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Enhanced root growth of the *Arabidopsis abi5* mutant under high salinity is associated with reduced NO levels in *Arabidopsis* seedlings

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Key massage

The impaired endogenous NO levels in *abi5-7* mutant confer salt tolerance of *Arabidopsis* plants resulting from the reduced transcript level of *NIA2* and resulting low NR activity.

Abstract

Many environmental factors and stresses influence plant growth and development. High salt stress is expected to have a damaging effect on more than 50% of fully arable land by 2050. Understanding the response of plants to excessive use of nitrogen fertilisers and salt stress is vital for increasing crop productivity. However, the effect of excessive nitrate application on plant growth is controversial and not well-understood; therefore, we investigated the effect of a high nitrate supply and high salt stress on plant growth performance. We showed that *abi5-7* plants exhibited tolerance to adverse environmental conditions of high nitrate and high salinity stress. Particularly, *abi5-7* plants showed lower endogenous nitric oxide levels than Col-0 plants because of their lower nitrate reductase activity, resulting from the decreased transcript levels of *NIA2*, a gene encodes nitrate reductase. The supply of excess nitrate reduced the plant's salt stress tolerance, in which nitric oxide appeared to play a crucial role. Therefore, discovering regulators, such as ABI5, that can regulate nitrate reductase activity and understanding the molecular functions of these regulators are important for implementing gene-editing techniques to modulate transcription factor activities, leading to the optimal accumulation of nitric oxide to increase the productivity of crops exposed to various stress environments.

Keywords: abscisic acid, *Arabidopsis*, *ABI5*, nitrate reductase, nitric oxide, salt stress tolerance

Abbreviations

ABA, abscisic acid; DAF-FM DA, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; HNHS, high nitrate and high salinity; L-NNA, Nω-nitro-L-arginine; MDA, malondialdehyde; MS, Murashige Skoog; NO, nitric oxide; NR, nitrate reductase; ROS, reactive oxygen species; SNP, sodium nitroprusside; TBA, 2-thiobarbituric acid, TCA, trichloroacetic acid

Introduction

Plant growth and development are typically affected by many environmental factors and stresses (Lu et al. 2015). Among these, salt stress is crucial for determining plant growth and productivity. Salinity is predicted to confer deleterious effects on more than 50% of total arable lands by 2050 (Ashraf and Wu 2011). Salt stress accompanied by oxidative stress also has various harmful effects on plant growth and development at the physiological, biochemical, and molecular levels. In addition to ion toxicity and osmotic stress, salinity stress results in hormonal imbalances in plants (Taiz and Zeiger 2010).

Plants must take up various nutrients for growth and development, including macro- and micronutrients (White and Brown 2010). Nitrogen is an essential element for fundamental cellular metabolism in plants. In agriculture, plants directly take up nitrogen that has been exogenously applied to a field, mainly in nitrate (NO_3^-) based forms (Goel and Singh 2015). However, excessive application of nitrate fertilisers often increases threats to the surrounding environment because nitrogen fertilizer unabsorbed by plant roots is easily leached into the soil by runoff or lost through volatilisation and denitrification (Liu et al. 2014a). Although corn yield can be increased by 4% by increasing the provision rate of nitrogen fertiliser by 30%, this increase in fertiliser usage results in 53% more nitrate lost through leaching (Donner and Kucharik 2003). During assimilation, nitrate taken up by plant roots is reduced to ammonium by nitrate reductase encoded by *NIA1* and *NIA2* (Hao et al. 2010; Olas and Wahl 2019). In response to salinity, different approaches for nitrate assimilation have been demonstrated in different species or plants with different genotypes. For instance, nitrate reductase (NR) activity in *Bruguiera parviflora* (Parida and Das, 2004), rice (*Oryza sativa L.*) (Anuradha and Ram Rao 2003) and maize (*Zea mays L.*) (Baki et al. 2000) were decreased when plants were exposed to salt stress. In contrast, nitrate provision at high concentrations can also negatively affect plant growth. For example, high nitrate accumulation leads higher nitric oxide (NO) levels in plant cells, which, together with superoxide, catalyse the conversion of peroxynitrite ($ONOO^-$) by nitrate reductase (Durner and Klessig 1999; Lamattina et al. 2003). However, the impact of excessive nitrate application on plant growth is controversial and poorly understood.

The interactions between high nitrate and phytohormones, such as abscisic acid (ABA), in stress responses have been widely studied (L et al. 2001; De Smet et al. 2003; Kiba et al. 2012). Application of both high nitrate (>30 mM) and exogenous ABA represses lateral root growth in *Arabidopsis* (De Smet et al. 2003; Deak and Malamy 2005). Previous studies indicated that high nitrate, as an osmolyte that alters the plant cell osmotic potential (Deak and Malamy 2005), leads to the release of ABA in its active form (Ondzighi-Assoume et al. 2016) which subsequently affects leaf gas exchange and stomatal function (Guo et al. 2003b). ABA-insensitive mutants have been shown to reduce the high nitrate-induced inhibition of plant growth (Signora et al. 2001).

ABI5 functions as a transcription factor in response to abiotic stress in the ABA signalling pathway and as an integrator of ABA with other phytohormones (Skubacz et al. 2016). Chang et al. (2019) revealed that *ABI5* functions in the salt tolerance of *Arabidopsis* plants under the regulation of ABF3. It has been suggested that *ABI5* negatively regulates photosynthesis by inducing chlorophyll degradation (Shkolnik-Inbar et al. 2013). Together with *ABI4*, *ABI5* is involved in lateral root growth and acts as a negative regulator of lateral root formation under abiotic stress (Skubacz et al. 2016). Repression of *ABI5* expression levels helps maintain the necessary stomatal aperture for gas exchange (Kang et al. 2018).

Based on these previous studies, we investigated how high nitrate levels affect plant growth, particularly in the presence of high salinity. We found that *abi5-7* plants showed tolerance to unfavourable environmental conditions of high nitrate and high salinity (HNHS) stress. Particularly, *abi5-7* plants showed impaired endogenous NO levels compared to *Arabidopsis thaliana* Columbia-0 (Col-0) plants because of the reduced transcript level of *NIA2* and resulting low NR activity.

Results

Oversupply of nitrate reduces salt tolerance of Arabidopsis Col-0

Recent studies showed that application of nitrate at high concentrations causes toxicity in plants (Durner and Klessig 1999; Lamattina et al. 2003). To understand the roles of nitrogen nutrients in plant growth and development, particularly under high nitrate conditions in the presence of high salinity (175 mM NaCl), we examined the growth performance of Col-0 under HNHS conditions. As shown in Fig. 1A, Col-0 grown in 30 mM nitrate were smaller than those grown in 5 mM nitrate

(control condition) with or without high salinity. Particularly, without salt, high nitrate had no significant effect on primary root growth but it reduced the chlorophyll content of Col-0 compared to in control plants. Exposure of Col-0 to high salinity led to a remarkable decrease in primary root elongation and the levels of photosynthetic pigments (Fig. 1B, C). Additionally, to determine if the variation in potassium content influenced plant development in response to varying nitrate levels, we examined the growth of Col-0 plants grown in high potassium conditions by increasing the total K^+ ion concentration in N5 medium upto 10, 20, 50 and 100 mM using KCl and K_2SO_4, and no noticeable difference in plant growth between these conditions was observed (Fig. S1A). The effect of high salinity on plant growth inhibition was greater in the presence of a high nitrate concentration. In agreement with a previous report (Lee et al. 2021), we found that Col-0 is more sensitive to high salinity in the presence of high nitrate concentrations.

Disruption of ABI5 alters sensitivity to high nitrate concentration under high salinity

As ABA is well-known to be associated with plant stress responses (Chang et al., 2019), we screened several ABA-related mutants and confirmed that *abi4-1* and *abi5-7* plants grew better than Col-0 under HNHS conditions. We examined the growth performance of Col-0, *abi4-1*, and *abi5-7* plants in response to HNHS conditions by providing 5 and 30 mM of KNO_3 as the sole nitrogen source combined with 175 and 200 mM of NaCl for two weeks. As shown in Fig. 2A, similar to Col-0, there were no significant phenotype differences between *abi5-7* plants grown under N5 and N30 media, but all plants showed significant phenotypic alterations under high salt stress. In agreement with a previous study (Shkolnik-Inbar et al. 2013), we found that although *abi4-1* plants were slightly smaller than Col-0 plants grown in N30 medium, *abi4-1* plants were tolerant to salt stress, particularly under high nitrate supplementation. As expected, *abi5-7* plants were tolerant to high-salt stress compared with Col-0 plants, especially under HNHS conditions. We then examined other stress-related growth parameters, such as the fresh weight, number of lateral roots, and chlorophyll content in the presence or absence of salt stress combined with different nitrate concentrations (Fig. 2B–E). Similar to Col-0, *abi4-1* and *abi5-7* plants did not grow well when they were subjected to high-salt stress; nevertheless, the growth reductions of *abi5-7* plants were significantly smaller than those of *abi4-1* and Col-0 plants. Particularly, *abi5-7* had greater fresh weights and longer primary root lengths, better lateral root development, and higher chlorophyll contents under the inhibitory effects of high nitrate than those of Col-0 and

abi4-1, particularly in the presence of high salinity. These results indicate that ABI5 plays essential roles in response to high-salt stress and that knocking out *ABI5* in Col-0 reduces the sensitivity to high salt stress, especially under HNHS conditions.

As shown in Fig. 2, *abi5-7* plants exhibited better growth performance than *abi4-1* in HNHS, and thus we chose *abi5-7* plants for further analyses. We then determined the transcript levels of *ABI5* in Col-0 plants exposed to N5, N30, N5S175, and N30S175 conditions. As shown in Fig. 2F, *ABI5* transcription was kept at low level in the absence of salt stress. In contrast, *ABI5* was highly expressed following exposure of Col-0 to 175 mM NaCl. Specifically, *ABI5* was expressed at a similar level in salt-treated Col-0 grown under normal and high nitrate conditions at 6 h; however, at 24 h after the treatments, the transcript level of *ABI5* in Col-0 exposed to normal nitrate and high-salt stress was continuously increased by two-fold, whereas those in Col-0 treated with HNHS stress remained unchanged. We also evaluated the expression of two other ABA-insensitive genes, *ABI3* and *ABI4*, which were shown to collaborate with ABI5 in the control of abiotic stress responses (Nakamura et al. 2001; Reeves et al. 2011). The results revealed lower expression of these genes in *abi5-7* plants (Fig. S1B).

Ion homeostasis improved salt tolerance in abi5-7 under high nitrate conditions

Salt stress causes disturbances in ion homeostasis in plants, and plant salt tolerance can be explained by improvements in the Na^+/K^+ ratio (Jiang et al. 2013). In high-salinity environments, plants accumulate higher Na^+ contents and suffer depletion of tissue K^+ ions, which eventually leads to cellular damage. Therefore, we examined whether the improved Na^+/K^+ homeostasis conferred salt stress tolerance to *abi5-7*. Notably, the K^+ content decreased when plants were subjected to saline conditions, and high-nitrate supplements induced K^+ levels in plants. As shown in Fig. 3A, there were no differences in Na^+/K^+ homeostasis in Col-0 and *abi5*-7 plants in the absence of high-salt stress, whereas salt stress caused changes in intracellular Na^+/K^+ homeostasis in both Col-0 and *abi5-7* plants. Particularly, plants grown under normal nitrate and high-salt stress conditions showed higher Na^+/K^+ ratios than those grown under HNHS-stress conditions. Nevertheless, *abi5-7* plants had a lower Na^+/K^+ ratio than Col-0 plants grown under high-salt conditions. These observations suggest that the lack of *ABI5* function confers salt tolerance by improving the Na^+/K^+ ratio.

MDA, a secondary lipid peroxidation product, is an indicator of cell membrane injury induced by salt stress (Wu et al. 2017; Zhou et al. 2017). We found that MDA levels in *abi5-7* cells were lower than those in Col-0 plants under saline conditions (Fig. 3B). Additionally, anthocyanin and proline are valuable salt-induced markers that accumulate to protect plants exposed to an adverse environment (Jeon et al. 2020). The anthocyanin and proline contents in *abi5-7* plants were lower than those in Col-0 plants (Fig. 3C, D). Moreover, the transcript levels of the osmotic stress markers *RD29A* and *COR15R* were significantly higher in response to high nitrate conditions in Col-0 and *abi5-7* plants than under normal nitrate conditions (Fig. 3E). Under high salt stress, their expression was strongly upregulated when 175 mM NaCl was added to the growth media, but *abi5-7* plants showed lower expression levels of *RD29A* and *COR15A* compared to Col-0 plants. These findings indicate that *abi5-7* is less affected by salt-induced osmotic and ionic stresses.

Nitrate accumulation and NR activity were altered in abi5-7 plants

Next, we compared nitrate accumulation and NR enzyme activity in Col-0 and *abi5-7* plants incubated with normal and high nitrate concentrations with or without high-salt stress. As shown in Fig. 4A, significantly larger amounts of nitrate accumulated in Col-0 and *abi5-7* plants grown under 30 mM NO_3^- conditions, and 175 and 200 mM NaCl treatments led to decreases in nitrate accumulation in Col-0 and *abi5-7* plants, whereas *abi5-7* plants showed lower intracellular nitrate contents compared to in Col-0 under salt stress conditions. Nevertheless, nitrate accumulation in plants grown in N5S200 was higher than that in plants grown in N5S175 and the nitrate contents were not affected by an increase of NaCl concentration from 175 to 200 mM when plants were grown in the presence of 30 mM NO_3^-. In addition, the lower accumulation of nitrate in *abi5-7* plants resulted in lower NR enzyme activities compared with those of Col-0 grown under stress conditions (Fig. 4B). Similar to nitrate accumulation, high-salt stress treatments disturbed the NR activities in both Col-0 and *abi5-7* plants, and the NR activities of Col-0 and *abi5-7* plants treated with 30 mM NO_3^- were higher than those in Col-0 and *abi5-7* plants grown under 5 mM NO_3^- conditions, respectively.

To investigate the effects of *ABI5* function on the nitrate-related signalling pathway under high-salt stress conditions, we investigated the expression levels of nitrate-related genes in Col-0 and *abi5-7* plants under 5 and 30 mM NO_3^- conditions in the presence of 175 mM NaCl. First,

because of the lower nitrate contents in *abi5-7* plants, we examined the transcript levels of the nitrate transport genes *NRT1.1* and *NRT2.1*. The results showed that salt application decreased the transcript levels of *NRT1.1* and *NRT2.1* (Fig. 4C). However, high nitrate increased the transcript level of *NRT1.1* while reducing that of *NRT2.1*. It appears that the transcript levels of these genes in *abi5-7* plants were remarkably lower than those in Col-0 under HNHS conditions. Long-distance nitrate transport from roots to shoots mediated by two important nitrate transporters, *NRT1.5* and *NRT1.8*, contributes to nitrate reallocation and plant acclimation to adverse environments (Fan et al. 2007; Han et al. 2016). We found that under high nitrate treatment without salt stress, but not in other treatments, the transcript level of *NRT1.5* in Col-0 was higher than that in *abi5-7* plants and no difference in transcript levels of *NRT1.8* between Col-0 and *abi5-7* plants under identical conditions was observed (Fig. 4C).

The lower NR activities in *abi5*-7 plants led us to examine the transcript levels of *NIA1* and *NIA2*. As shown in Fig. 4D, there was no difference in the transcript levels of *NIA1* and *NIA2* in Col-0 and *abi5-7* plants under normal nitrate conditions. Although we found that salt stress inhibited accumulation of the *NIA1* transcript, the transcript levels of *NIA2* were significantly enhanced in both plants. High nitrate treatment also resulted in higher transcript levels of *NIA2*, but not *NIA1*, both in the presence and absence of salinity. Moreover, *abi5-7* plants had lower transcript levels of these genes compared to Col-0 plants under high nitrate and salt treatments. These findings may explain why *abi5-7* plants had lower NR activity compared to Col-0 under HNHS conditions.

