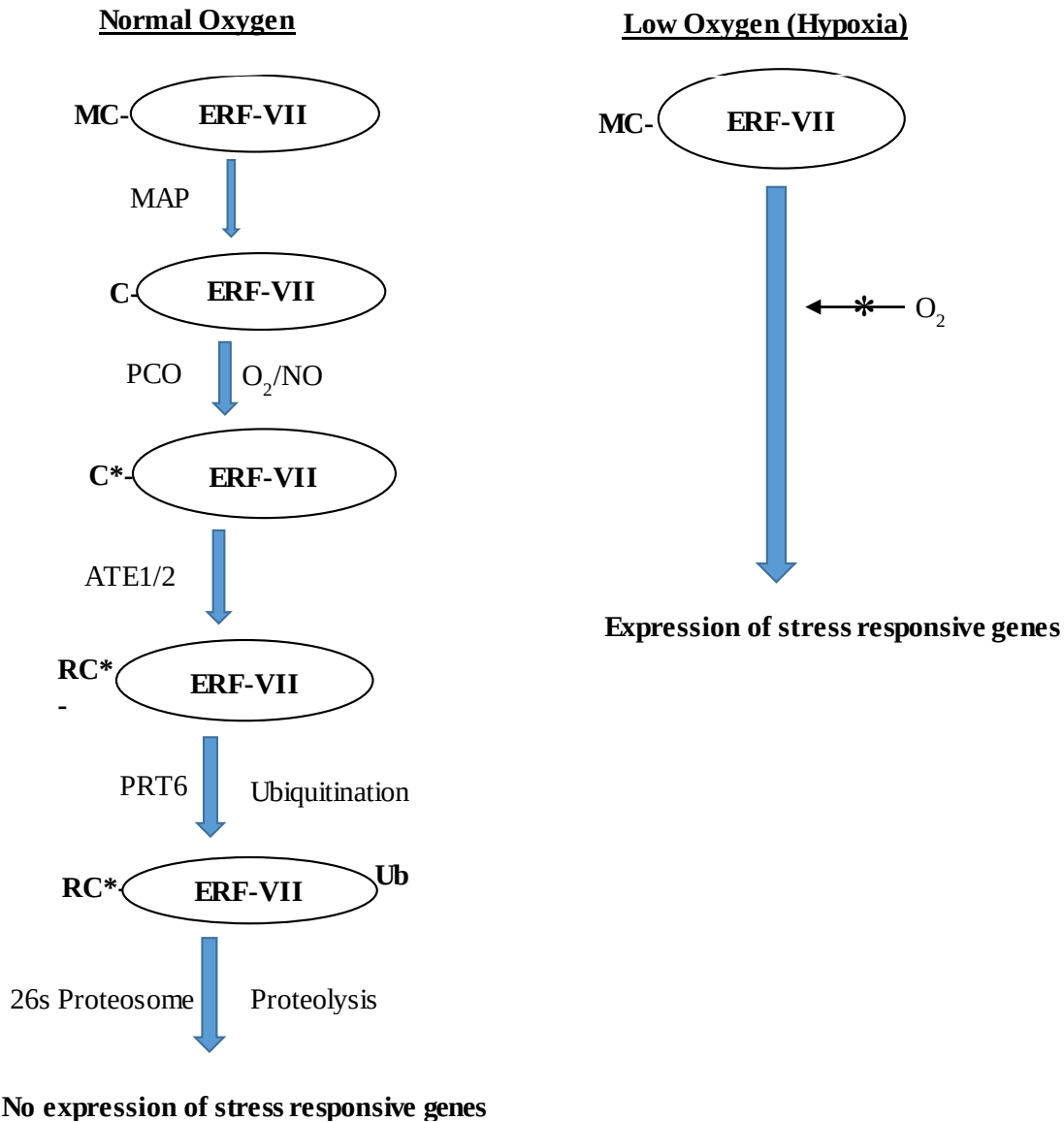
prt6 mutation increases mRNA accumulation of key transcription factors involved in ABA-dependent (ABFs/AREBs) and independent (DREB2s) pathways. Ethylene and BR play a crucial role in limiting Na^+ uptake into the roots and transport into the shoots of plants and inducing the expression of dehydrins such as *RD29A* and *RD29B*. Our study revealed that the *prt6* mutation enhances the responsiveness to both ethylene and BR, resulting in restricted Na^+ accumulation in roots and shoots and increased expression of dehydrin genes.

