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Note

A high concentration of nitrate causes temporal inhibition of lateral root growth by suppressing cell proliferation

Yoko Okushima, Hiroyuki Inamoto, Masaaki Umeda*

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan
* E-mail: mumeda@bs.naist.jp Tel: +81-743-72-5592 Fax: +81-743-72-5599

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Abstract Root development is highly affected by nutrient supply under various environmental conditions. A high concentration of nitrate is known to inhibit lateral root growth after emergence from the parent root, implying that cell proliferation ceases at a specific stage in lateral root development. However, the mechanism by which external nitrate availability modulates the cell cycle remains to be elucidated. In this study, we analyzed cell cycle regulation during high-nitrate-mediated inhibition of lateral root growth in *Arabidopsis*. The expression of mitotic reporter genes, such as those for *CDKB2;1* and *CYCB1;1*, was suppressed in emerged lateral root primordium of seedlings grown under high nitrate conditions, which temporally arrested the outgrowth of the primordium for the first 3 days. In contrast, the expression of *CDKA;1*, which encodes an ortholog of yeast Cdc2/Cdc28p, was not affected by external nitrate availability. These results indicate that the cell cycle in emerged lateral root primordia is possibly arrested at G1/S under high nitrate conditions but that the primordia retain the competence for cell division. The expression of an auxin response marker was reduced in stunted lateral root primordia under high nitrate conditions, but exogenous auxin application could not suppress growth inhibition. This suggests that reduced auxin accumulation and/or signaling are not the primary cause of high-nitrate-mediated inhibition of lateral root growth.

Key words: Auxin, cell cycle, cyclin-dependent kinase, lateral root, nitrate.

Plant root systems perform a range of functions, including the uptake of water and nutrients and the structural anchoring of the plant to the soil. In dicots, lateral root formation is crucial for maximizing these root functions. The process of lateral root formation in *Arabidopsis* can be divided into 4 stages: (1) activation of pericycle cells at the xylem poles, (2) formation of the lateral root primordium, (3) emergence of the lateral root primordium from the primary root epidermis, and (4) activation of the meristem to allow continuous elongation of the lateral root (De Smet et al. 2003; Malamy and Benfey 1997; Zhang and Forde 2000). The plant hormone auxin plays an important role in controlling all stages of lateral root development (Benkova et al. 2003; De Smet et al. 2006).

Lateral root growth is regulated by both intrinsic developmental programs and environmental conditions, including nutrient availability (Lopez-Bucio et al. 2003; Malamy 2005). Nitrate (NO_3^-) is the primary source of nitrogen available to plants and has been shown to affect lateral root development (Walch-Liu et al. 2006). For example, a locally enriched supply of nitrate promotes lateral root elongation specifically within the nitrate-rich zone (Zhang and Forde 1998, 2000). In contrast, a uniformly high concentration of nitrate (above 10 mM) represses lateral root development prior to the activation of the lateral root meristem (Zhang and Forde 1998, 2000; Zhang et al. 1999). *Arabidopsis* plants harboring a mutation in the nitrate reductase gene are much more sensitive to higher nitrate conditions than wild-type plants, suggesting that nitrate, rather than its metabolites, is responsible for the inhibition of lateral root growth (Zhang et al. 1999).

Root elongation depends largely on cell division in the root apical meristem. Cell division is controlled by the enzyme activity of cyclin-dependent kinase (CDK) complexes composed of catalytic CDK and regulatory cyclin subunits. In *Arabidopsis*, the A- and B-type CDKs (CDKA and CDKB) have been inferred to be crucial for cell cycle progression. CDKAs are functional homologs of yeast Cdc2/Cdc28p and are expressed constitutively throughout the cell cycle. By contrast, CDKBs are plant-specific and are classified into 2 subtypes, CDKB1 and CDKB2, whose expression is specific to the late S-to-M phase and to the G2-to-M phase, respectively (Menges et al. 2005; Segers et al. 1996). Based on the oscillation of

Abbreviations: GUS, β-glucuronidase; IAA, indole-3-acetic acid; NAA, 1-naphthylacetic acid

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their expression and kinase activity during the cell cycle, CDKAs are assumed to participate in both the G1/S and G2/M transitions, while CDKBs are probably involved in the G2/M transition (Inze and De Veylder 2006; Van Leene et al. 2010).