ABI5 is a key factor for sensing environmental conditions during seed germination and regulates two late embryogenesis abundant proteins, *EM1* and *EM6*, in concert with *ABI3* (Tezuka et al. 2013). We observed that high salt treatment strongly increased the levels of *EM1* transcript in both genotypes; however, the transcript levels of *EM1* in *abi5-7* plants were significantly lower than those in Col-0 plants under high nitrate conditions, even in the absence of salinity. Nevertheless, those of *EM6* did not significantly differ between Col-0 and *abi5-7* plants (Fig. 4D).

ABA plays a central role in abiotic stress responses, and its biosynthetic pathways are tightly controlled by these stresses (Kim et al. 2010; Sah et al. 2016; Vishwakarma et al. 2017). Thus, we analysed the ABA contents in tested plants. As shown in Fig. 4E, salt stress resulted in higher ABA

levels in Col-0 and *abi5-7* plants, which were slightly decreased in combination with high nitrate. However, under salinity stress, ABA levels were significantly higher in *abi5-7* compared to in Col-0.

Reduction of NR activity alters endogenous NO levels in abi5-7 plants

We next examined whether NO is involved in the responses to HNHS conditions. We used a cell-permeable fluorescent probe, DAF-FM DA, and examined NO levels in Col-0, *abi5-7*, *nia2-1*, and *nia2-2* plants exposed to HNHS. As shown in Fig. 5A, salt stress induced higher NO accumulation in both Col-0 and *abi5-7* plants, whereas NO levels were lower in *abi5-7* plants than in Col-0 plants. The differences in NO levels in Col-0 and mutants were more evident under HNHS conditions. However, plants lacking NIA1 showed NO levels similar to those of Col-0 under HNHS conditions (Fig. S2). Our results indicate that in *abi5-7* plants under HNHS conditions, NO levels were lower because of reduced NR activity.

Next, we determined ROS accumulation in Col-0 and *abi5-7* exposed to HNHS stress, specifically H_2O_2 and O_2^- levels, by performing 3′,3′-diaminobenzidine and nitroblue tetrazolium staining assays because NO also induces oxidative stress. We predicted that ROS accumulation was also lower in *abi5-7* plants than in Col-0 plants. Plants grown in high-nitrate conditions showed higher ROS production in their shoots, which was increased when plants were exposed to salt stress and further increased under HNHS. Additionally, *abi5-7* and *nia2* plants showed lower ROS accumulation than Col-0 plants under identical treatments (Fig. S3A, B). It appears that *abi5-7* suffers less inhibitory effects of salt stress than Col-0, especially under high-nitrate conditions. This reasoning is supported by the fact that *abi5-7* plants exhibited higher total protein contents than Col-0 plants (Fig. 5B). We also examined the protein content of *nia2* plants, which were lower than that of Col-0 under 5 mM NO_3^- conditions with or without salinity, possibly because of decreased NR activity in *nia2* plants.

To further examine the relationship between NO levels and tolerance of *abi5-7* plants to HNHS, we examined the growth performance of Col-0, *abi5-7*, and *nia2* plants. As shown in Fig. 6, *abi5-7* plants exhibited the highest tolerance to HNHS among the tested plants. Similar to Col-0 and *abi5-7* plants, *nia2* plants were smaller when grown under high-nitrate conditions, and there was no visible difference in the growth performance of all plants under non-salt stress conditions. Under high salinity, *nia2* plants grew better than Col-0 plants under high nitrate conditions but could not mimic the growth phenotype of *abi5-7* plants. Although there was no significant

difference in growth performance between Col-0 and *nia2* plants under high nitrate without high salinity (Fig. 6A–D), the deficiency of NIA2 led to a significant decrease in the chlorophyll content (Fig. 6E). However, similar to *abi5-7* plants, *nia2* plants were less sensitive to salt stress compared to Col-0 plants, with *nia2* plants showing a higher fresh weight, longer primary root length, and higher chlorophyll content, particularly under high-nitrate conditions. However, knockout of *NIA1* did not contribute to the tolerance of plants to HNHS stress (Fig. S4A–E). Particularly, *nia1* had a lower fresh weight and chlorophyll content compared to Col-0 (Fig. S4B, E); no difference was observed between *nia1* and Col-0 in the number of lateral roots under high salinity (Fig. S4D); however, under high salinity, *nia1* plants showed longer primary root lengths compared to Col-0 plants (Fig. S4C).

To further understand the role of NO in the tolerance of *abi5-7* in HNHS, we examined the effects of external NO on the growth performance of Col-0, *abi5-7*, and *nia2* plants using sodium nitroprusside (SNP) and Nω-nitro-L-arginine (L-NNA), known as NO donors and nitric oxide synthase (NOS) inhibitors, respectively. As shown in Fig. 7, addition of L-NNA (25 μM) to the media improved the growth performance of Col-0 and *abi5-7* plants under high salinity by increasing the fresh weights and chlorophyll contents (Fig. 7B, E) but not the primary root lengths and lateral root formations (Fig. 7C, D). Inhibition of NOS activity in *nia2* plants resulted in higher sensitivity to salt stress by decreasing the growth performance. In contrast, the harmful effects of high-salt stress on plant growth, particularly under high nitrate conditions, were exacerbated by SNP supplementation at 10 μM or higher in all tested plants. The fresh weights, primary root length, and chlorophyll contents of *abi5-7* subjected to 10–25 μM SNP were lower than those of Col-0 grown under high salinity.

Discussion

Up to 70% of plant growth and productivity can be affected by various environmental factors, such as salinity, drought, and high temperature (Mantri et al. 2012). To survive under these negative environmental conditions, plants use complex and sophisticated responses at the molecular, cellular, and physiological levels, including complex networks that support the recognition and transmission of environmental stress signals (Mittler 2006; Nakashima et al. 2009). A deeper understanding of the mechanisms that support plant environmental stress tolerance will enable the

development of crops with an improved ability to withstand constantly changing environmental conditions, and ultimately contribute to increased yields.

Nitrogen fertilisers are widely used in agriculture because nitrogen is an indispensable nutrient for plant survival; however, these fertilisers are often overused (Donner and Kucharik 2003). We examined whether an oversupply of nitrogen would increase the tolerance to salt stress in plants. We observed that high nitrate concentrations (30 mM NO_3^-) inhibited plant growth in the presence or absence of high-salt stress (Fig. 1). To understand plant responses under HNHS conditions at the molecular level, we evaluated *abi5-7* plants showing better growth than Col-0 under HNHS conditions. ABA is a key stress signalling hormone that not only participates in regulating plant growth and development, but also is involved in the response to osmotic stresses, including high salinity (Ali et al. 2020). The phenotypes of ABA-insensitive mutants *abi4-1, abi5* in response to high nitrate conditions (Signora et al. 2001), salt stress conditions (Shkolnik-Inbar et al. 2013) and the higher ABA contents in *abi5-7* plants exposed to salt stress in comparison with those in Col-0 (Fig. 4D) strengthened the considerable functions of ABA in developmental and physiological processes. Previous studies indicated that ABI5 is a master regulator of seed development and germination (Ali et al. 2020). In the ABA-dependent pathway, *ABI3* and *ABI4* positively regulate *ABI5* expression during seed germination and early seedling development (Finkelstein and Lynch 2000; Lopez-Molina et al. 2002; Bossi et al. 2009), and function together with *ABI5* in abiotic stress regulation (Nakamura et al. 2001; Reeves et al. 2011). ABI5 proteins are degraded in the absence of abiotic stress (Skubacz et al. 2016). We found that *ABI5* transcripts accumulated at high levels under salinity stress (Fig. 2F) and that *abi5-7* plants grew much better than Col-0 under high-nitrate and HNHS conditions (Fig. 2A–E), which agrees with previous results (Signora et al. 2001). However, unexpectedly, the anthocyanin and proline contents, which help plants withstand salt stress, were lower in *abi5-7* plants than in Col-0 plants, suggesting that loss of *ABI5* reduced stress caused by HNHS conditions in these plants.

We next examined why *abi5-7* plants were less stressed than Col-0 plants under HNHS conditions. First, we examined whether *abi5-7* accumulates less sodium than Col-0, as under the adverse effects of salt stress, ion over-accumulation results in a severe Na^+/K^+ imbalance that inhibits plant growth. In addition, *abi4-1* plants, a homologous gene of *ABI5*, exhibited resistance to salt stress because of the reduced sodium accumulation in their shoots (Shkolnik-Inbar et al. 2013) (Fig. 2A–E). Additionally, *abi5-7* plants exhibited a lower Na^+ to K^+ ratio compared to Col-

0 plants (Fig. 3A), indicating that the better salt stress tolerance of *abi5-7* plants is similar to the mechanism used by *abi4-1* plants. Na^+ appears to enhance nitrate uptake and nitrate reallocation to shoots (Kaburagi et al. 2014). Our study also suggests that there is a positive correlation between Na^+ and NO_3^- transport in plants because the nitrate content in *abi5-7* plants was significantly lower than that in Col-0 under N5S175 and N30S175 conditions (Fig. 4A). High nitrate application led to enhanced nitrate accumulation in both Col-0 and *abi5-7* plants (Fig. 4A). Moreover, this difference in nitrate content appears to have arisen from differences in the expression levels of nitrate transport proteins, given that there was a significant difference between the expression levels of *NRT1.1* and *NRT2.1* in the Col-0 and *abi5-7* plants after high nitrate treatments; these are two major components that are highly associated with pivotal functions in root nitrate uptake (Ye et al. 2019) (Fig. 4C). Moreover, *NRT1.5* and *NRT1.8* play essential roles under salinity stress (Li et al. 2010; Chen et al. 2012) but did not differ between Col-0 and *abi5-7* plants under HNHS conditions (Fig. 4C). As reported previously (Álvarez-Aragón and Rodríguez-Navarro 2017), our results support that the nitrate content in plants plays a decisive role in the absorption of sodium from the surrounding environment.

Because the nitrate contents were lower in *abi5-7* plants than in Col-0 plants, without a significant difference in the transcript levels of nitrate transport genes, we next examined the activity of NR. As demonstrated previously (Krishna Rao and Gnanam 1990; Gouia et al. 1994), NR activity was decreased when plants were subjected to salt stress conditions (Fig. 4B). We found that the lower NR activity in *abi5-7* plants was partly due to the lower transcript levels of *NIA1* and *NIA2* in *abi5-7* plants than in Col-0 plants (Fig. 4D). Although the effects of salinity on nitrogen metabolism have been reported, the impact of high-salt stress on NR activity remains unclear. NR is an enzymatic source of NO production and plays crucial roles in nitrate reduction and assimilation in plants (Meyer et al. 2005). We found that the transcripts of *NIA1* and *NIA2* increased when high nitrate was provided (Fig. 4D). Although the transcript levels of *NIA1* were reduced under high-salinity conditions, those of *NIA2* were increased, showing further increases under high-nitrate and high-salt conditions. NR enzymes also contribute to NO production, in addition to reducing nitrate to nitrite. Previous studies showed that NO has both positive and negative roles in the regulation of guard cell ABA signalling (Sun et al. 2019) in stomatal closure stimulated by ABA in *Arabidopsis*, which was investigated in *Arabidopsis nia1/nia2* knock-out

mutants (Neill et al. 2008). Moreover, through ERF-regulated transcription of *ABI5* (Gibbs et al. 2014) and degradation of the ABI5 protein (Albertos et al. 2015), studies explored the function of NO in the control of seed germination in Arabidopsis. Endogenous NO levels in *Arabidopsis* are increased via distinct mechanisms involving enzymatic and non-enzymatic pathways, among which NR is one of the most important enzyme-based NO biosynthesis pathways in plants and is encoded by two crucial genes, *NIA1* and *NIA2*, as well as NO-associated (*AtNOA1*) (Rockel et al. 2002; Sudhamsu et al. 2008). Therefore, we examined whether *abi5-7* mutation causes alterations in NO levels, as NO is a well-known modulator of several phytohormones, such as ABA which has important roles in the growth of post-germinative seedlings (Lopez-Molina et al. 2001). Although NO acts as a signalling molecule with multiple biological functions (Neill et al. 2003; Crawford and Guo 2005; Wang et al. 2010), it can disrupt various cellular reactions, leading to inhibition of plant growth and development depending on its concentration, plant species, and developmental stages (Zhao et al. 2007; Bai et al. 2012). Particularly, high accumulation of nitrate in plants can cause severe toxicity to plant growth via NR activity which catalyses the conversion of nitrite into peroxynitrile ($ONOO^-$) (Reddy and Menary 1990; Durner and Klessig 1999; Lamattina et al. 2003). Both *NIA1* and *NIA2* mediate ABA-induced NO generation in *Arabidopsis* (Desikan et al. 2002), with *NIA2* responsible for 90% of the total NR activity in plants (Desikan et al. 2002; Kolbert and Erdei 2008; Hao et al. 2010; Vitor et al. 2013). Therefore, we examined whether the lower sensitivity of *abi5*-7 plants to HNHS was associated with a lower NO content. We demonstrated that high nitrate application resulted in higher endogenous NO levels, which were strongly increased in plants subjected to high-salt stress and further enhanced under HNHS stress. However, as expected, *abi5-7* plants showed significantly lower endogenous NO levels compared to Col-0 plants, likely because of their lower NR activity (Fig. 5A). Moreover, the lower endogenous NO levels in *nia2* plants (Fig. 5A) confirmed that NR-dependent NO production contributes to the enhanced tolerance of *abi5-7* plants under HNHS conditions. To confirm the roles of endogenous NO in plants under salinity stress, we examined the growth performance of Col-0, *abi5-7*, and *nia2* plants under HNHS conditions in the presence of NO donor or NOS inhibitor (SNP and L-NNA, respectively). Our results showed that L-NNA improved growth performance under the adverse effects of HNHS (Fig. 7). *abi5-7* plants exposed to the NO scavenger mimicked the sensitive phenotype of Col-0 under salt stress or HNHS conditions (Fig. 7). The better growth of plants under salt stress in the presence of L-NAA demonstrated that not

only NR-dependent NO, but also NOS-dependent NO also were important in the salt stress tolerance of plants. Taken together, our results demonstrate that supplying excess nitrate does not increase the plant's salt stress tolerance, but rather reduces it. NO appears to play a crucial role in the underlying mechanisms. Therefore, the discovery of regulators such as ABI5 that can regulate NR activity and understanding its molecular functions are very important, as *ABI5* can be subjected to gene editing techniques to modulate its TF activities, leading to optimal accumulation of NO to increase the productivity of crops exposed to various stress environments.

Materials and Methods

Plant material and growth conditions and phenotypic screening

Arabidopsis thaliana Columbia-0 (Col-0) was used as the wild-type plant. T-DNA-tagged mutants including *nia1-1* (SALK_071547C), *nia1-2* (SALK_ 148487C), *nia2-1* (SALK_138297c), *nia2-2* (SALK_088070), and *abi4-1* (CS8104) were purchased from the *Arabidopsis* Biological Resource Center (Columbus, OH, USA). PCR screening was conducted to obtain homozygous mutants using the LBb1.3 primer and full-length forward and reverse primers of related genes, as listed in Supplementary Table S1. The *abi5-7* mutant was obtained as previously described (Nambara et al., 2002). The surface-sterilised seeds were kept in the dark at 4°C for 3 days before being sown in Murashige Skoog (MS) medium without nitrogen medium and containing 2% sucrose, 5 mM of KNO_3 as the sole nitrogen source, and 0.5% Phytagel agar, pH 5.8. Seedlings were incubated in a growth chamber as previously described (Nguyen et al. 2016) under a 16-h light/8-h dark cycle, 23 ± 1°C, 50–55 μmol photons m^{-2} s^{-1}, and approximately 70% humidity. Four-day-old germinated seedlings were transferred to fresh nitrogen-free MS medium supplemented with 5 and 30 mM of KNO_3 combined with NaCl at 0, 175, and 200 mM (N5 as the control condition, N30, N5S175, N30S175, N5S200, and N30S200, respectively) and grown for an additional 2 weeks. In the phenotypic experiments investigating effects of exogenous nitric oxide on plant growth, 10, 25, 50 and 100 μM sodium nitroprusside (SNP) and 25 μM Nω-nitro-L-arginine (L-NNA) was supplemented into the above media. All plates were sealed with Parafilm and placed vertically in a growth chamber. After 2 weeks, primary root elongations, fresh weights and lateral roots were measured, and plants were collected to measure the chlorophyll content.

