Nitrate reductase-dependent NO production is critical for *Arabidopsis* roots response to ABA

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ABSTRACT. The exogenous application of abscisic acid (ABA) to inhibit root growth has been reported but its signal mechanisms are unclear. In this study, the ABA response was investigated using *Arabidopsis thaliana* mutants *noa1* and *nia1,nia2*. The post-germination root growth of the *nia1,nia2* mutants was more sensitive to ABA than that of both wild-type (WT) and the *noa1* mutant. The sensitivity of root growth was restored after ten days. Similarly, the root cells of the *nia1,nia2* mutants produced endogenous NO later than those in the WT and *noa1* seedlings under ABA treatment. After several days, the fluorescence of NO reemerged followed by a recovery of root growth in the *nia1,nia2* mutants. On the other hand, pharmacologic analysis showed that exogenous nitro iron hydrogenated sodium, an NO donor, partially restored root growth under ABA treatment. And tungstate, a target inhibitor of nitrate reductase (NR), significantly reduces ABA-induced NO production and stops root growth of WT seedlings. In addition, the ABA treatment enhanced NR activity, which decreased with time. In conclusion, NO production is critical for root growth under ABA treatment. The early source of NO induced by ABA is primarily the NR pathway. Other NO sources appear to be involved later in the regulation of root growth.
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Abbreviations: ABA, abscisic acid; **NO,** nitric oxide; **WT,** wild-type; **NR,** nitrate reductase; **NOS,** nitric oxide synthase; **VD-toxins,** *Verticillium dahliae* toxins; **DAF-2DA,** 4,5-diaminofluorescein diacetate.

1. Intrdduction

The root system is very important in the plant lifecycle and is strongly influenced by endogenous signals and exogenous stimuli (Kopyra et al., 2003; Almaghrabi, 2012). As a universal gaseous molecule, NO is involved in numerous plant growth and developmental processes, including root elongation, seed germination, stomatal movement, and stress responses (Lamattina et al., 2003; Arasimowicz et al., 2007; Zhao et al., 2007). NO is reportedly involved in nitrate-induced inhibition of root elongation in *Zea mays* (Zhao et al., 2007). Moreover, the reduction of endogenous NO concentrations resulting from inhibition of NOS activity could underpin Al-induced arrest of root elongation in *Hibiscus moscheutos* (Tian et al., 2007). On the other hand, the exogenous supply of NO can effectively promote root growth in cucumber (Neill et al., 2003). NO possesses several resources such as nitric oxide synthase (NOS)-like enzymes, NR, and other non-enzymatic pathways (Desikan et al., 2002; Guo et al., 2003; Crawford et al., 2006; Wilson et al., 2008). Up to now, the mechanisms responsible for NO synthesis in plants remain controversial. We have also previously reported that VD-toxins trigger stronger NO burst primarily from the NR pathway in *A. thaliana* (Shi et al., 2008).

The phytohormone ABA is a universal and important signaling molecule with multiple functions in regulating plant development and stress responses (Leung et al., 1998; Umezawa et al., 2010). The exogenous application of ABA can inhibit root growth and this phenomenon has widely reported under normal and stress conditions (Sharp et al., 2004; Bai et al., 2009). ABA is closely associated with other signals in terms of its signal transduction and mediation of physiologic processes (Bright et al., 2006; Umezawa et al., 2010). For instance, the triple mutant *nia1, nia2,noa1-2* shows decreased seedling establishment and enhanced ABA sensitivity (Lozano-Juste and León, 2010). In addition, ABA and NO are closely related to stress-induced stomatal closure, which is very important for plant adaptation to various stresses (Ribeiro et al., 2009). *Atnos1* (*noa1*) guard cells have significantly impaired NO generation in response to ABA compared with the WT plants, but to a lesser degree than those of *nia1,nia2* mutants (Guo et al., 2003; Bright et al., 2006). Therefore, the diversity of ABA responses may require multiple ABA receptors, such as ABAR/CHLH, GPCRs (GTG1 and GTG2), PYR/ RCARs and so on (Shen et al., 2006; Pandey et al., 2009; Ma et al., 2009; Park et al., 2009; Hubbard et al., 2010).