To reveal how the cell cycle is modulated in response to external nitrate supply, we investigated high-nitrate-mediated inhibition of lateral root elongation. Previous reports demonstrated that the inhibitory effect is seen most clearly when seedlings are grown on a medium containing 50 mM KNO_3 and a low concentration of sucrose (0.5%) (Zhang and Forde 1998; Zhang et al. 1999). Therefore, in this study, we used the same growth conditions. Seedlings of wild-type (Col-0) *Arabidopsis* were grown on a medium containing 1 or 50 mM KNO_3 for 7 days, and the growth of the primary and lateral roots, as well as the density of the emerged lateral roots, were examined. As previously reported, the number of lateral roots was not different between the two KNO_3 concentrations (Zhang and Forde 1998; Zhang et al. 1999). However, the primary roots of seedlings grown in 50 mM KNO_3 were slightly longer (Figure 1A, B). Under 50 mM KNO_3 conditions, lateral root growth was suppressed during the first 3 days after emergence (Figure 1C, D); this observation is different from the previous report that no lateral root is visible even 14 days after germination under the same growth conditions (Zhang and Forde 2000).

To gain insights into the molecular basis of high-nitrate-mediated inhibition of lateral root elongation, we examined the expression of cell cycle genes using β-glucuronidase (GUS) reporter lines. Transgenic plants harboring *pCDKA;1:CDKA;1-GUS* (Adachi et al. 2009) displayed strong and uniform GUS activity in primary root meristems under both 1 and 50 mM KNO_3 conditions (Figure 2). Strong expression was also observed in unemerged and emerged primordia and in meristems of lateral roots of seedlings grown in 1 or 50 mM KNO_3 (Figure 2). These observations suggest that the accumulation of CDKA;1 in primary and lateral root meristems is barely affected by external nitrate supply. We then examined the expression of the *pCDKB2;1:CDKB2;1(nt)-GUS* reporter line to examine the expression of CDKB2;1, which is specifically expressed in the late G2 and M phases (Adachi et al. 2006). As shown in Figure 2, GUS signals showed a patchy pattern across the root meristem, and neither the intensity nor the number of GUS spots in the primary root meristems was significantly affected by the KNO_3 concentrations (Figure 2). However, the number of GUS-positive cells was significantly lower in emerged lateral root primordia grown in 50 mM KNO_3 but not in unemerged primordia or in meristems of elongating lateral roots (Figure 2). Similarly, the expression of *pCYCB1;1:CYCB1;1(nt)-GUS*, a reporter line for the mitotic cyclin CYCB1;1