In planta imaging of NO

In planta, NO accumulation was analysed using the NO-specific fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) (Invitrogen, Carlsbad, CA, USA) as described by (Guo et al. 2003a) with minor modifications. Briefly, 9-day-old seedlings were treated with various combinations of 5 and 30 mM NO_3^- and 175 mM NaCl and then incubated with 5 μM DAF-FM DA in 20 mM HEPES-NaOH (pH 7.5) for 30 min in the dark, followed by three washes with 20 mM HEPES-NaOH (pH 7.5) for 5 min each time. The seedlings were then incubated for an additional 1 h in the dark before visualisation on a laser confocal scanning microscope (LSM 700; Zeiss, Oberkochen, Germany) at excitation and emission wavelengths of 488 nm and 515–565 nm, respectively. The images were processed and analysed using Zeiss LSM 700 software. ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the relative fluorescence intensity**.**

Chlorophyll assay

Frozen samples (100 mg) were ground into a fine powder in liquid nitrogen using a mortar and pestle and the chlorophyll content was measured as described by (Ni et al. 2009). The powdered samples were transferred into a 1.5-mL e-tube and 700 μL of 80% acetone was added. The sample was incubated at 4°C for 30 min in the dark to protect the chlorophyll from light damage. The supernatant was collected by centrifugation (3,000 rpm, 4°C for 15 min) and then transferred into a 96-well plate. The chlorophyll content was determined using a Multi-Detection Microplate Reader (HIDEX Sense, HIDEX, Turku, Finland), and the absorbance was measured at 663 and 645 nm. The following equations were used to estimate chlorophyll concentrations: Chl $a + b$ (mg/g) = [8.02 × (A663) + 20.20 × (A645)] × V/1000 × W (V: volume of the extract; W: weight of fresh leaves).

Quantitative reverse transcription PCR

From 9-day-old seedlings that had been treated with 5 and 30 mM NO_3^- with or without salt stress at 175 mM NaCl, total RNA was extracted using TRIzol reagent (Invitrogen) as previously described (Lee et al. 2017). A RevertAid First cDNA Synthesis Kit (Thermo Fisher Scientific,

Waltham, MA, USA) was used to synthesise first-strand cDNA. Real-time qPCR was performed with an FX connect Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using BlasTaq^{TM} 2X qPCR MasterMix (Applied Biological Materials, Richmond, British Columbia, Canada). *AtActin2* was used as an internal control for expression normalisation. All primers used are listed in Supplementary Table S1.

Nitrate content assay

The nitrate content was measured as described by (Zhao and Wang 2017). Samples (100 mg) of 9-day-old seedlings were frozen in liquid nitrogen and ground into a fine powder. The powdered samples were transferred into 1.5 mL tubes. After adding 1 mL deionised water, the tube was boiled at 100°C for 1 h. The sample was centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant (100 µL) was transferred into a 15-mL Falcon tube, to which 0.4 L of salicylic acid-sulfuric acid was added and the mixture was mixed well by vortexing. After incubation at 25°C for 30 min, 9.5 mL of 8% (w/v) NaOH solution was added to the tube and the tube was cooled to room temperature (approximately 30 min). The nitrate content was evaluated by measuring the absorbance at 410 nm using a Multi-Detection Microplate Reader (HIDEX Sense), and a standard curve obtained with KNO_3 solution was used for quantification.

NR activity

NR activity was determined using previously described methods (Smarrelli and Campbell 1983). Briefly, samples (100 mg) of 9-day-old seedlings were ground in liquid nitrogen and homogenised in 0.5 mL of extraction buffer containing 25 mM potassium phosphate buffer with 10 mM potassium nitrate and 0.05 mM EDTA. The mixture was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was transferred to a new tube. Fresh extraction buffer (1.8 mL) was prepared with 100 µL 2.0 of β-NAD^+, and the mixture was mixed well and equilibrated to 30°C. The extracts (100 µL) were added and immediately mixed by swirling before incubation at 30°C for exactly 2 min. The reaction was stopped by adding 1 mL of 58 mM sulphanilamide solution prepared in 3 M hydrochloric acid and 1 mL 0.77 mM N-(1-naphthyl) ethylenediamine dihydrochloride solution. The mixture was mixed well and incubated at 25°C for an additional

10 min. The absorbance at 540 nm was used to calculate the NR activity using a multi-detection microplate reader (HIDEX Sense).

Protein concentration measurement

Nine-day-old seedlings grown on the control medium were treated with 5 and 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, samples were collected and total protein was extracted using a PRO-PREP^{TM} Kit (iNtRON Biotechnology, Gyeonggi-do, Korea). Protein concentrations were measured using a BCA Protein Assay Kit (Merck Millipore, Billerica, MA, USA).

ROS accumulation determination

3′,3′-Diaminobenzidine and nitroblue tetrazolium were used to detect hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in shoots, as previously described (Jiang et al. 2013; Yang et al. 2014). The relative intensity of staining was analysed using ImageJ software (NIH).

Determination of anthocyanine contents

The anthocyanin content in the treated samples was measured as described previously (Lee et al. 2021). A four-day-old seedling sample (50 mg) treated with HNHS stress was ground into a fine powder with liquid nitrogen and extracted in extraction buffer (300 µL methanol containing 5% HCl v/v) overnight at 4°C. Next, 200 µL of distilled water and 500 µL of chloroform were added, followed by centrifugation at 13,000 rpm for 20 min. The supernatant was transferred to a fresh tube, and the anthocyanin content was analysed using a Multi-Detection Microplate Reader (HIDEX Sense) at absorbance wavelengths of 353 and 650 nm.

Determination of proline content

Samples (100 mg) of 9-day-old seedlings treated with HNHS stress were ground into a fine powder and used to measure the proline content as described in a previous study (Bates et al. 1973). Powder samples were extracted with 1 mL sulfosalicylic acid (3% v/v). After centrifugation, 200 µL of the supernatant was exposed to reaction buffer (glacial acetic acid 80% v/v, phosphoric acid 6% v/v,

70.17 mM ninhydrin). After 1 h of incubation at 100°C, the reactions were stopped at 4ºC. Toluene (200 µL) was added, and the mixtures were vortexed. The proline content was analysed using a Multi-Detection Microplate Reader (HIDEX Sense) at an absorbance of 520 nm.

Determination of malondialdehyde content

The malondialdehyde (MDA) content was determined using 2-thiobarbituric acid (TBA) measurements as previously described (Zhang and Huang 2013). Briefly, samples (100 mg) of treated seedlings were ground in liquid nitrogen and transferred into a 1.5-mL e-tube, to which 1 mL of 0.1% trichloroacetic acid (TCA) (w/v) was added. The samples were kept on ice until they were homogenised with frequent vortexing. Next, 500 µL of supernatants after centrifugation at 13,000 rpm for 10 min at 4°C were transferred into another tube containing 1.5 mL of fresh 0.5% TBA (w/v)/20% TCA (w/v) solution at a 4-fold dilution. The mixtures were incubated at 80°C for 30 min with frequent inversion. After incubation, the reaction was immediately stopped by placing the samples on ice, followed by centrifugation at 13,500 rpm at 4°C for 5 min to settle any TBA precipitate. The absorption of the reaction mixtures at 532 and 600 nm was measured using a multi-detection microplate reader (HIDEX Sense).

Measurement of sodium and potassium ion contents

Na^+ and K^+ concentrations were determined using inductively coupled plasma-optical emission spectrometry (Agilent 710 ICP-OES, Agilent Technologies, Santa Clara, CA, USA), as described by (Choi and Gilroy 2015). Briefly, 100 mg of plant materials after pre-treatment with different NO_3^- and NaCl concentrations were washed a few times with deionised water to remove any Na^+ and K^+ passively adhering to their surface. The samples were digested in a separate glass test tube containing 0.6 mL of concentrated HNO_3 at 150°C until the plant tissues were entirely dissolved. Next, 0.4 mL of $HClO_4$ was added and incubated at 180°C until the total sample volume was lower than 0.5 mL. The extracts were cooled to room temperature before bringing the sample volume to 5 mL. The concentrations of elements were quantified using a standard curve obtained with Na and K standard solutions, and the Na^+ to K^+ ratio was calculated.

Abscisic acid content measurement

A four-day-old seedling sample (200 mg) treated with HNHS stress was used to examine ABA content levels. The ABA content was determined using a commercial kit (Phytodetek Elisa kit, PDK 09347, Agdia, Inc., Elkhart, Indiana, United States) according to the manufacturer's instructions (Liu et al. 2014b; Jeong et al. 2018).

Gene accession numbers

The gene sequences reported in this article can be found in the Arabidopsis Information Resource (www.arabidopsis.org) under the following accession numbers: *ABI5* (AT2G36270), *ABI4* (AT2G40220), *ABI3* (AT3G24650), *NIA1* (AT1G77760), *NIA2* (AT1G37130), *EM1* (AT3G51810), *EM6* (AT2G40170), *NRT1.1* (AT1G12110), *NRT2.1* (AT1G08090), *NRT1.5* (AT1G32450), *NRT1.8* (AT4G21680), *ABF3* (AT4G34000), *COR15A* (AT2G42450), *RD29A* (AT5G52310), *AtNOA1* (AT3G47450), *ACTIN2* (AT3G18780).

Statistical analyses

The treatments in each experiment were divided into three groups for statistical analyses, with the absence of salt stress as the first group and presence of NaCl at 175 and 200 mM as the second and third groups, respectively. Statistical analyses were performed using two-way ANOVA, followed by Tukey's test for comparison of means, with a minimum confidence level of 95%. Different letters on the graphs (a, b, c,…) indicate significant differences at $p < 0.05$. The error bars represent the SE of the three biological replicates.

Supplementary information

Figure S1. Col-0 plants were not phenotypically affected by high concentration of potassium.

Figure S2. The transcript levels of *ABI5* in Col-0 exposed to salt stress with or without high nitrate provision.

Figure S3. Endogenous NO levels in the root tips of *nia1* mutants in response to HNHS stress.

Figure S4. ROS accumulations in *abi5-7* and *nia2* mutants in HNHS conditions.

Figure S5. The growth performances of *nia1* mutants under HNHS conditions

Table S1. List of primers used in this study

Author Contribution statement

QL conducted plant growth performance tests and measured plant growth parameters, *in planta* NO and ROS accumulation, extracted and determined the total protein concentrations, analysed Na^+/K^+ ion concentrations, calculated Na^+/K^+ ratios, statistical analyses, and wrote the manuscript. HT conducted a nitrate reductase assay, nitrate content assay and quantitative RT-PCR. DN and YL conducted MDA, proline and anthocyanin content measurements. E-HC designed the experiments, perfomed statistical analyses. HL designed the experiments and wrote the manuscript, and all authors commented on previous versions of the manuscript. All authors reviewed and agreed with the content of the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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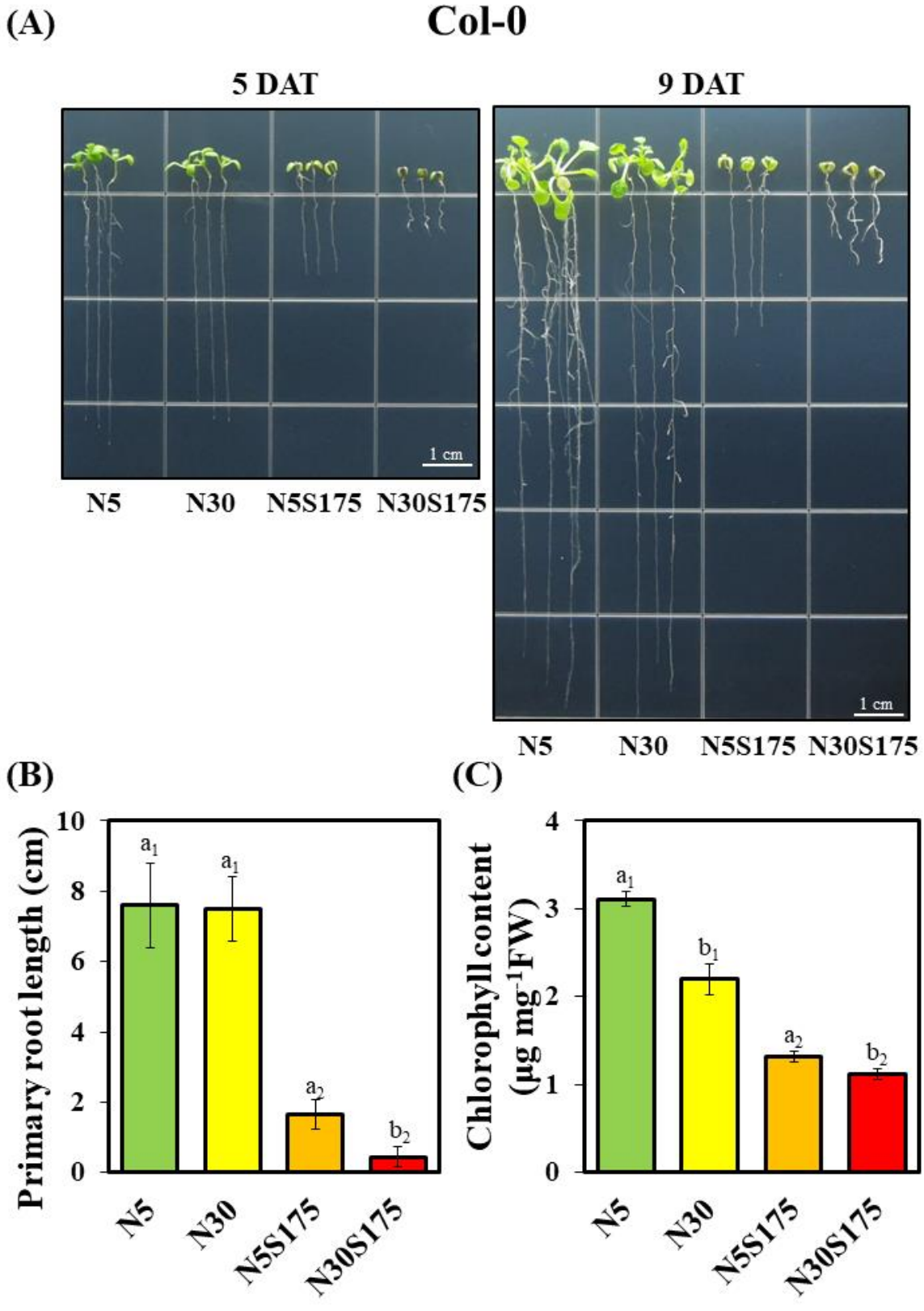
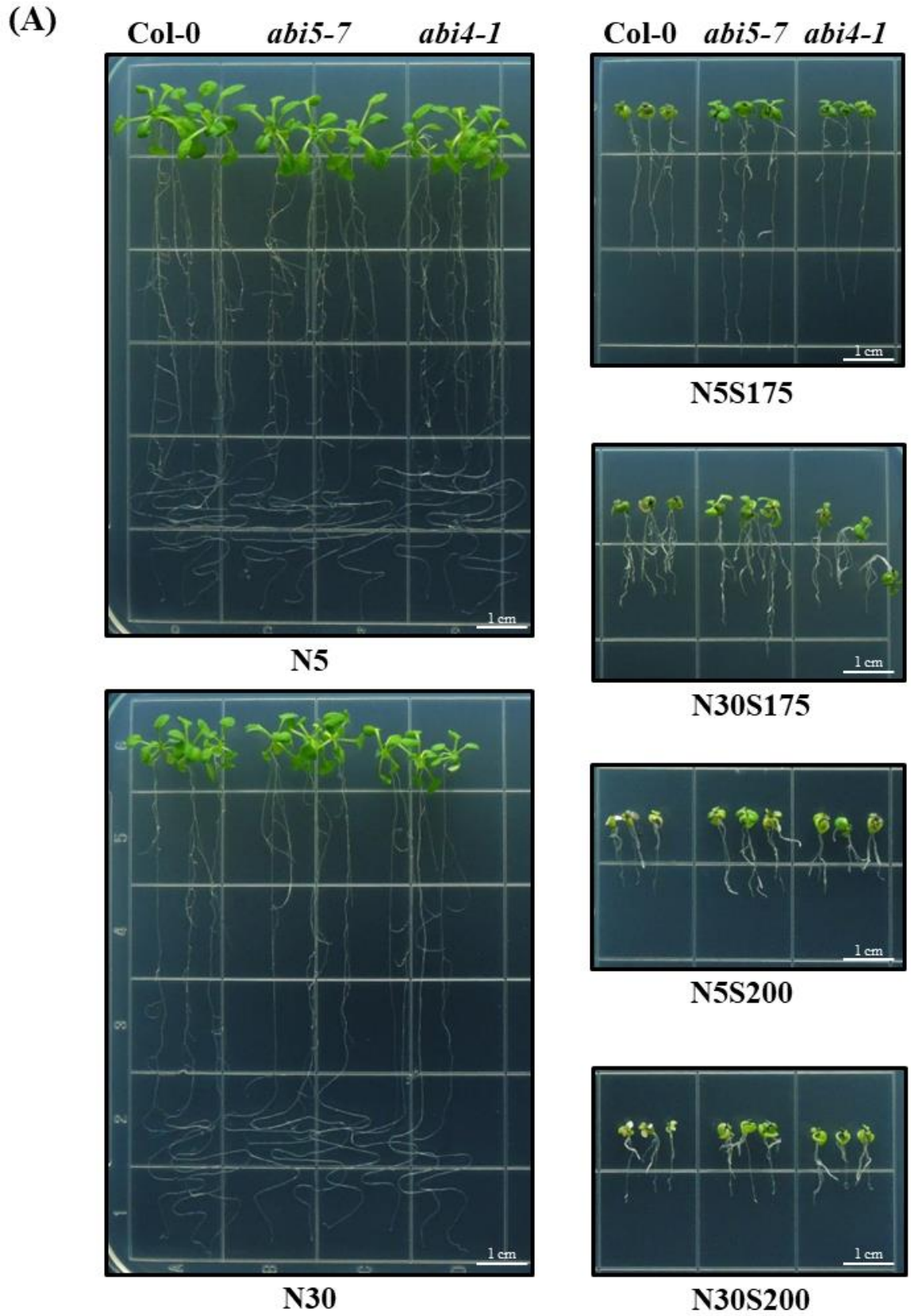