To elucidate the underlying molecular mechanisms responsible for NO production and its function in *A. thaliana* seedlings responses to ABA, WT seedlings and *Atnoa1*, and *nia1,nia2* mutants of *A. thaliana* were used as donors to investigate the ABA response.

2. Material and Methods

Plant materials and growth conditions

A. thaliana ecotype Columbia (Col-0) was used as the WT control. NR-deficient mutant *nia1,nia2* and NOS-associated mutant *noa1* were obtained from Prof. Nigel M. Crawford and Dr. Fang-Qing Guo. *Arabidopsis* mutant *noa1* was identified that had impaired NO production, organ growth, and ABA-induced stomatal movements (Guo et al., 2003). n*ia1, nia2* double mutants have only0.5% of wild-type shoot NR activity and display very poor growth on media with nitrate as the only form of nitrogen (Wilkinson and Crawford, 1993). The seeds were surface-sterilized in 10% sodium hypochlorite and rinsed five times in sterile water. The seeds were then sown into MS medium containing 3% sucrose and 0.7% agar for normal germination or into the same basic medium supplemented with ABA treatment at indicated concentrations. After storage at 4 °C for 72 h, the seeds were germinated at 22 °C with 14 h light (100 M m^{-2} s^{-1}) and 10 h dark and 70% relative humidity. The Petri dishes were arranged vertically in a growth chamber. After six or seven days of culturing in MS medium, the seedlings were harvested for experiments.

Chemicals and ABA treatments

Sterilized mutant and WT seeds were germinated in MS medium added with sodium tungstate (Na_2WO_4, NR inhibitors), or S-nitroso-N-acetyl-DL penicillamine (SNAP, an NO donor) and/or 0, 0.5, 1.0, 1.5, 2.0 μmol L^{-1} ABA. The seedlings were used for further analysis at the desired time points, and seedlings grown in basic MS medium without treatments were used as controls. All chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

Growth measurement

To examine the effect of ABA and SNAP on root elongation, mutants and WT, seedlings were planted in MS medium containing ABA or ABA/SNAP for varying time periods (7, 11, 17 d). The seedlings were then photographed and their primary root lengths were measured.

NO assays

Endogenous nitric oxide was visualized using the specific NO fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA), according to the method described by Shi et al.(2008, 2009). Seven-day-old seedlings planted in MS medium with or without 0.5 μmol L^{-1}ABA treatment were placed in an Eppendorf tube containing a loading buffer (10 mmol L^{-1} Tris–KCl, pH 7.2) supplemented with DAF-2DA at a final concentration of 10 mmol L^{-1} and maintained at room temperature in the dark for 10 min. The roots loaded with DAF-2DA were observed and photographed using fluorescence microscopy (Olympus BX51, Japan). Images of NO-induced fluorescence were processed and analyzed using Zeiss LSM software image J version 1.37v.

Assay of nitrate reductase activities

NR activity was measured in *A. thaliana* seedlings with or without ABA treatment according to references (Bradford,1976; Allegre et al., 2004; Shi et al., 2008). The seedlings grown on MS medium supplemented with or without 0.5 μmol L^{-1}ABA for 6, 12, and 18 days were harvested and immersed with 1 ml of 0.1 mol L^{-1} phosphate buffer (pH 7.5), 0.1 mol L^{-1} KH_2PO4, 14 mmol L^{-1} mercaptoethanol containing anti-protease cocktail (Merck KGaA, Darmstadt, Germany), and 1 mmol L^{-1} chymostatin, in the presence of 200 μl of 1.4 mmol L^{-1} NADH and 200 μl of 0.1 mol L^{-1} KNO_3 at 30℃ in the dark. After 1 h, the reaction was stopped with 100 μl of 1 mol L^{-1} zinc acetate, 1 ml of 1% sulphanilic acid, and 1 ml of 0.2% naphthyl ethylene diamine. Nitrite concentration was measured at 540 nm. NR activities were determined by the difference between the quantity of nitrite in seedlings extracts after 1 h of reaction and the initial quantity. Experiments were repeated at least three times.