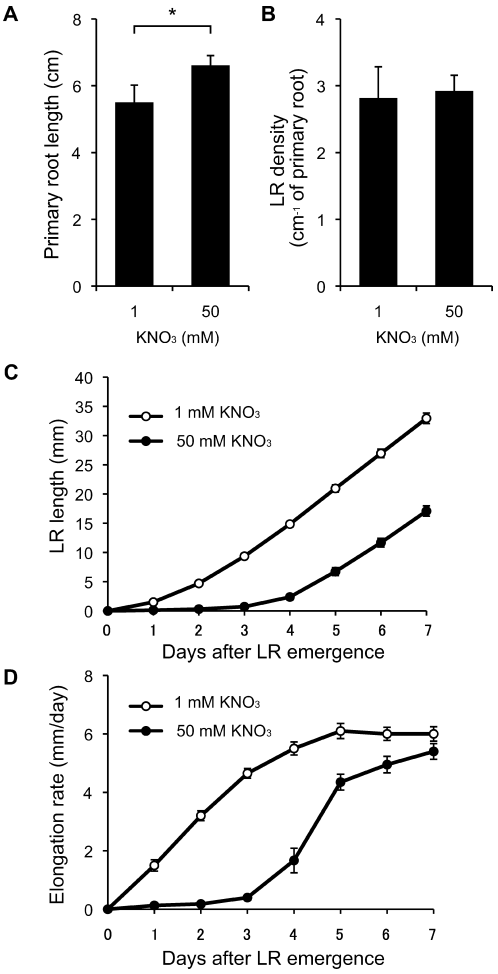

Figure 1. Effect of nitrate availability on primary and lateral root growth. Four-day-old seedlings grown on a medium containing 10 μM NH_4NO_3 as a nitrogen source were transferred to a fresh medium containing 1 or 50 mM KNO_3 instead of NH_4NO_3. (A) Primary root length of seedlings grown on media with the indicated concentrations of KNO_3 for 7 days. Bars and error bars represent means±SD ($n \geqq 10$). Asterisk indicates significance at $P<0.001$. (B) Density of lateral roots (LRs) of seedlings grown on media containing 1 or 50 mM KNO_3 for 7 days. Numbers of emerged lateral roots were scored using a stereomicroscope. Bars and error bars represent means±SD ($n>10$). (C, D) Lateral root (LR) growth was measured at 24-h intervals for 7 days after primordia emergence from the primary root. Length (C) and elongation rate (D) of lateral roots.

(Colon-Carmona et al. 1999), was suppressed in emerged lateral root primordia when plants were grown in the presence of 50 mM KNO_3 (Figure 2). These results suggest that high nitrate conditions temporarily inhibit lateral root growth by suppressing cell proliferation, and that this inhibition is caused by cell cycle arrest at a stage other than G2/M, possibly at G1/S.

Since auxin is an important regulator of lateral root development (Benkova et al. 2003; De Smet et al. 2006), we examined the effect of a high KNO_3 concentration on the auxin response. We used *Arabidopsis* seedlings carrying the *DR5:GUS* construct (the GUS coding sequence under the control of a synthetic auxin-inducible

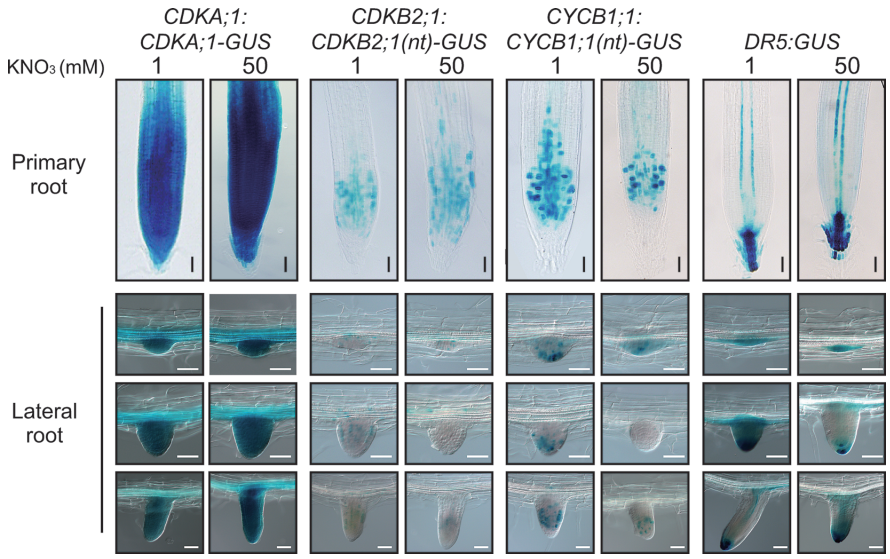

Figure 2. Expression patterns of *pCDKA;1:CDKA;1-GUS*, *pCDKB2;1:CDKB2;1(nt)-GUS*, *pCYCB1;1:CYCB1;1(nt)-GUS*, and *DR5:GUS* in primary roots and in emerging lateral roots at three distinct stages. Seedlings were grown on a medium containing 1 or 50 mM KNO_3 for 7 days and subjected to GUS staining. The roots were observed under a light microscope (Olympus BX50) equipped with a digital camera control unit (DP71; Olympus). Bars = 50 μm.

promoter), which can monitor auxin accumulation and distribution (Ulmasov et al. 1997). In either the presence of 1 or 50 mM KNO_3, *DR5:GUS* was primarily expressed in the quiescent zone and the columella cells of the primary root tips (Figure 2). However, in emerged lateral root primordia, *DR5:GUS* expression was suppressed in the presence of 50 mM KNO_3 (Figure 2). These data suggest that high nitrate supply reduces the accumulation of, or the response to, auxin in stunted lateral root primordia. This prompted us to examine the effect of exogenous auxin supply on lateral root elongation. However, neither IAA nor NAA (10–200 nM) could rescue the growth inhibition caused by 50 mM KNO_3 during the first 3 days after emergence of the primordium (Figure 3).