(A)
Col-0
5 DAT
9 DAT
1 cm
1 cm
N5
N30
N5S175
N30S175
N5
N30
N5S175
N30S175
(B)
Primary root length (cm)
10
8
6
4
2
0
a1
a1
a2
b2
N5
N30
N5S175
N30S175
(C)
Chlorophyll content (μg mg-1FW)
4
3
2
1
0
a1
b1
a2
b2
N5
N30
N5S175
N30S175

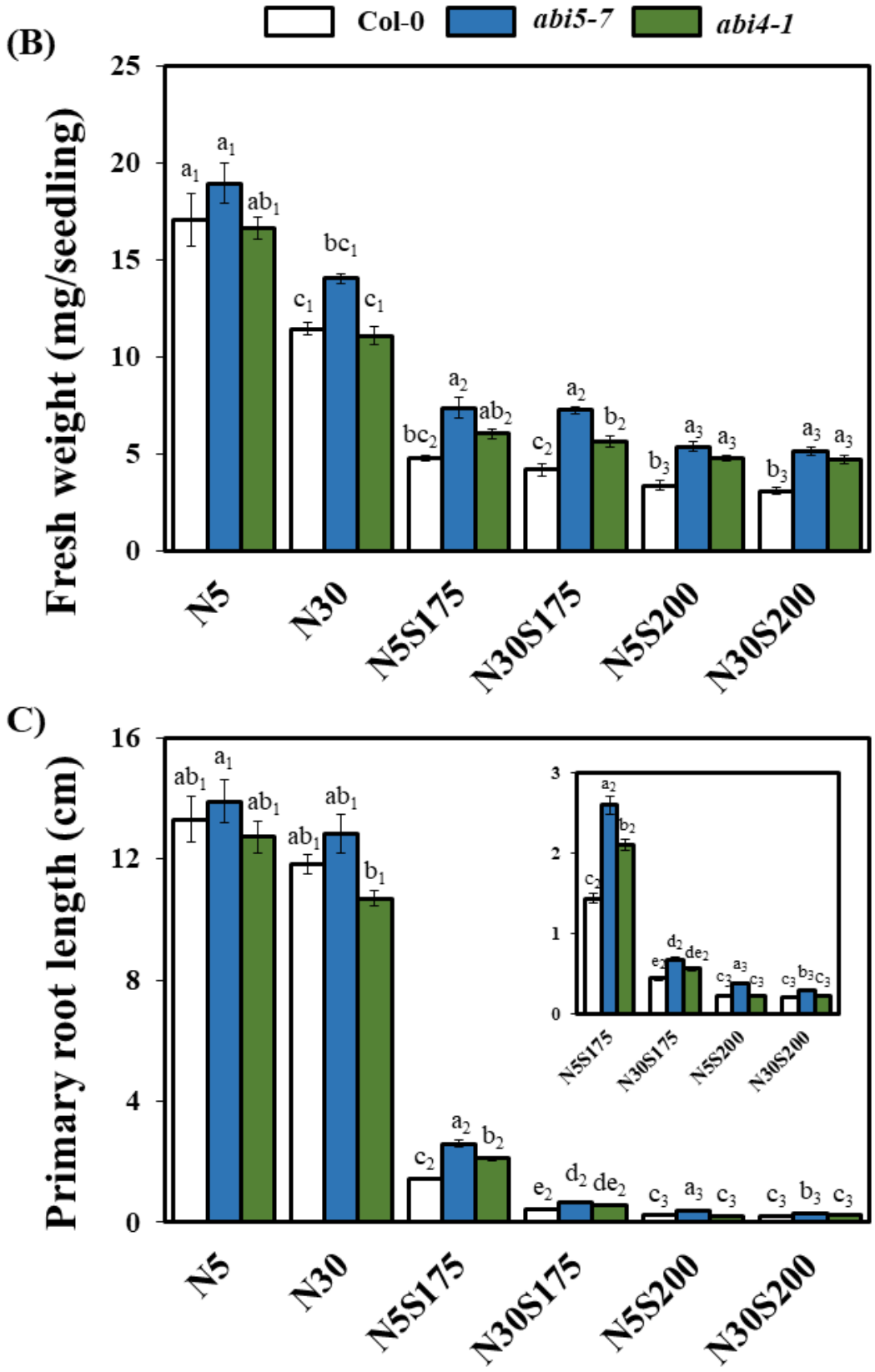
730

Figure 1. Col-0 showed a sensitive phenotype to HNHS tress.

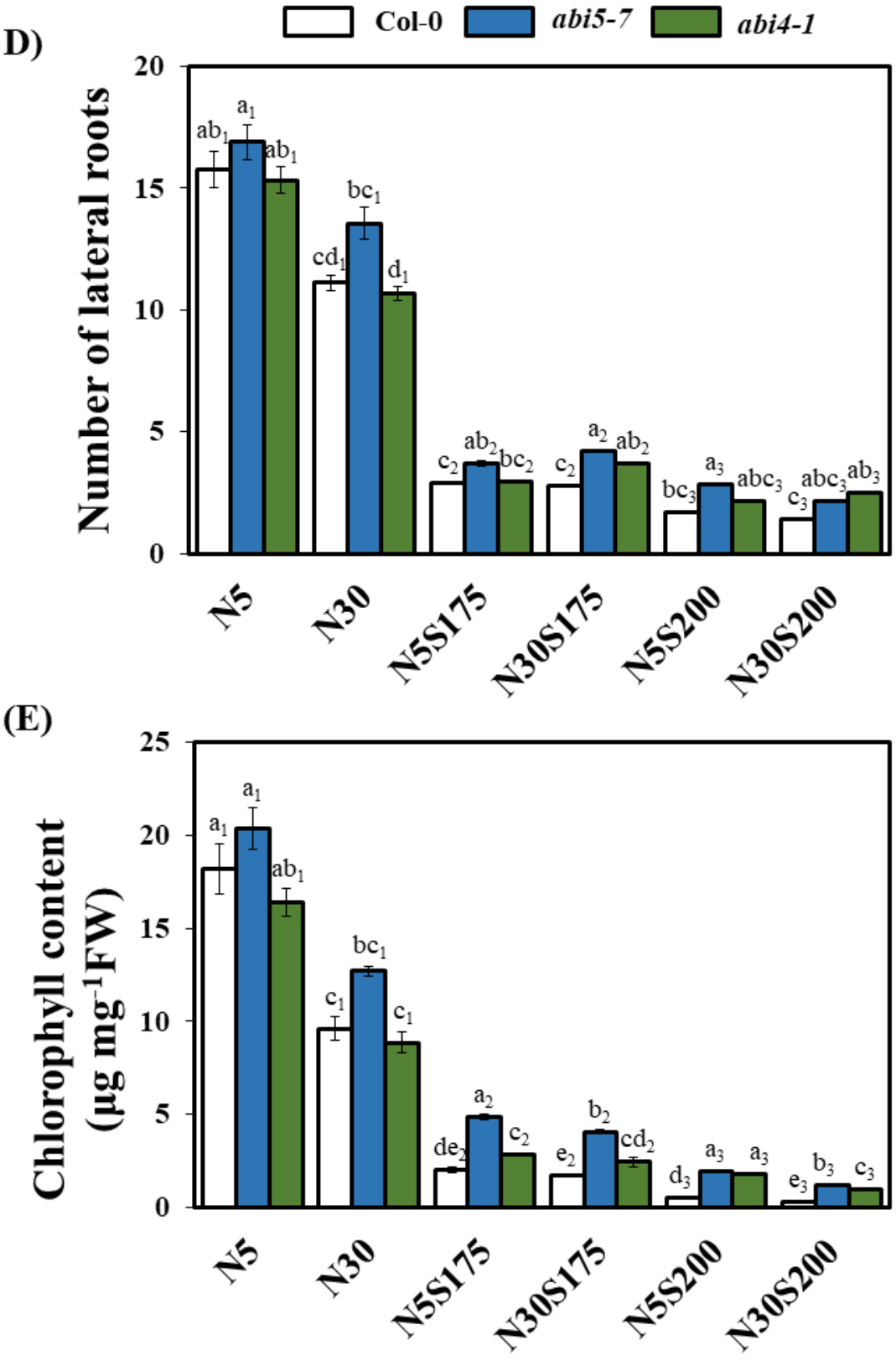
Four-day-old Col-0 grown on 5 mM NO_3^- agar medium was transferred to 5 and 30 mM NO_3^- media in the presence of 0 or 175 mM NaCl. After 9 days, the phenotype was established, and seedlings were collected for the phenotypic assay. Primary root growth and the chlorophyll content were measured as physical plant parameters. The error bars on top of each treatment represent the standard errors of three independent replicates. Different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (group 2).


(A)
Col-0
abi5-7
abi4-1
Col-0
abi5-7
abi4-1
1 cm
N5S175
N5
N30S175
N5S200
N30
N30S200

739


(B)
Col-0
abi5-7
abi4-1
Fresh weight (mg/seedling)
25
20
15
10
5
0
N5
N30
N5S175
N30S175
N5S200
N30S200
C)
Primary root length (cm)
16
12
8
4
0
3
2
1
0
N5S175
N30S175
N5S200
N30S200

740


D)
Col-0
abi5-7
abi4-1
Number of lateral roots
20
15
10
5
0
ab1
a1
ab1
bc1
cd1
d1
c2
ab2
bc2
c2
a2
ab2
bc3
a3
abc3
c3
abc3
ab3
N5
N30
N5S175
N30S175
N5S200
N30S200
(E)
Chlorophyll content
(µg mg-1FW)
25
20
15
10
5
0
a1
a1
ab1
bc1
c1
c1
de2
a2
c2
e2
b2
cd2
d3
a3
a3
e3
b3
c3
N5
N30
N5S175
N30S175
N5S200
N30S200