Measurement of protein content

Protein Content of the enzyme extracts was measured according to the method of Bradford [26] . Bovine serum albumin (BSA, Sigma) was used as a standard. The assay reagent is made by dissolving 100 mg of Coomassie Blue G250 in 50 mL of 95% ethanol. The solution is then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. BSA at a concentration of 1000 μg/mL in distilled water is used as a stock solution. Pipet BSA stock solution between 10 and 300 μg of protein into coated wells. Add 200μL of Coomassie Blue G250 solution and mix well avoiding foaming. Distilled water provide the reagent blank. For the calibration curve, measure the A595 of the standards against the reagent blank between 2 min with ELIASA（Bio-RAD Model 550） repetitively. Pipet above seedlings extracts into coated wells, and measure the A595 of the samples repetitively. Calculate the total protein concentratio in excell. Experiments were repeated at least three times.

3. Results

Effect of ABA on seedlings establishment of WT, *nia1, nia2* and *noa1* mutants

To determine whether endogenous NO is involved in the ABA response during seed germination and seedlings establishment, the seeds of *nia1,nia2*, *noa1*, and WT were planted in ABA-containing medium and the phenotypes were screened. Within seven days, the germination rates of both the WT and mutants were almost close to 100% under ABA treatment at indicated concentrations. The post-germination growth was restrained from root elongation following the increase of ABA

concentration beyond 0.5 μmol L^{-1}, whereas at less than 0.5 μmol L^{-1}, ABA appears to promote root prolongation. The results indicate that ABA, as a ubiquitous phytohormone, has the dual effect of contributing to growth at low levels and inhibiting development at high levels (Figure 1). Therefore, 0.5 μmol L^{-1} ABA was used in further experiments. The *nia1,nia2* mutants were more sensitive to ABA (0.5 μmol L^{-1}) than *noa1* and WT, which exhibited retarded growth (Figure 1 and 2). Interestingly, the enhanced sensitivity of the *nia1,nia2* seedlings recovered after about 10 days. Similarly, the average seedling growth rate suggests that 0.5 μmol L^{-1} ABA slightly inhibited root prolongation in the *noa1* mutant and the WT, whereas it significantly inhibited root prolongation in the *nia1,nia2* mutants within 10 days. The average growth rate of the *nia1,nia2* mutants increased to almost the same as that of the two other genotypes after 11 to 14 days (Figure 2A and 2B).

Effect of ABA on endogenous NO levels in the root tips of the WT, *nia1,nia2* and *noa1* seedlings

To explore the effect of ABA on endogenous NO level, *nia1,nia2*, *noa1*, and WT *A. thaliana* seedlings grown in ABA-containing medium at different times were used to detect NO levels through labeling with DAF-2DA, a NO-specific probe. As shown in Figure 3 A, a bright green fluorescence of NO was detected in the root tips of WT and *noa1* 7-day-old seedlings grown in ABA-treated medium in contrast with the weak fluorescence in the control. Slightly faded yellow fluorescence was observed in the root tips of the *nia1,nia2* mutants, which indicates that it was seriously impaired compared with that in *noa1* and WT seedlings. The data show that exogenous ABA application induces NO production and that the *nia1,nia2* mutants cannot produce NO because of NR deficiency. NO fluorescence in the *nia1,nia2* seedlings was enhanced after 11 days and the fluorescence intensity remained at similar levels in the three genotypes after 17 days. The results indicate that at an early stage, the ABA-induced NO probably originated from an NR-dependent pathway, which is later replaced by other NO sources.

Effect of inhibitor of NR on root elongation and NO production

To determine the source of the ABA-induced NO in the roots, the effects of sodium tungstate, a target inhibitor of NR, on root elongation and NO production were investigated in WT seedlings. A marked reduction in the root elongation of the 7-day-old seedlings was observed when tungstate was present in the ABA-treated medium (Figure 4). The addition of tungstate to the ABA-treated medium significantly inhibited the ABA-induced NO production (Figure 4). Interestingly, the roots of the 11-day-old seedlings initiated elongation accompanied by increased NO fluorescence (Figure 4). These results suggest that the ABA-induced inhibition of root elongation likely occurs via NO production.