Several studies have shown that a developmental checkpoint operates just after emergence of the lateral root primordium from the parent root (Beeckman and Friml 2010; De Smet et al. 2003; De Smet et al. 2006); thus, primordial outgrowth ceases under unfavorable growth conditions. We found that the expression of *pCDKB2;1:CDKB2;1(nt)-GUS* and *pCYCB1;1:CYCB1;1(NT)-GUS* in emerged lateral root primordia was suppressed in 50 mM KNO_3, which temporally arrested the outgrowth. The expression of *pCDKB2;1:GUS,* a reporter construct monitoring *CDKB2;1* promoter activity, was also repressed in stunted lateral root primordia by high nitrate supply (data not shown), suggesting that suppression of CDKB2 expression is regulated at the transcriptional level. In contrast, the expression of CDKA;1 did not appear to be affected by external nitrate availability. It is known that *CDKA;1* expression reflects the competence for cell division (Hemerly et al. 1993).

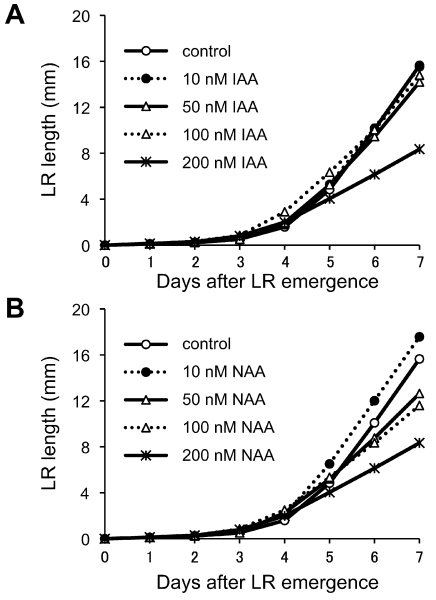

Figure 3. Effect of exogenous auxin application on high-nitrate-mediated inhibition of lateral root elongation. Four-day-old seedlings were transferred to a fresh medium containing 50 mM KNO_3. The indicated concentrations of IAA (A) or NAA (B) were supplied to the medium, and the length of the lateral roots (LRs) was measured at 24-h intervals for 7 days after emergence of the primordia from the primary root. The error bars represent SD (n > 10).

Therefore, our results indicate that the stunted lateral root primordia continue to retain the ability to restart cell division. Indeed, in the presence of 50 mM KNO_3, lateral roots started to elongate at the fourth day after primordium emergence. A previous report also noted that lateral roots treated with 50 mM nitrate began to grow immediately after transfer to a medium containing 1 mM nitrate (Zhang and Forde 2000).

We found that the expression of *DR5:GUS* was reduced in stunted lateral root primordia in the presence of 50 mM KNO_3; a similar observation was also reported previously (Bao et al. 2007). However, the growth inhibition was not recovered by exogenous auxin application (although the effect of lower concentrations of auxin remains to be elucidated), implying that high nitrate conditions impair not only the auxin level but also other factor(s) that are prerequisite for activating lateral root primordia. Nevertheless, it remains an intriguing question as to how nitrate supply affects the amount and/or distribution of auxin. A recent study has demonstrated that NITRATE TRANSPORTER1;1 (NRT1.1), which was initially characterized as an influx carrier for NO_3^- uptake from soil (Tsay et al. 1993), uses both nitrate and auxin as substrates (Krouk et al. 2010). It was proposed that, at very low nitrate availability, NRT1.1 represses lateral root growth by promoting basipetal auxin transport out of the root tip, whereas 1 mM nitrate inhibits NRT1.1-dependent auxin transport, thus promoting lateral root elongation. However, since the expression of *DR5-GUS* was reduced in stunted lateral root primordia treated with 50 mM KNO_3 (Bao et al. 2007), NRT1.1-mediated auxin transport may be stimulated at high nitrate concentrations. Further studies will reveal whether NRT1.1 is also involved in balancing auxin and nitrate transport and inhibition of lateral root elongation under high nitrate conditions.

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