741

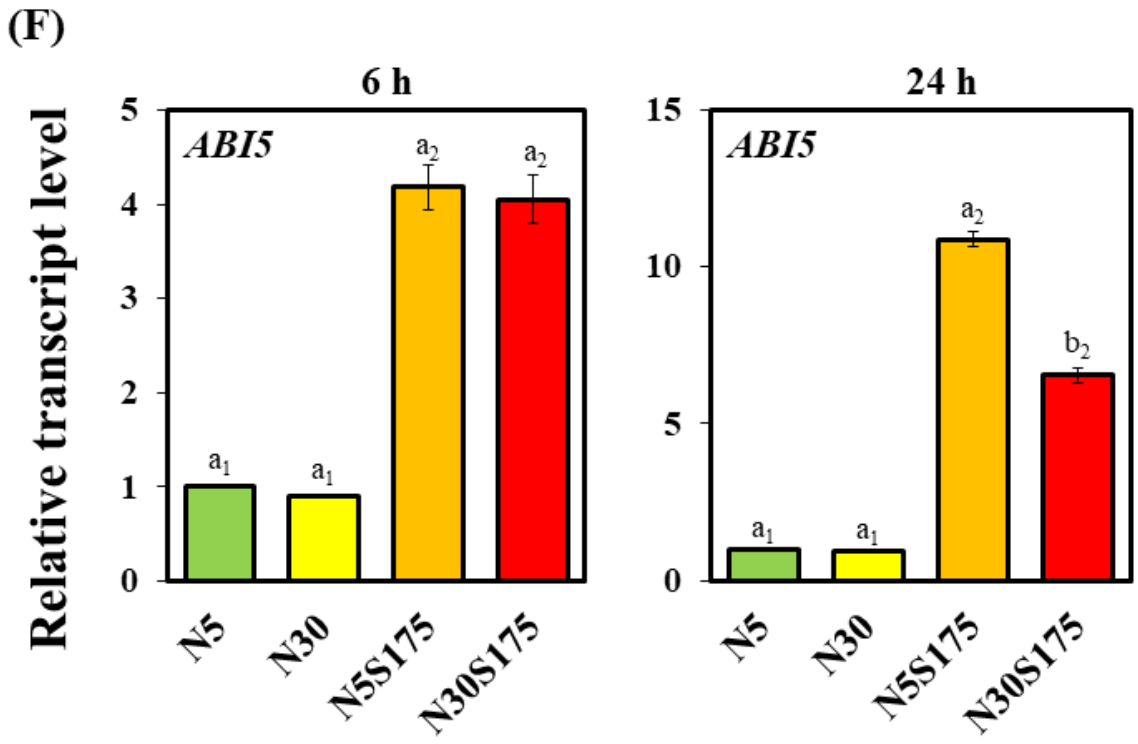
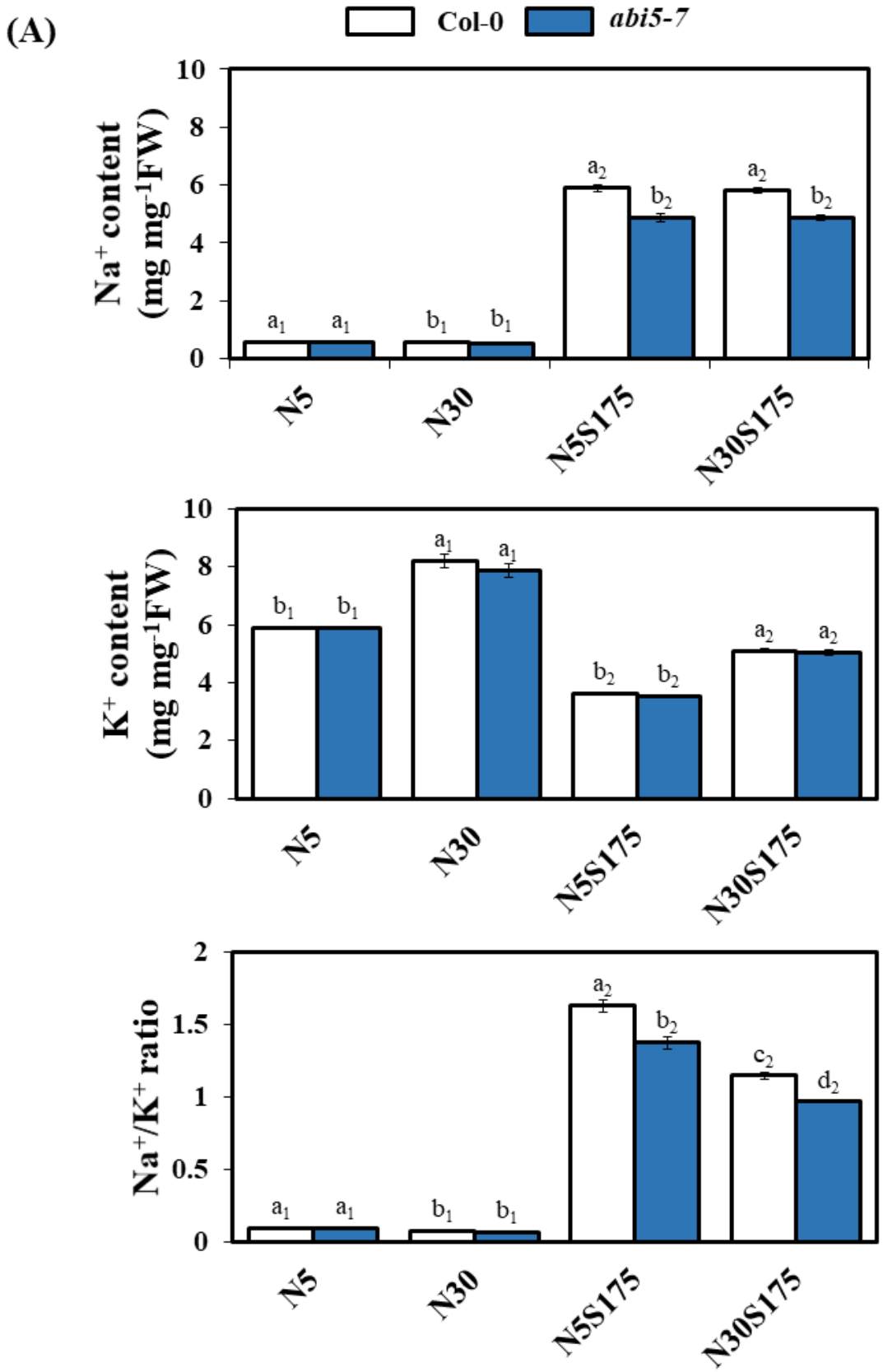
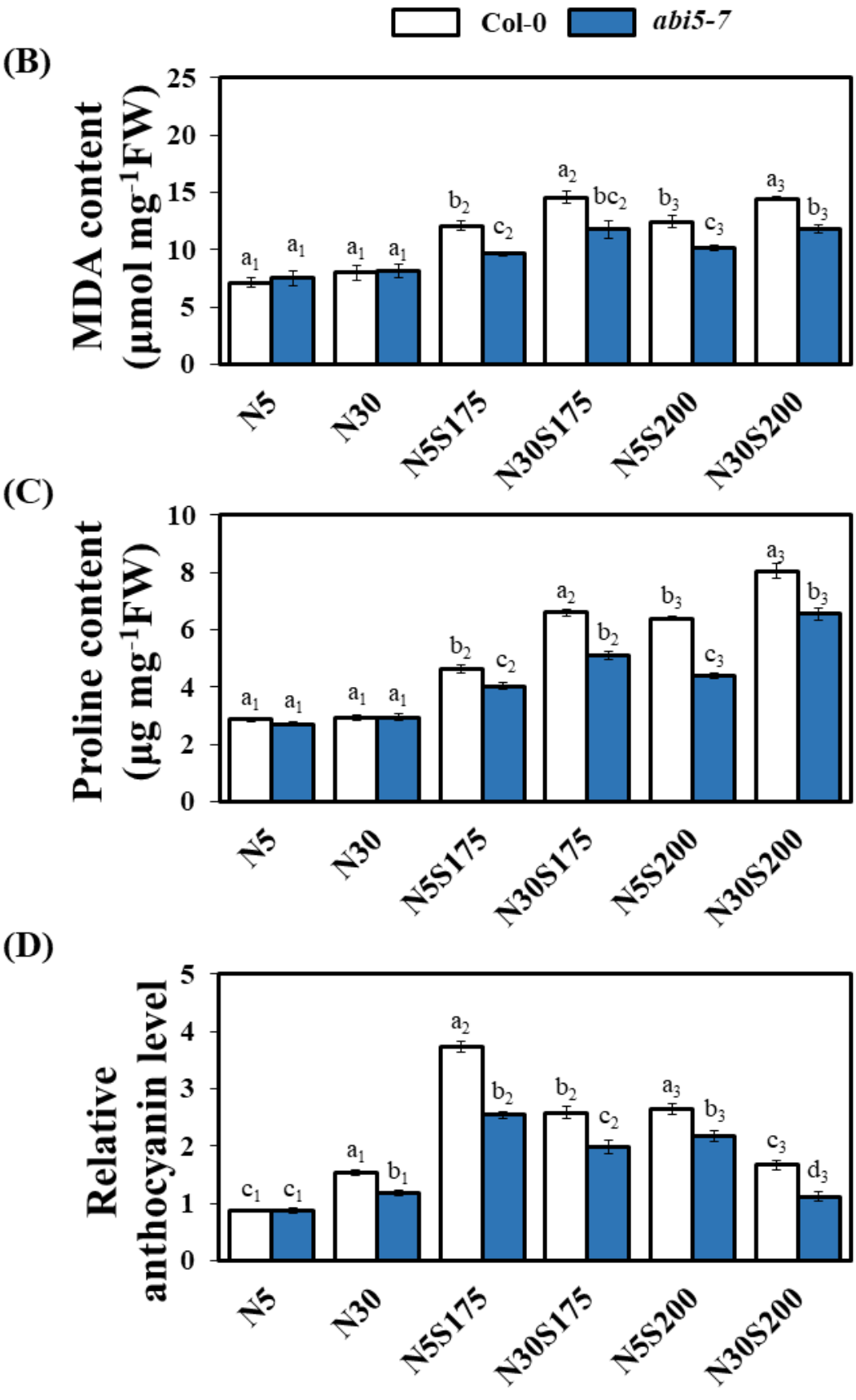


Figure 2. *abi5-7* mutant presented a tolerant phenotype to HNHS stress.

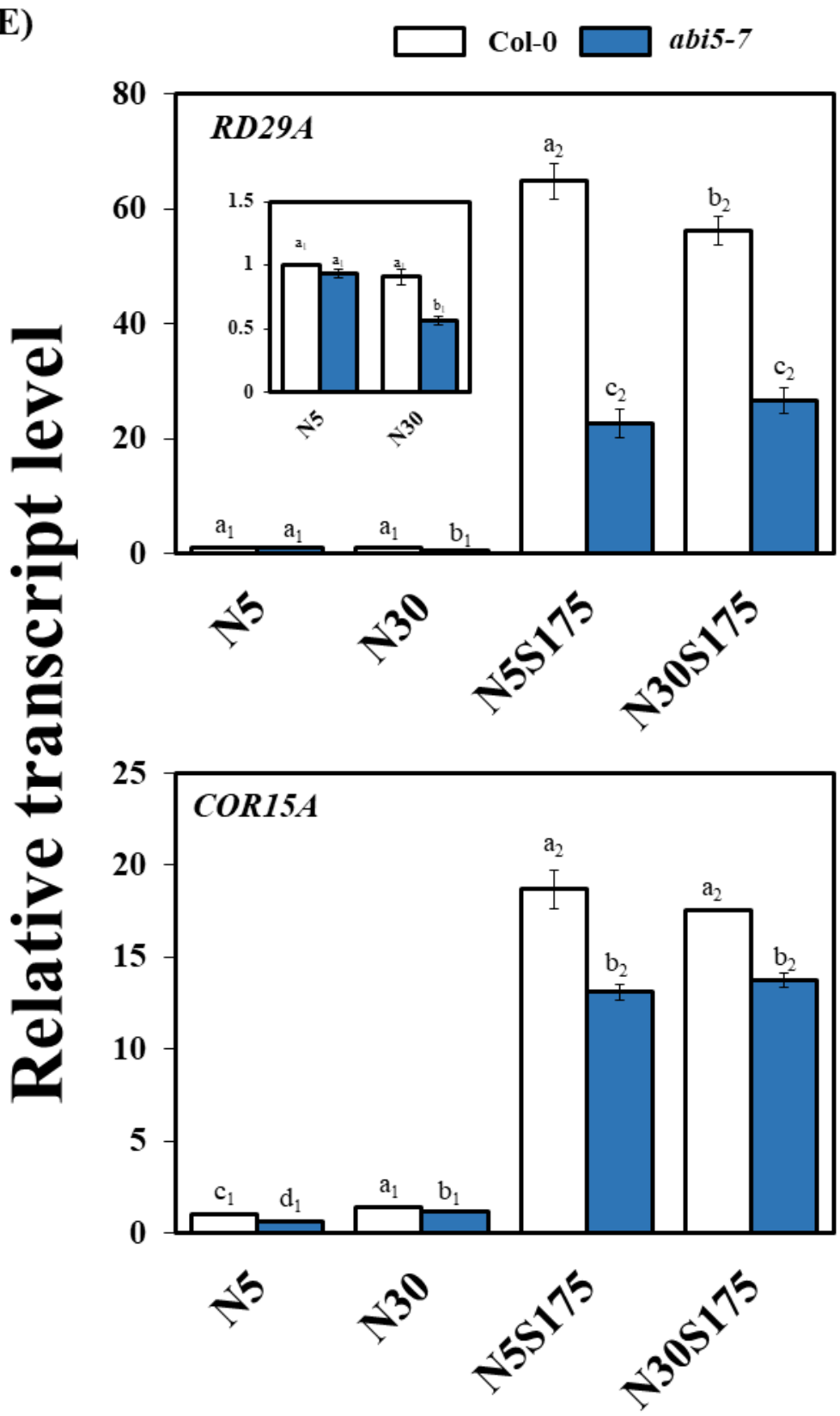
(**A**) Transcript levels of *ABI5* in Col-0 plants exposed to salt stress with or without the addition of high levels of nitrate. Nine-day-old Col-0 plants grown on 5 mM NO_3^- agar medium were treated with 5 and 30 mM NO_3^- solution in the presence of 0 or 175 mM NaCl. After 6 and 24 h of treatments, seedlings were collected, and the transcript levels of genes of interest were analysed, with *AtActin2* used as an internal control. The error bars on top of each treatment represent the standard error of three independent replicates. Different letters (a, b, or c) imply significant differences in two-way ANOVA ($P < 0.05$, Tukey's test). (**B**) Four-day-old Col-0, *abi4-1*, and *abi5-7* seedlings grown on 5 mM NO_3^- agar medium were transferred to 5 and 30 mM NO_3^- solution in the presence of 0, 175, and 200 mM NaCl. After two weeks, the phenotype was established, and seedlings were collected for the phenotypic assay. Fresh weights, primary root elongations, number of lateral roots and chlorophyll contents were measured as plant physical parameters. The error bars on top of each treatment represent the standard error of three independent replicates. Different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (group 2 and 3).


(A)
Col-0
abi5-7
Na+ content (mg mg-1FW)
K+ content (mg mg-1FW)
Na+/K+ ratio
N5
N30
N5S175
N30S175

759


Col-0
abi5-7
(B)
MDA content (μmol mg-1FW)
(C)
Proline content (μg mg-1FW)
(D)
Relative anthocyanin level
N5
N30
N5S175
N30S175
N5S200
N30S200

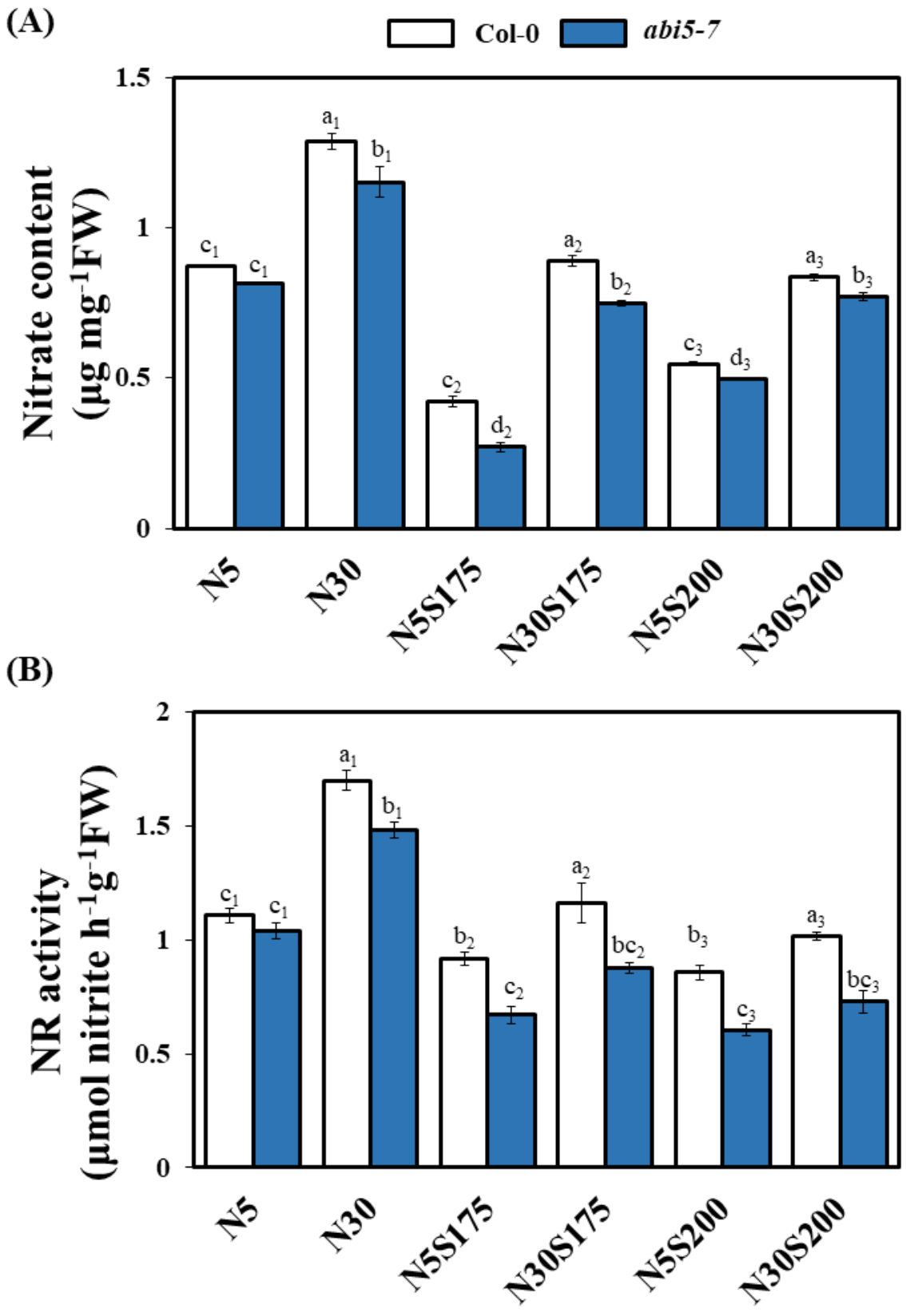
760


(E)
Col-0
abi5-7
Relative transcript level
RD29A
80
60
40
20
0
1.5
1
0.5
0
N5
N30
a2
b2
c2
a1
a1
a1
b1
N5
N30
N5S175
N30S175
COR15A
25
20
15
10
5
0
a2
b2
a2
b2
c1
d1
a1
b1
N5
N30
N5S175
N30S175