Effect of ABA on NR activity

To obtain further evidence of the source of ABA-induced NO, we investigated the effects of ABA on NR activity in WT seedlings at the indicated times. The addition of ABA triggered an increase in NR activity, particularly in 6-day-old seedlings. The NR activity tended to decreased gradually in 12 and 18d seedlings (Figure 5). The results illustrate that ABA activated the NR dependent pathway to release NO during the early developmental phase, whereas other NO-releasing pathways participated later in the process.

4. Discussions

In the present study, exogenous ABA application (beyond 0.5 μmol L^{-1}) suppressed post-germination root elongation of *A. thaliana* and induced the root tips to produce NO. ABA-induced suppression of root elongation was attenuated by SNAP. ABA-induced NO synthesis in the root cells of the *nia1,nia2* mutants was impaired and the plants were more sensitive than the WT and *noa1*. These results suggest that the effect of ABA on root inhibition may occur through its action on NO production. To explore further the sources of ABA-induced NO in the roots, the effects of ABA on NR activity and tungstate on root elongation were also investigated. Tungstate, as an inhibitor of NR synthesis, significantly decreased NO production and aggravated the suppression of root elongation induced by ABA. NR activity was increased by ABA treatment. These results indicate that ABA-induced NO was primarily from an NR-dependent pathway.

Both ABA and NO are reportedly involved in the root growth and stress response(Zhu 2002; Neill et al., 2003; Tian et al., 2007; Bai et al., 2009; Lozano-Juste and León, 2010). ABA and NO are closely related during many processes and systems. As previously reported, ABA-induced NO synthesis in the guard cells of *nia1,nia2* and *Atnos1* mutants are impaired, but NO production in *Atnos1* decreased less than in the *nia1,nia2* mutants (Desikan et al., 2002; Guo et al., 2003). Similarly, the *nia1,nia2,noa1-2* triple mutants exhibit decreased seedling establishment and enhanced ABA sensitivity (Lozano-Juste and León, 2010), which indicates that NO production is necessary for root elongation. However, the source of NO during the root response to ABA remains unclear.

Although increasing evidence suggests that NO and ABA play important roles in plants, the mechanisms responsible for NO synthesis and ABA signaling in plants remain controversial. Previous studies have reported that NO can be produced by NOS-like enzymes and by NR in plants (Desikan et al.,

2002; Guo et al., 2003; Zhao et al., 2007).

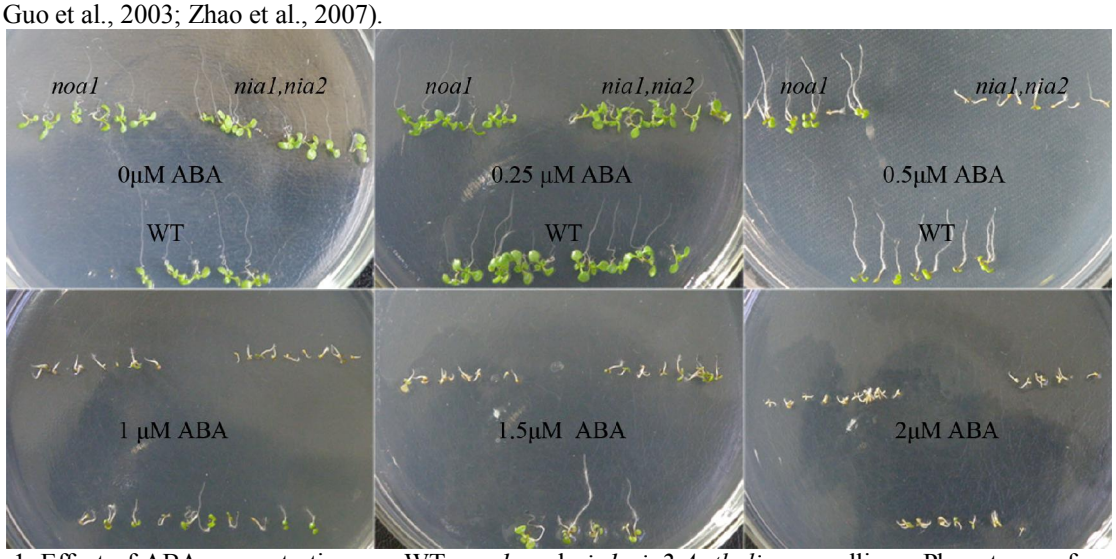

Figure 1. Effect of ABA concentrations on WT, *noa1*, and *nia1,nia2 A. thaliana* seedlings. Phenotypes of seedlings were photographed six days after growing on MS medium containing 0, 0.25, 0.5, 1.0, 1.5, and 2.0 μmol L^{-1}ABA.