In Chapter II, we further investigated the role of the PRT6/N-end rule pathway in adaptation to salinity stress in a commercially important crop, maize. In contrast to our results in Arabidopsis, our morphological and molecular analyses demonstrated that the *PRT6* gene functions as a positive regulator of salinity stress in maize at the seed germination, seedling, and adult plant stages. Under high salt, the *prt6* mutation reduced seed germination, primary root elongation, and shoot biomass growth. In addition, malondialdehyde (MDA) accumulation was significantly higher in *prt6* than wild-type plants under high salt, indicating that the mutant plants encountered more severe oxidative stress. Moreover, salinity stress reduced grain yield and yield-related components in this genotype. Altogether, our results provide new insight into the functional divergence of the *PRT6* gene to salinity tolerance between Arabidopsis and maize. The distinct function of *PRT6* gene in the two species may suggest that the pathway in Arabidopsis and maize regulates the fate of different target proteins. Identification of novel targets of the PRT6/N-end rule pathway in Arabidopsis and maize will provide useful information to elucidate the molecular mechanisms regulating distinct salinity tolerance.

Wheat is a major staple crop and is the most widely grown around the world. Application of nitrogenous fertilizers is essential for high grain yield and appropriate grain protein content in wheat. However, only 30-50% of the total N application is taken up by the plants and the remaining

N is lost to water bodies and atmosphere, causing water and air pollution. In Chapter III, a comparative analysis was conducted between two soft-red winter wheat genotypes, VA08MAS-369 and VA07W-415, to characterize the physiological and molecular traits associated with efficient nitrogen uptake and transport in wheat. Our agronomic data revealed that line 369 maintained yield and yield-related parameters, relative to line 415, under N deficient conditions. Higher nitrogen use efficiency (NUE) in line 369 at N limitation was attributed to higher nitrogen uptake efficiency (NUpE) in the genotype. Physiological and molecular analyses suggested that efficient N uptake in line 369 resulted from maintained development of root systems and enhanced mRNA accumulation of the nitrate transporters, especially high-affinity nitrate transporters, even under limited N. Consequently, this study determined key phenotypes which are closely associated with high N uptake efficiency in line 369 at low N, useful information for breeding of new wheat accessions with enhanced NUE. All results presented here were generated under controlled greenhouse conditions. Field analysis of the two genotypes in multiple locations and years is the next step for further understanding of the mechanisms underlying nitrogen uptake in wheat.

APPENDIX I: Supplementary Files for Chapter I



Supplementary Figure 1. Diagrammatic representation of the N-end rule pathway of targeted proteolysis. Under normoxia, the methionine residue of the Ethylene Response Factor (ERF)-VII transcription factors is first cleaved by methionine amino peptidase (MAP), exposing the cysteine residue. The exposed cysteine residue is later oxidized by plant cysteine oxidase (PCO) in the presence of oxygen and nitric oxide. The Arginine residue is subsequently added to the oxidized cysteine residue by arginyl tRNA transferase (ATEs). This modified N-terminal is recognized by the N-recogin PROTEOLYSIS-6 (PRT6) and targeted to degradation by the 26S proteasome. On the other hand, the degradation of the ERF-VII proteins is inhibited under low oxygen (hypoxia) because PCO requires molecular oxygen. As a result, ERF-VII transcription factors triggers the expression of hypoxia-responsive genes under low oxygen.

Supplementary Table 1. Primer sequences used for quantitative RT-PCR.