761

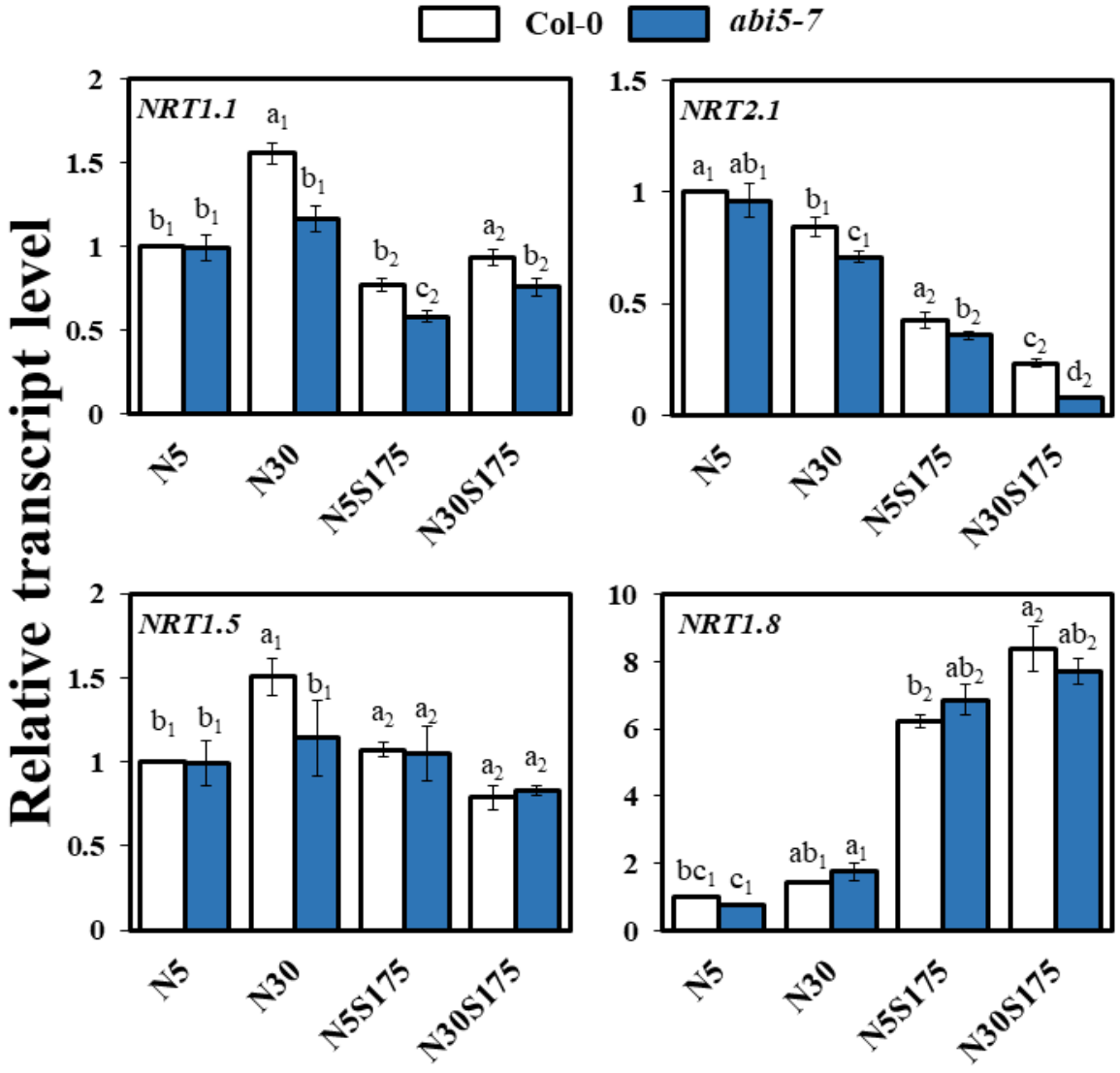
Figure 3. *abi5-7* suffered fewer HNHS-induced inhibitory effects than Col-0.

(**A**) *abi5-7* accumulated lower Na^+ resulting in lower Na^+/K^+ ratios than Col-0 under HNHS stress. Nine-day-old seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 48 h of treatment, samples were collected, and accumulation of Na^+ and K^+ was analysed using inductively coupled plasma-optical emission spectrometry; Na^+/K^+ ratios were calculated based on Na^+ and K^+ levels under identical conditions. The error bars on top of each treatment represent the SE of three independent replicates. (**B, C**) Accumulation of MDA and proline in *abi5-7* under HNHS conditions compared with those in Col-0. Nine-day-old Col-0, *abi5-7* seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0, 175, or 200 mM NaCl. After 24 h of treatment, seedlings were collected to quantify the MDA and proline levels. The error bars on top of each treatment represent the SE of three independent replicates. (**D**) Accumulations of anthocyanin in *abi5-7* under HNHS conditions compared with those in Col-0. Four-day-old Col-0, *abi5-7* seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- solution in the presence of 0, 175, or 200 mM NaCl. After 48 h of treatment, seedlings were collected for anthocyanin quantification. The error bars on top of each treatment represent the SE of three independent replicates. (**E**) Transcript levels of salt stress-responsive genes, *RD29A* and *COR15A*, in Col-0 and *abi5-7* plants exposed to HNHS conditions. Nine-day-old seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, seedlings were collected, and the transcript levels of the gene of interest were analysed, with *AtActin2* used as an internal control. The error bars on top of each treatment represent the SE of three independent replicates. Different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (group 2).

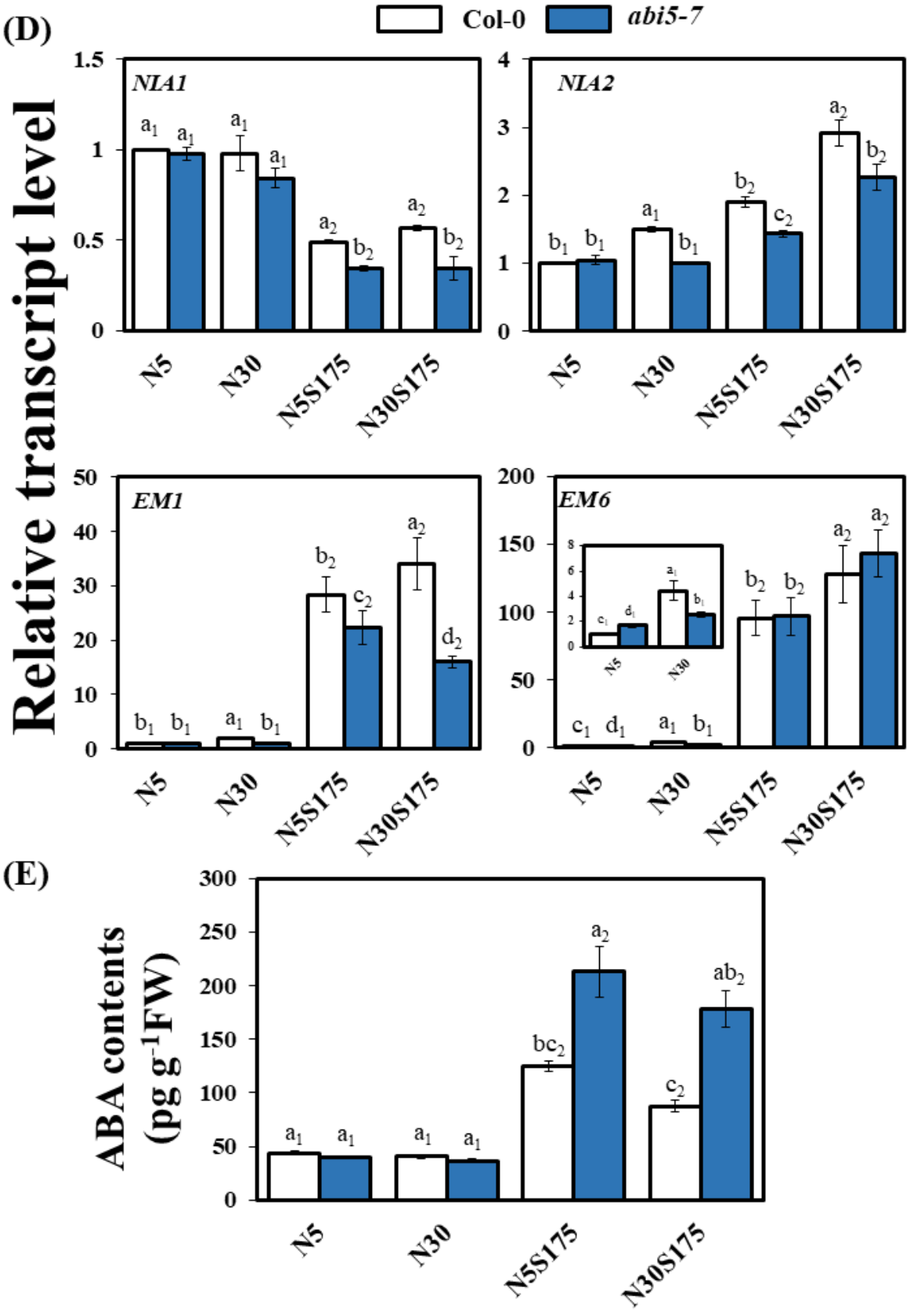

(A)
Col-0
abi5-7
Nitrate content (μg mg-1FW)
1.5
1
0.5
0
a1
b1
c1
c1
a2
b2
c2
d2
c3
d3
a3
b3
N5
N30
N5S175
N30S175
N5S200
N30S200
(B)
NR activity (μmol nitrite h-1g-1FW)
2
1.5
1
0.5
0
a1
b1
c1
c1
b2
c2
a2
bc2
b3
c3
a3
bc3
N5
N30
N5S175
N30S175
N5S200
N30S200

786

(C)


Col-0
abi5-7
Relative transcript level
NRT1.1
NRT2.1
NRT1.5
NRT1.8
N5
N30
N5S175
N30S175

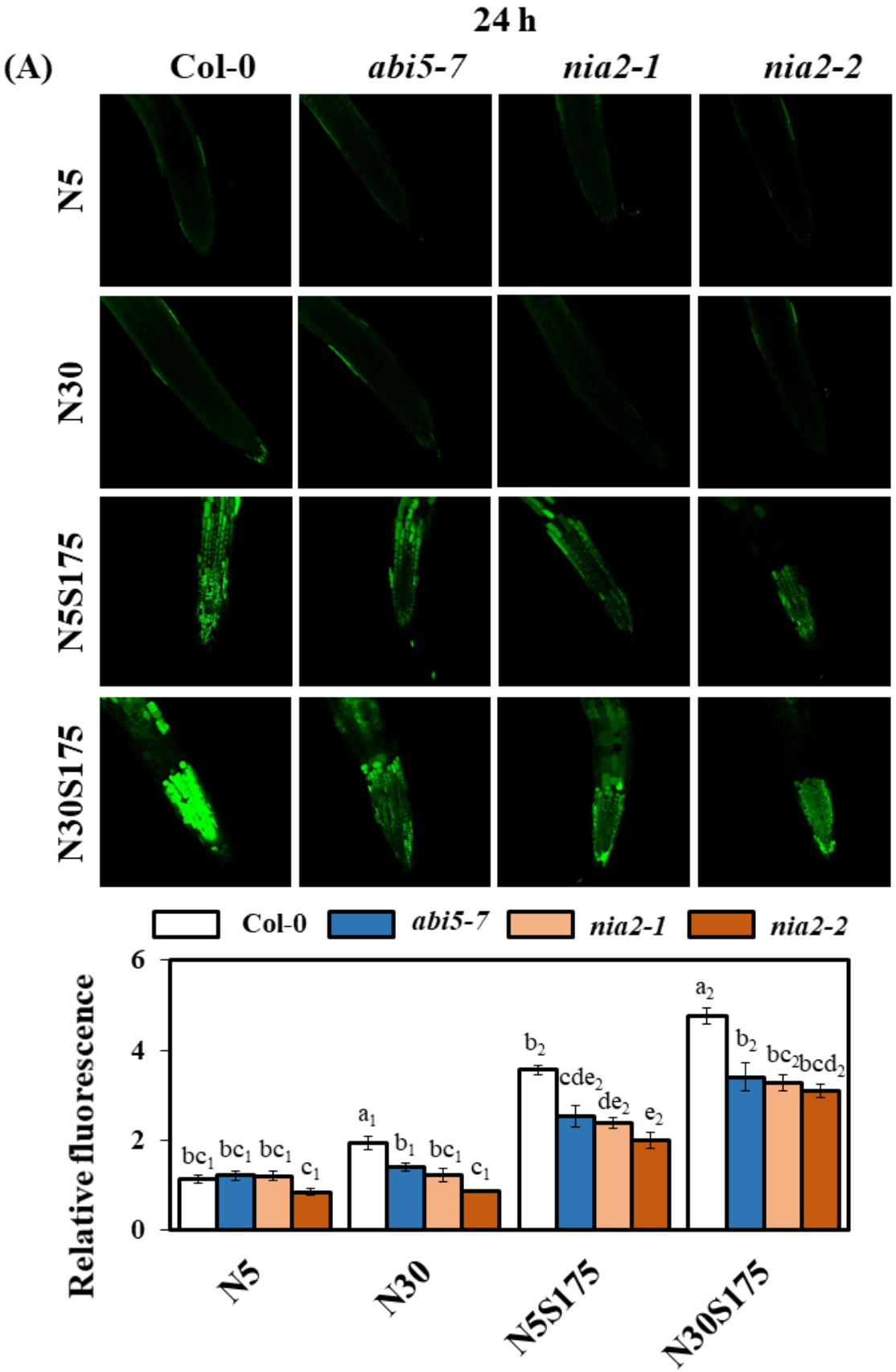
787


(D)
Col-0
abi5-7
Relative transcript level
NIA1
NIA2
EM1
EM6
N5
N30
N5S175
N30S175
(E)
ABA contents
(pg g-1FW)

788

Figure 4. *abi5-7* accumulated lower nitrate contents and had lower NR activity compared to Col-0 under HNHS conditions.

(**A, B**) Nitrate contents and NR activity were lower in *abi5-7* mutant than in Col-0. Nine-day-old investigated seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, samples were collected, and the nitrate contents and NR activity were analysed. The error bars on top of each treatment represent the SE of three independent replicates. (**C, D**) Transcript levels of nitrate transporters (*NRT1.1*, *NRT2.1*, *NRT1.5*, and *NRT1.8*), nitrate accumulation (*NIA1* and *NIA2*), ABI5 target gens (*EM1* and *EM6*), in Col-0 and *abi5-7* plants exposed to HNHS conditions. Nine-day-old seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, seedlings were collected, and the transcript levels of the gene of interest were analysed, with *AtActin2* used as an internal control. The error bars on top of each treatment represent the SE of three independent replicates. (**E**) The abscisic acid (ABA) content in *abi5-7* was higher than that in Col-0 plants. Nine-day-old investigated seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, samples were collected, and the ABA contents were analysed using Phytodetek Elisa kit (Agdia, Inc.) according to the manufacturer's instructions. The error bars on top of each treatment represent the SE of three independent replicates. Different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (group 2).