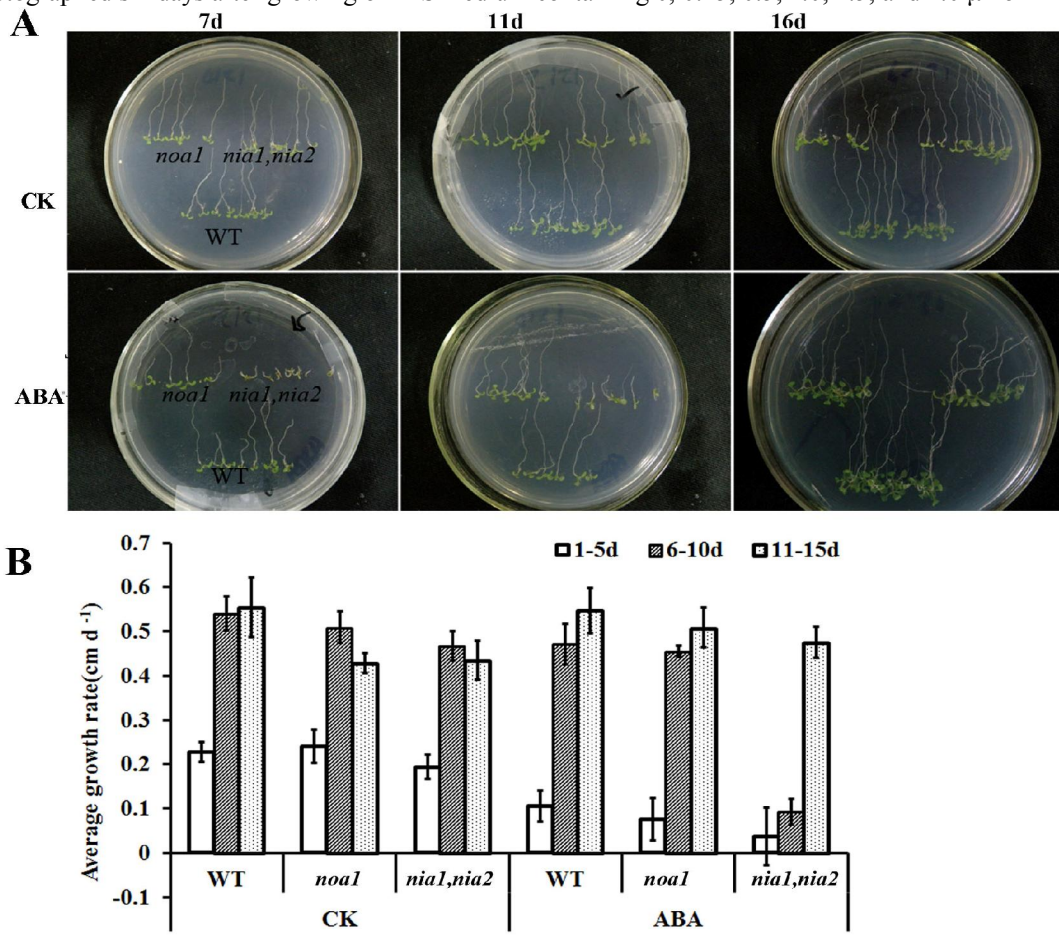

Figure 2. Effect of ABA on WT, *noa1* and *nia1,nia2 A. thaliana* seedlings. The seedlings grown on MS medium containing 0.5 μmol L^{-1} ABA were photographed and their primary root lengths were measured at varying time periods indicated in figure. A, Phenotype of the seedlings; B, Average root growth rate. CK is abbreviation of control. Each independent measurement had at least 20 seedlings, and each experiment was repeated at least three times. Values were given as means ± SE.