Gene name	Forward primer (5' ==> 3')	Gene ID	References
ABF1-F ABF1-R	TGCAGAAGAAACAGGCTGAA GACCGGT AAGGGT TCTTCTCA	AT1G49720	Yoshida et al., 2015
ABF3-F ABF3-R	CGTTCTCAACCTGCAACACA TCATAGGATGGTTATGAATCCAAG	AT4G34000	Yoshida et al., 2015
AREB1-F AREB1-R	GAGAGAAGGCCAAAGGAGAATGA CTTCAAGCTCCACGGTGT AAG	AT1G45249	Yoshida et al., 2015
AREB2-F AREB2-R	GCAACCTGGTGCTGGTATCC TGTTCTAGGCAACGTCAACGA	AT3G19290	Yoshida et al., 2015
DREB2A-F DREB2A-R	CAGTGTTCGCAACGGTTCAT AAACGGAGGTATTCGTAGTTGAG	AT5G05410	Sakuma et al., 2006
DREB2B-F DREB2B-R	CAGGCCGGA CAGTTGCA CCCAATACTGCTGCTCAAACCTCTA	AT3G11020	Sakuma et al., 2006
RD29A-F RD29A-R	TGGATCTGAAGAACGAATCTGATATC GGTCTTCCCTTCGCCAGAA	AT5G52310	Sakuma et al., 2006
RD29B-F RD29B-R	GCAAGCAGAA GAACCAATCA CTTTGGATGCTCCCTTCTCA	AT5G52300	Yoshida et al., 2015
RAB18-F RAB18-R	TCGGTCGTTGTATTGTGCTTTTT CCAGATGCTCATTACACACTCATG	AT1G43890	Yoshida et al., 2015
SOS1-F SOS1-R	TGGAGGAAGCGACCGATTCTC CGATAACGAGAAGAGCGACAG	AT2G01980	Cheng et al., 2016
HKT1-F HKT1-R	TCTGGTCTCATCTGGCTCCTAATC CACCGTCACTCCAAGAAGAACAC	AT4G10310	Cheng et al., 2016
HRE1-F HRE1-R	GGCCTCTGCCTTATCCCTCTGT GCGTAAACCCGTCTCAGTGAGTG	AT1G72360	Licausi et al., 2010
HRE2-F HRE2-R	GAAGCGTAAACCCGTCTCAGT TTTGCTCGGGCACGAATCT	AT2G47520	Licausi et al., 2010
RAP2.2-F RAP2.2-R	AATCCGTGGCACGAAAAGC GGATACGACGCTAGGGTTCTTCT	AT3G14230	Papdi et al., 2015
RAP2.3-F RAP2.3-R	CGTCATCGCCACGATCAA TGAGAGACAACGCAGACCTTCTT	AT3G16770	Papdi et al., 2015
RAP2.12-F RAP2.12-R	CGCTGCGGAAGGTT CAGTT CAGCCCATTTTCCCCAAGGA	AT1G53910	Paul et al., 2016
ADH1-F ADH1-R	TATTCGATGCAAAGCTGCTGTG CGAACTTCGTGTTTCTGCGGT	AT1G77120	Paul et al., 2016
PDF1.2-F PDF1.2-R	CACCTTATCTTCGCTGCTCTT GCCGGTGCGTCGAAAG	AT5G44420	Van der Does et al., 2013
ACO2-F ACO2-R	CCTCCTCTCAACCACTCTATTGTC CATCTTCATAAACATAAACACCCCACAC	AT1G62380	Dong et al., 2015
ERF1-F ERF1-R	ATTCTTTCTCATCCTCTTCTTCT CGAATCTCTTATCTCCGCCG	AT3G23240	Mao et al., 2016
BAS1-F	CCAAGGACCATGTCGTTAAGC	AT2G26710	Schneider et al., 2012