24 h
(A)
Col-0
abi5-7
nia2-1
nia2-2
N5
N30
N5S175
N30S175
Col-0
abi5-7
nia2-1
nia2-2
Relative fluorescence
0
2
4
6
bc1 bc1 bc1 c1
a1 b1 bc1 c1
b2 cde2 de2 e2
a2 b2 bc2 bcd2
N5
N30
N5S175
N30S175

809

810

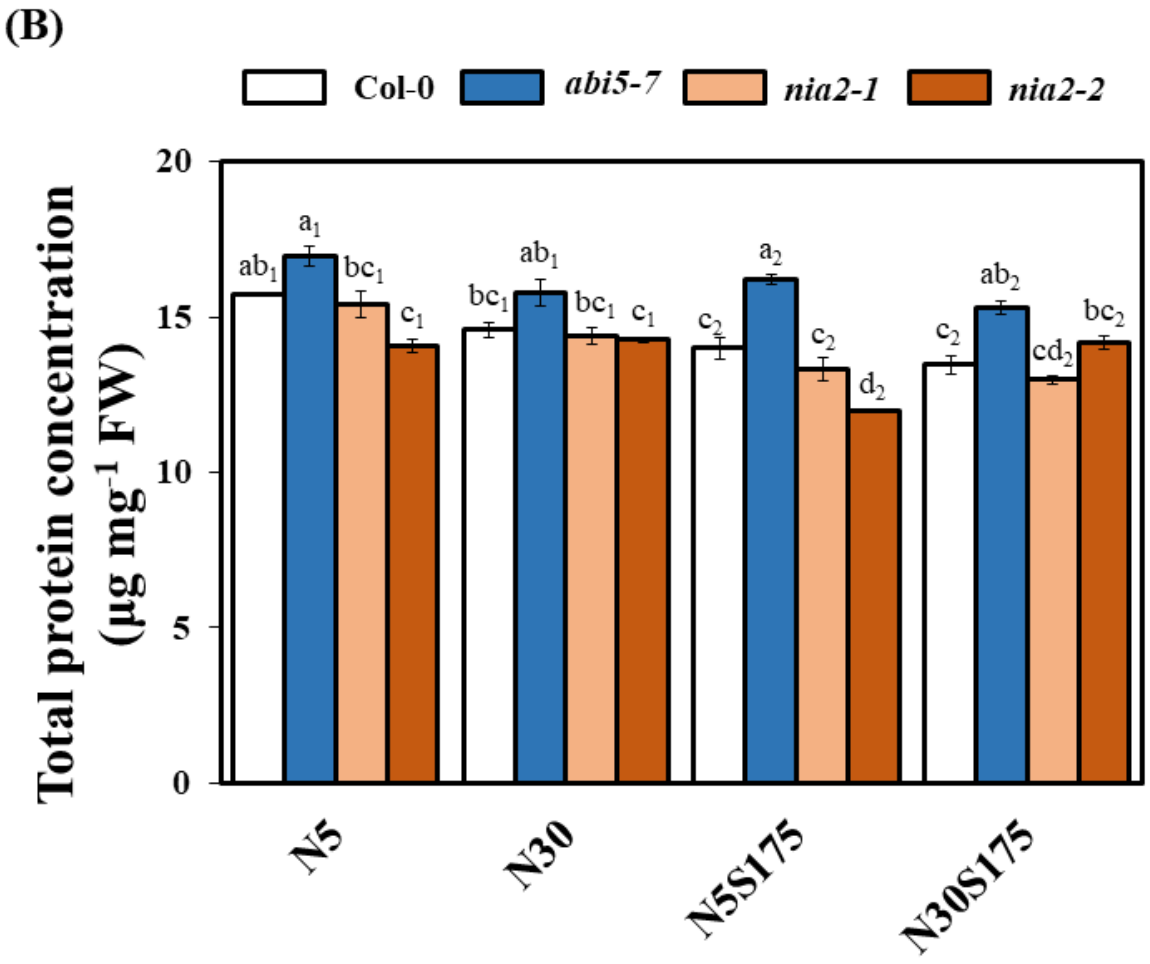
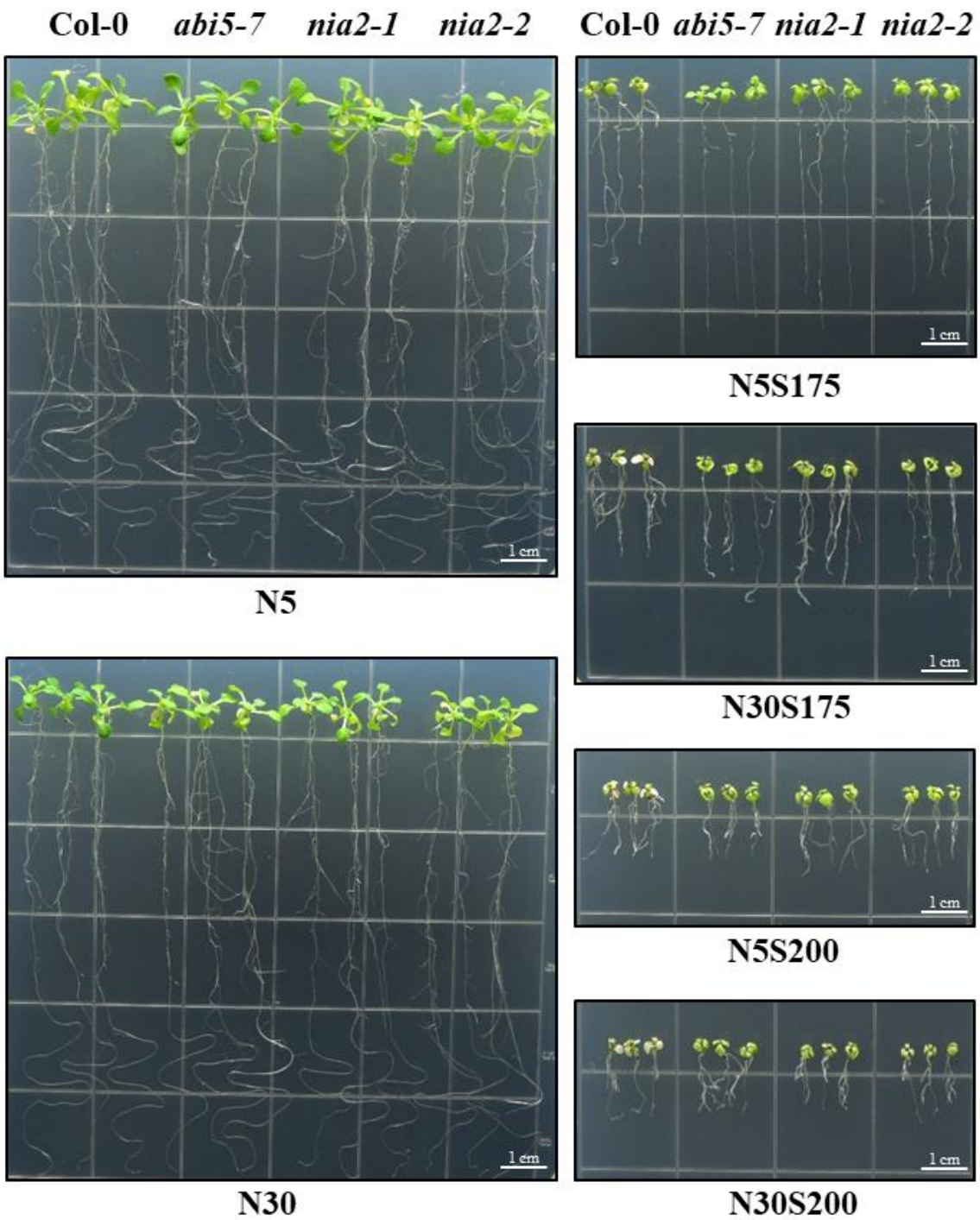

(B)
Col-0
abi5-7
nia2-1
nia2-2
Total protein concentration (µg mg-1 FW)
20
15
10
5
0
ab1
a1
bc1
c1
bc1
ab1
bc1
c1
c2
a2
c2
d2
c2
ab2
cd2
bc2
N5
N30
N5S175
N30S175

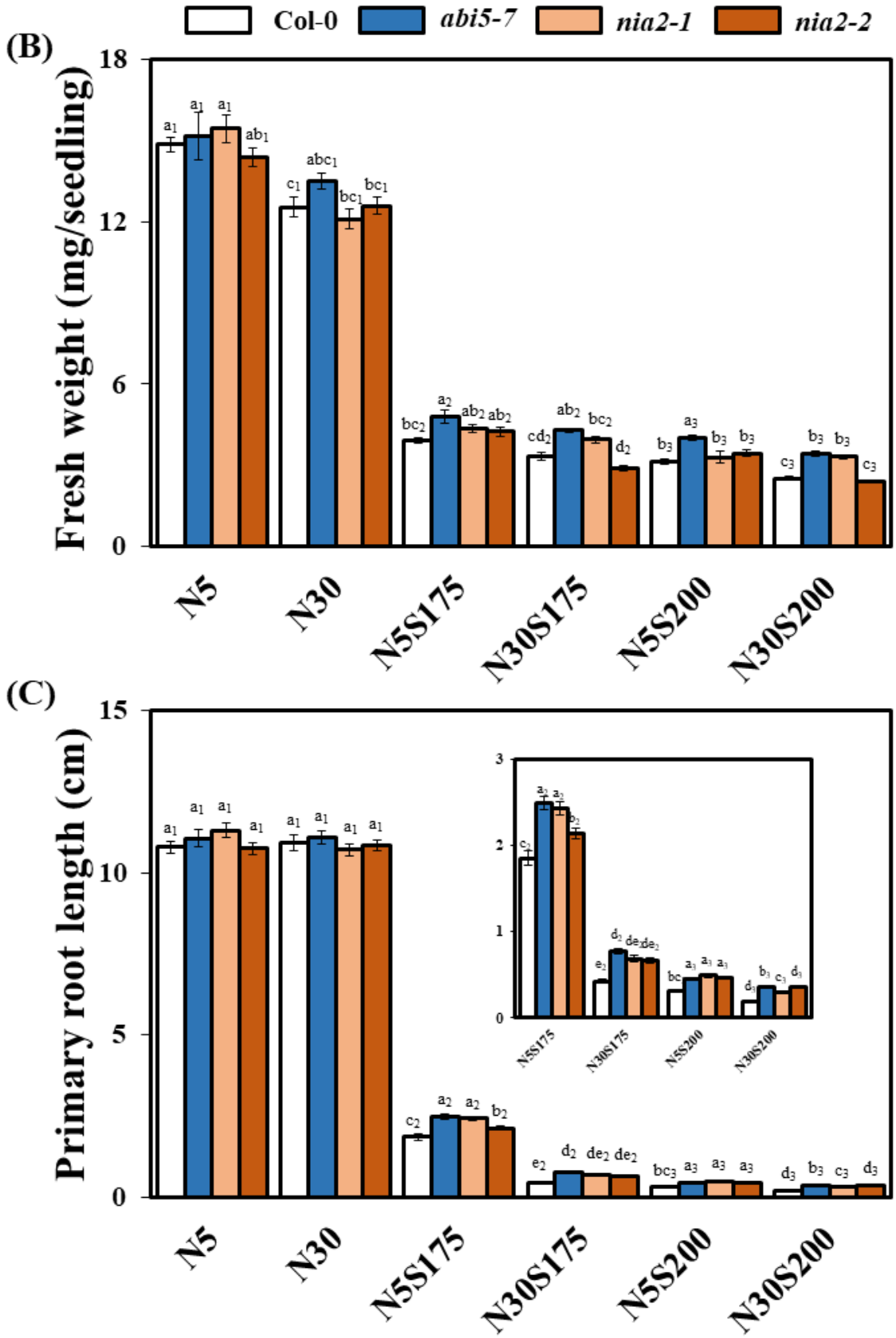
Figure 5. Endogenous NO levels in *abi5-7* were lower than those in Col-0, which was related to NR activity.

(**A**) *abi5-7* and *nia2* mutants accumulated lower endogenous NO levels compared to Col-0. Nine-day-old seedlings grown in 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, samples were collected, and *in planta* NO accumulation was analysed with a NO-specific fluorescent probe, DAF-FM DA, using a laser confocal scanning microscope with a excitation and emission wavelengths of 488 nm and 515–565 nm, respectively. Relative fluorescence was measured using the ImageJ software. The error bars on top of each treatment represent the SE of three independent replicates. (**B**) Total protein concentration among the genotypes under HNHS conditions. Nine-day-old seedlings grown on 5 mM NO_3^- agar medium were treated with 5 or 30 mM NO_3^- in the presence of 0 or 175 mM NaCl. After 24 h of treatment, samples were collected, and total protein was extracted; the protein concentrations were measured in a BCA assay. The error bars on top of each treatment represent the SE of three independent replicates. Different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (group 2).

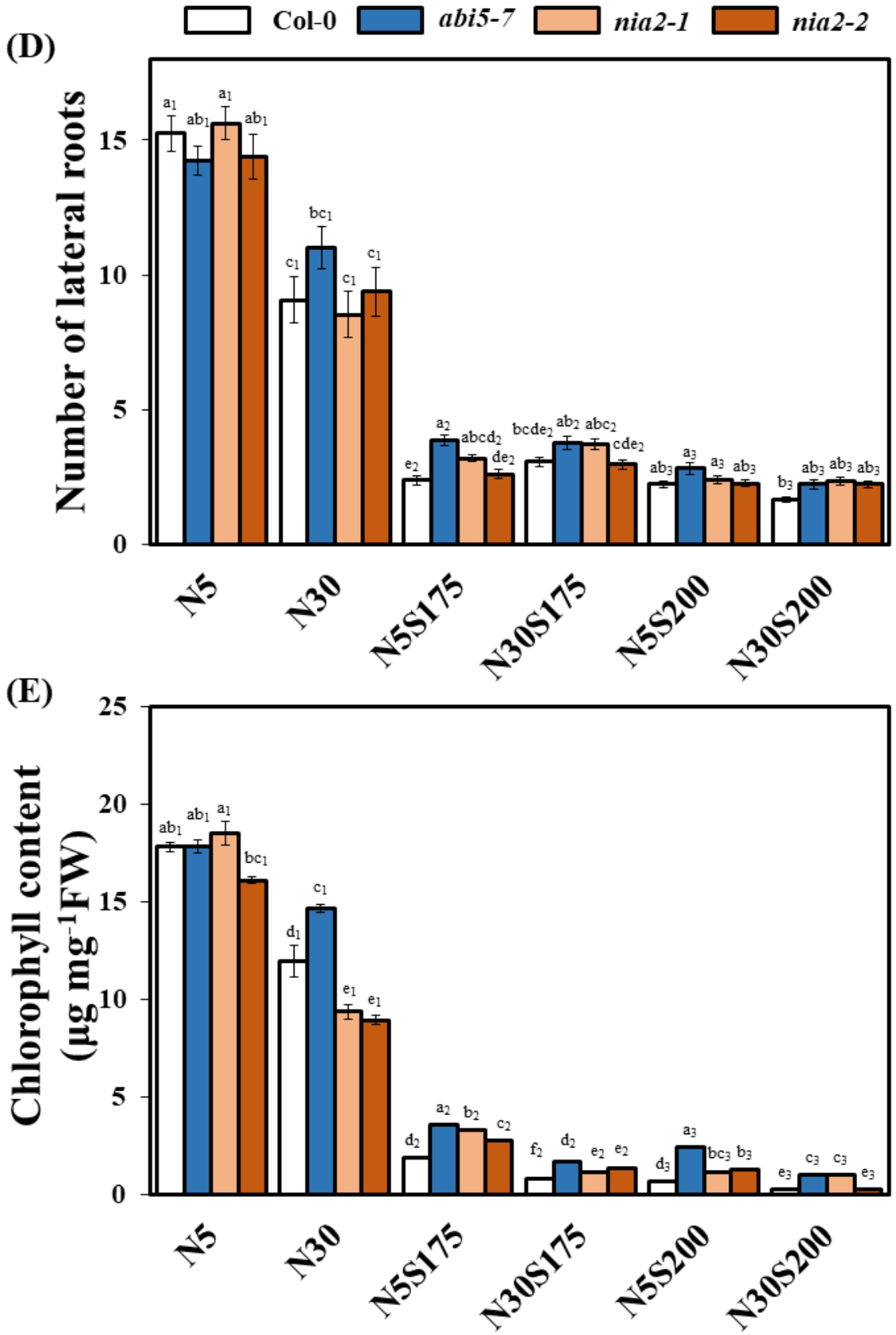
(A)