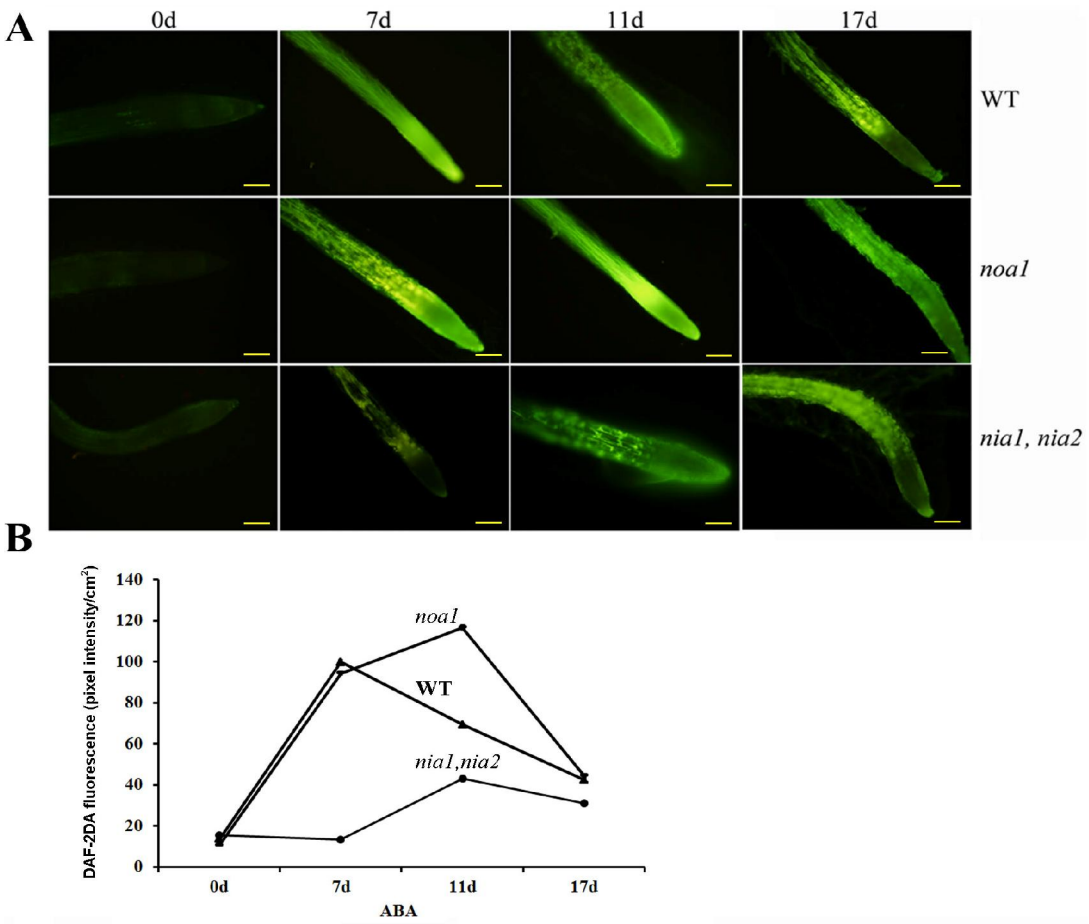

Figure 3. ABA-induced endogenous NO dynamics in the roots of WT, *noa1*, and *nia1,nia2 A. thaliana* seedlings. A, Real-time image of DAF-2DA fluorescence; B, Time course of DAF-2DA fluorescence intensity. Zero-day-old seedlings are control seedlings grown on medium lacking ABA for 7d. Each independent measurement had at least 10 seedlings, and each experiment was repeated at least three times. Values were given as means ± SE. Scale bars represent 100 μm.