BAS1-R	CCTGAAGTATAGCAAGATTCTGACC		
PAE8-F	GGGCATCATTACAGGAGAT	AT4G19420	Xie et al., 2015
PAE8-R	CAGCAGAACAGCCAGACAAA		
XTH33-F	ATACCCTCATTTGGAACCTCCACC	AT1G10550	Dong et al., 2014
XTH33-R	CACTTAACTCCACGTGAGCAACG		
Saur_AC1-F	CAGAAGAAGAAGAGATATGTGGTG	AT4G38850	Zhu et al., 2013
Saur_AC1-R	GTATTGTTAAGCCGCCCATTG		
18S RNA-F	AAACGGCTACCACATCCAAG	AT2G01010	Yoshida et al., 2015
18S RNA-R	CCTCCAATGGATCCTCGTTA		
UBQ10-F	AGATCCAGGACAAAGGAAGGTATTC	AT4G05320	Lin et al., 2013
UBQ10-R	CGCAGGACCAAGTGAAAGAGTAG		

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APPENDIX II: Supplementary Files for Chapter III

Supplementary Table 1. Primer sequences used for quantitative RT-PCR.

Gene name	Primer sequence (5' ==> 3')	Gene ID	Reference
TaNRT2.1-6B F TaNR2.1-6B R	GTGGTGCTGTATTTGGCGTCG AAGAAGTTGCGTTAGCCCTGC	Traes_6bs_19784bbef	He et al 2015
TaNRT2.2-6D F TaNR2.2-6D R	TTCCAGTGTAGACTTGTGTAATGC CTCAAAGTTGCTGTAAATTCAGACC	Traes_6DS_9184F1B4B	He et al 2015
TaNPF2.1 F TaNP2.1 R	CGG TGG TAC AGG TTC AAG AAG TC CGG AGT ATA KGA AGC CCC AAA AG	AK334628;TC280584;HF544999	Buchner 2014
TaNPF2.2 F TaNP2.2 R	GTG GAC CTR TTCTACCTT GTC AC AGC CTT CTT GGG GCT TTC ATC G	BJ244453;CV780655;BJ250261;HF545000	Buchner 2014
TaNPF6.3 F TaNP6.3 R	CAY GGG AGC AAC GAC GGC TG ATG CGT TTC TCCTTG TAC ACG TAG	BG907608;TC391493;HF544987	Buchner 2014
TaNPF7.1-6D F TaNP7.1-6D R	CTACAAGACCTGCGCCATCTTC GATGAGGTATAGCCGCGAGGAG	BJ279017;HX175323;TC380559;HF544992	He et al 2015
TaNPF7.2-6B F TaNP7.2-6B R	CCCAGCAGTCAAAGCAAACACTG GGAGGAACACCACAGGTTTAC	TC334619;BU099863;TC433104;HF544993	He et al 2015
TaAMT1 F TaAMT1 R	ATGGCTAACACAGAAGCATCAA TTTCTTTTCGTGGCCTCTACTTC	TRIAE_CS42_6AL_TGACv1_472556_AA1523540.1	Bajgain, 2018
TaAMT2 F TaAMT2 R	GGACTACCTGTGCAACCAAGTTC CGAAGCCGAAAGAGGTAGTAGAA	TRIAE_CS42_6BL_TGACv1_501472_AA1617880.1	Bajgain, 2018
TaAMT3 F TaAMT3 R	CAGTATGGCAATGGAGAGTTCA CACCGATATATGCATTTTGTGC	TRIAE_CS42_6DL_TGACv1_526515_AA1685900.1	Bajgain, 2018
TaAMT4 F TaAMT4 R	GGACTACCTGTGCAACCAAGTTC CGAAGCCGAAAGAGGTAGTAGAA	TRIAE_CS42_6DL_TGACv1_527195_AA1700270.1	Bajgain, 2018
TaAMT5 F TaAMT5 R	GGACTACCTGTGCAACCAAGTTC CGAAGCCGAAAGAGGTAGTAGAA	TRIAE_CS42_6AL_TGACv1_473845_AA1533160.1	Bajgain, 2018
Ta Actin (3 like) F Ta Actin (3 like) R	GAC GCA CAA CAG GTA TCG TGT TG AGC GAG GTC AAG ACG AAG GAT G	Ta.28253.1.S1_at;TC441720	Buchner 2014
β-TaTubulin qRT-F β-TaTubulin qRT-R	ACCGCCAGCTCTTCCACCCT TCACTGGGGCATAGGAGGAA	U76558	He et al., 2015

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