Col-0 abi5-7 nia2-1 nia2-2
Col-0 abi5-7 nia2-1 nia2-2
1 cm
N5
N5S175
N30
N30S175
N5S200
N30S200

827


Col-0
abi5-7
nia2-1
nia2-2
(B)
Fresh weight (mg/seedling)
18
12
6
0
N5
N30
N5S175
N30S175
N5S200
N30S200
(C)
Primary root length (cm)
15
10
5
0
N5
N30
N5S175
N30S175
N5S200
N30S200

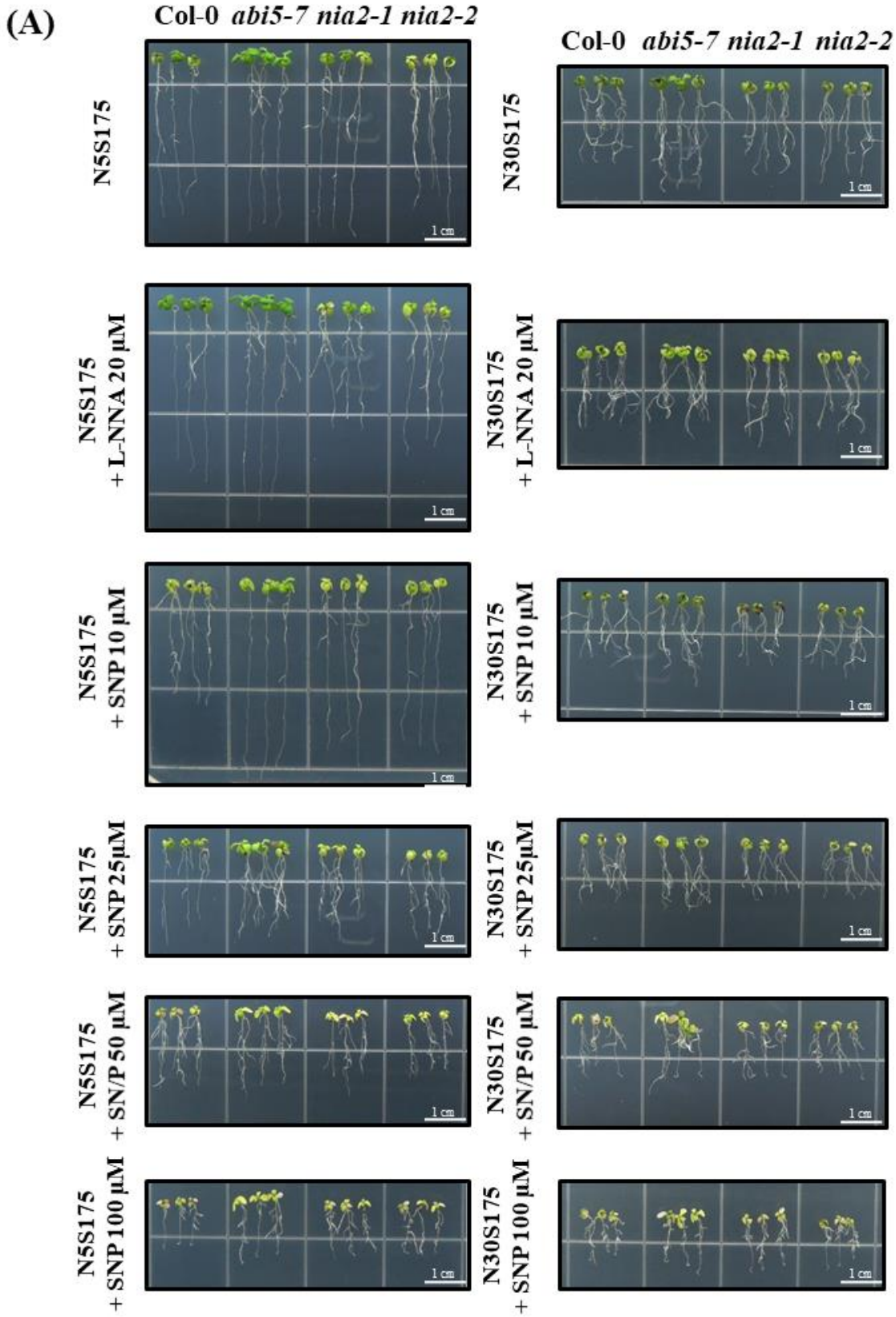
828


(D)
Col-0
abi5-7
nia2-1
nia2-2
Number of lateral roots
0
5
10
15
N5
N30
N5S175
N30S175
N5S200
N30S200
(E)
Chlorophyll content (μg mg-1FW)
0
5
10
15
20
25
N5
N30
N5S175
N30S175
N5S200
N30S200

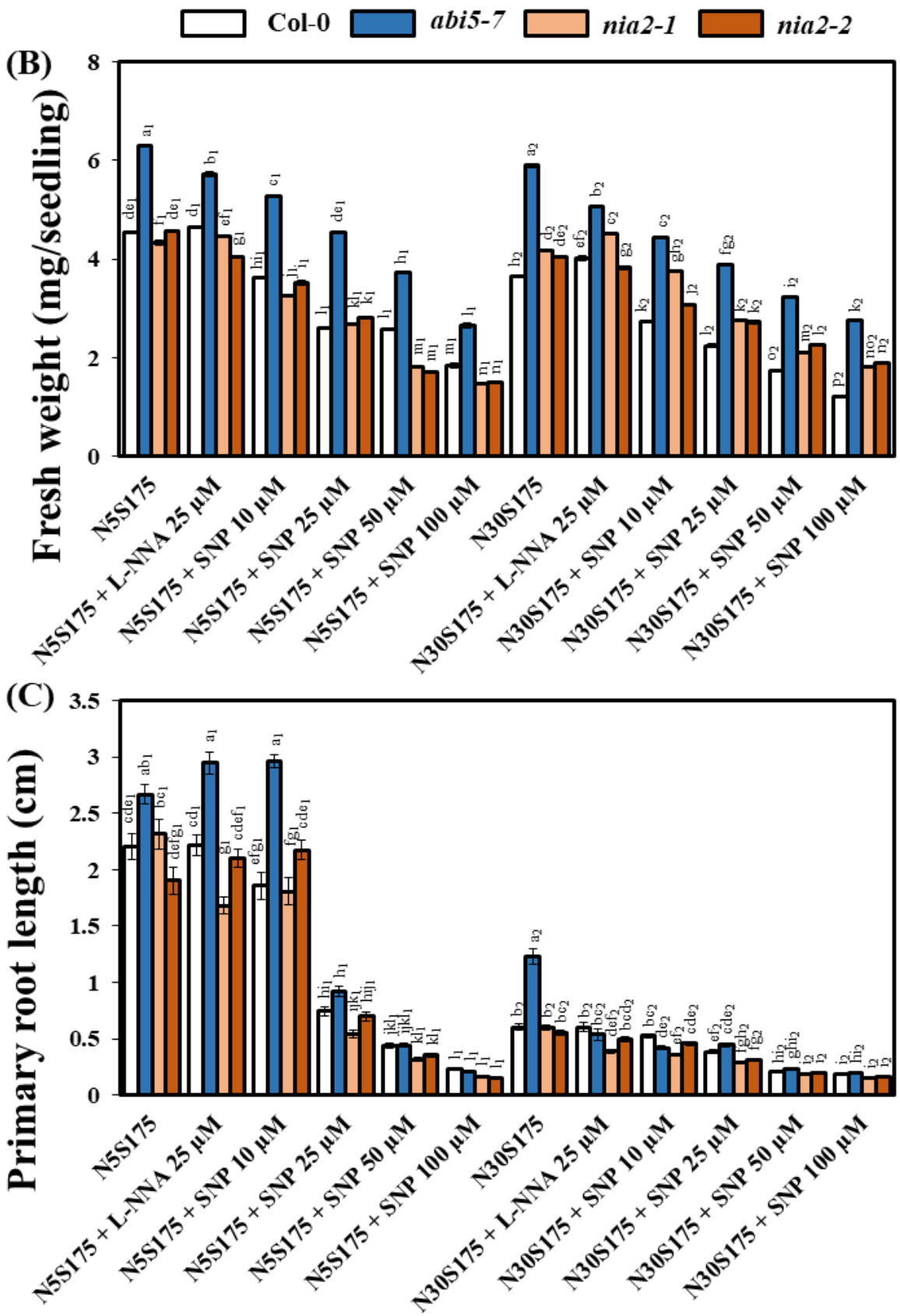
829

Figure 6. *NIA2* may be involved in the tolerance of *abi5-7* mutant to HNHS stress through the phenotype of *nia2* mutants.

Four-day-old Col-0, *abi5-7*, and *nia2* seedlings grown on 5 mM NO_3^- agar medium were transferred to 5 or 30 mM NO_3^- in the presence of 0, 175, and 200 mM NaCl. After two weeks, the phenotype was established, and seedlings were collected for the phenotypic assay. Fresh weight, primary root elongation, number of lateral roots, and chlorophyll content were measured as plant physical parameters. The error bars on the top of each treatment represent the SEs of three independent replicates. Different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (groups 2 and 3).


(A)
Col-0 abi5-7 nia2-1 nia2-2
Col-0 abi5-7 nia2-1 nia2-2
N5S175
N30S175
N5S175 + L-NNA 20 µM
N30S175 + L-NNA 20 µM
N5S175 + SNP 10 µM
N30S175 + SNP 10 µM
N5S175 + SNP 25µM
N30S175 + SNP 25µM
N5S175 + SN/P 50 µM
N30S175 + SN/P 50 µM
N5S175 + SNP 100 µM
N30S175 + SNP 100 µM
1 cm

840


Col-0
abi5-7
nia2-1
nia2-2
(B)
Fresh weight (mg/seedling)
0
2
4
6
8
(C)
Primary root length (cm)
0
0.5
1
1.5
2
2.5
3
3.5
N5S175
N5S175 + L-NNA 25 µM
N5S175 + SNP 10 µM
N5S175 + SNP 25 µM
N5S175 + SNP 50 µM
N5S175 + SNP 100 µM
N30S175
N30S175 + L-NNA 25 µM
N30S175 + SNP 10 µM
N30S175 + SNP 25 µM
N30S175 + SNP 50 µM
N30S175 + SNP 100 µM

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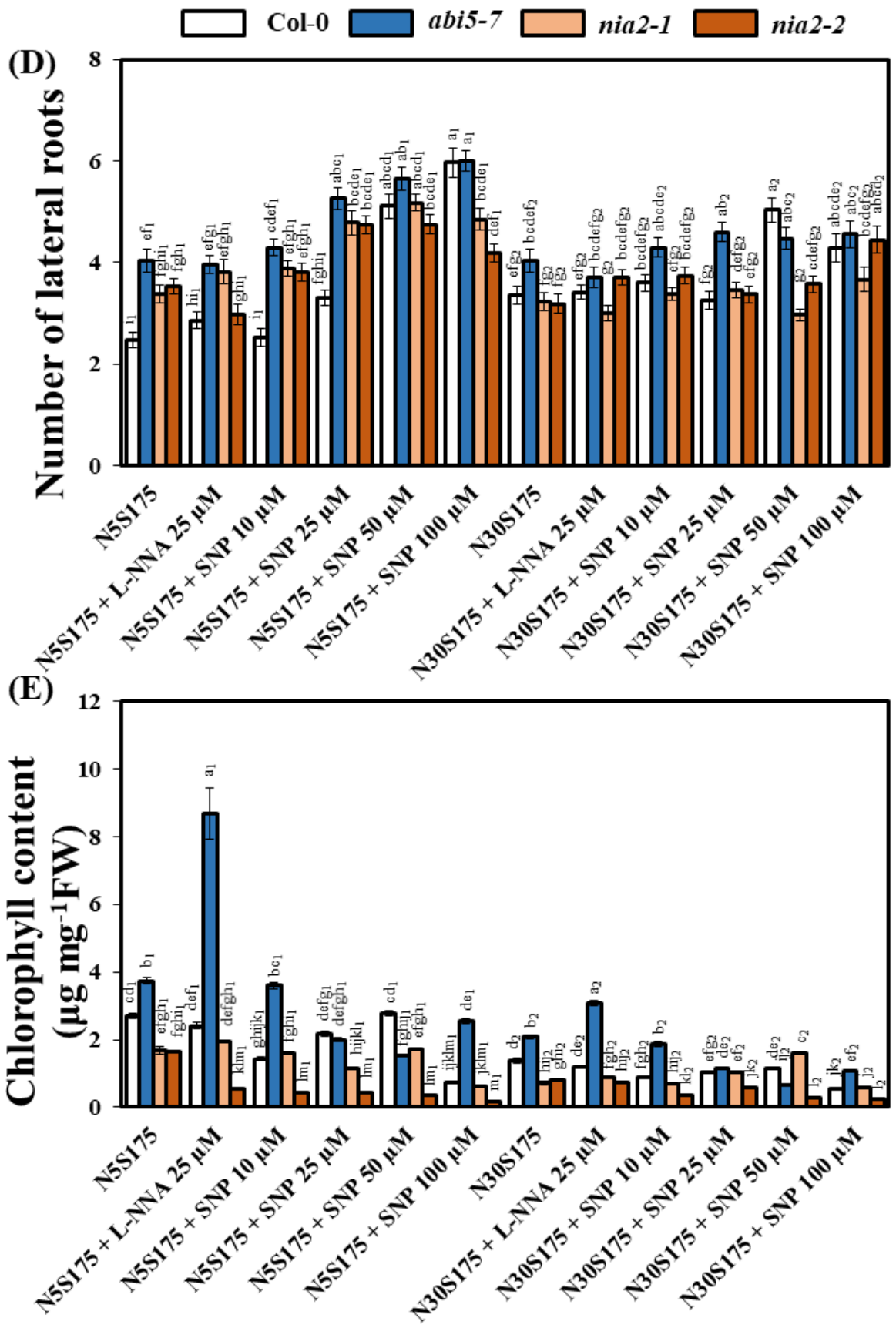
Col-0
abi5-7
nia2-1
nia2-2
(D)
Number of lateral roots
(E)
Chlorophyll content
(μg mg-1FW)
N5S175
N5S175 + L-NNA 25 μM
N5S175 + SNP 10 μM
N5S175 + SNP 25 μM
N5S175 + SNP 50 μM
N5S175 + SNP 100 μM
N30S175
N30S175 + L-NNA 25 μM
N30S175 + SNP 10 μM
N30S175 + SNP 25 μM
N30S175 + SNP 50 μM
N30S175 + SNP 100 μM

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Figure 7. External NO application inhibited *abi5-7* growth under HNHS conditions.

Four-day-old Col-0, *abi5-7*, and *nia2* seedlings grown on 5 mM NO_3^- agar medium were transferred to 5 or 30 mM NO_3^- in the presence of 175 mM NaCl. After two weeks, the phenotype was established, and seedlings were collected for the phenotypic assay. Fresh weight, primary root elongation, number of lateral roots, and chlorophyll content were measured as plant physical parameters. The error bars on top of each treatment represent the SEs of three independent replicates. The different letters (a, b, or c) indicate significant differences in two-way ANOVA ($P < 0.05$, Tukey's test) in which the treatments were divided into sub-categories, including the absence (group 1) and presence of salinity (groups 2 and 3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ABI5SupplementaryDataPlantCellReport20220402.pdf