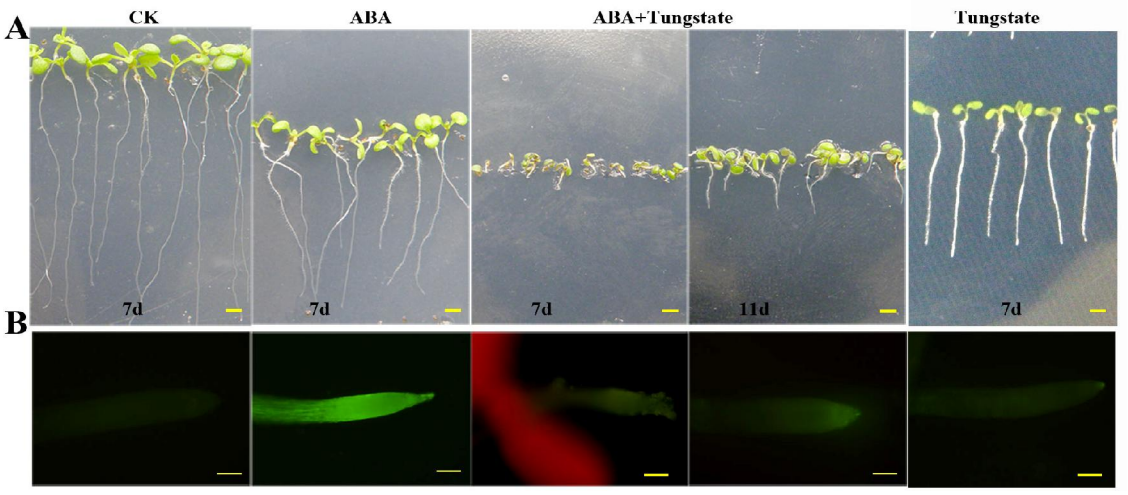

Figure 4. Effects of ABA and NR inhibitors on WT *A. thaliana* seedlings. The seedlings grown on MS medium containing 0.5 μmol L^{-1} ABA and 0.1 μmol L^{-1} sodium tungstate(NR inhibitors)were photographed at indicated time. A, Phenotype of the *A. thaliana* seedlings, Scale bars represent 1 mm ; B, Endogenous NO levels in the root tips, Scale bars represent 100 μm.

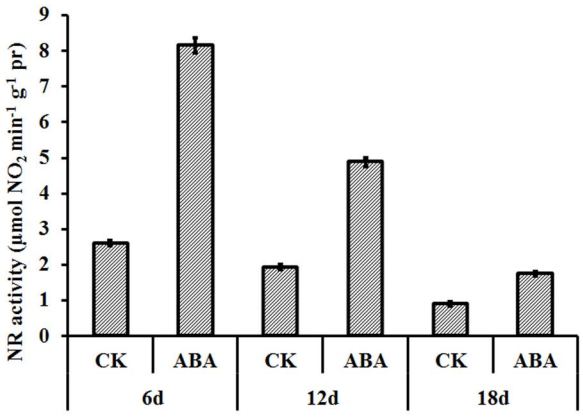

Figure 5. Effect of ABA on NR activity in *A. thaliana* seedlings. Seedlings grown in MS medium containing 0.5 μmol L^{-1} ABA for 6, 12, and 18 days were harvested and used to determine NR activity. The seedlings growing on medium lacking ABA are control, and CK is abbreviation of control. Each independent measurement had at least 20 seedlings, and each experiment was repeated at least three times. Values were given as means ± SE.

Accordingly, several ABA receptors are present in plants (Shen et al., 2006; Pandey et al., 2009; Ma et al., 2009; Park et al., 2009; Hubbard et al., 2010). Therefore, the sources of NO production in response to ABA should be correspondingly diverse. Our results not only prove that ABA-induced NO production is critical for root elongation, but also that the NR-dependent pathway is the major source of NO during the early stage of seedling establishment. The result is consistent with the reports by Desikan et al. (2002) and Zhao et al. (2007). ABA-induced-NO promotes the root elongation according to our results, which is adaptive response to exogenous ABA signal. This is consistent with the previous reports (Pagnussat et al., 2002). And it should be a mechanism of counteraction between ABA and NO in ABA-inhibited roots. As we know both ABA and NO have dose effect with low concentration of promotion while high concentration of inhibition. One important finding in the present study is that the roots of the *nia1,nia2* mutants resumed normal growth after about 10 days (Figure 2). Similarly, the suppression effect of tungstate was alleviated after 11 days (Figure 4). The results show that although NR is the major enzyme, it is not the only source of ABA-induced NO. Another pathway is involved later in the process, which is the adaptive response to exogenous stimuli.

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