1 **Melatonin alleviates aluminum-induced root growth inhibition by interfering with nitric oxide**
2 **production in *Arabidopsis***

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10 **Running title: Melatonin alleviates Al toxicity in *Arabidopsis***

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12 **Highlights**

13 •Nitric reductase- and nitric oxide synthase-dependent NO production contributed to Al-induced root
14 growth inhibition in *Arabidopsis*.

15 •Al inhibits root growth by NO-mediated reduction of the cell division cycle and the quiescent center
16 cellular activity.

17 •External application of melatonin alleviated Al-induced root growth inhibition.

18 •Melatonin alleviated Al toxicity through interfering with NO production.

19 •SNAT-mediated melatonin synthesis played a critical role in Al resistance.

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Abstract

Root growth inhibition is the primary symptom of aluminum (Al) toxicity. In this study, the roles of nitric oxide (NO) and melatonin, two ubiquitous signal molecules involved in diverse plant physiological processes and stress responses, were investigated in *Arabidopsis* responses to Al toxicity. The results showed that nitric reductase- and nitric oxide synthase-dependent NO production contributed to Al-induced root growth inhibition through interfering with the root cell cycle progression and the quiescent center cell homeostasis. The Al-induced NO production was decreased by exogenous melatonin, which was associated with abolishment of Al-induced root elongation and cell cycle arresting, and with the quiescent center activity returning to the Al-free control levels. The expression of *AtSNAT* (*Arabidopsis thaliana* serotonin N-acetyltransferase) was down-regulated by Al, which coincided with decreased melatonin accumulation in *Arabidopsis*. Pharmacological and genetic analyses further confirmed that a decrease in melatonin synthesis enhanced NO production and Al sensitivity. Taken together, the results presented here suggest that melatonin alleviates Al-induced root growth inhibition by interfering with NO-mediated reduction of cell division cycle progression and the quiescent center cellular activity in *Arabidopsis* roots.

Key Words: Al toxicity; melatonin; nitric oxide signaling; root growth; *Arabidopsis*.

1. Introduction

Acid soils ($\text{pH}_{\text{water}} < 5.5$) that limit crop production are found worldwide, occupying up to 40% of arable land (Kochian et al., 2004; Rengel and Zhang, 2003). Aluminum (Al) toxicity is a major factor limiting plant growth and crop yield in acidic soils. The root tip is the primary target of Al toxicity. Excess Al causes a rapid (minutes to hours) inhibition of root growth through interference with cell elongation (Barcelo and Poschenrieder, 2002), whereas long-term inhibition of root growth is likely due to Al inhibition of cell division in the apical meristematic zone (Kochian, 1995). For instance, after a 12-day treatment with 25-175 μM Al, primary root growth in *Arabidopsis* was inhibited by blocking the induction of the mitotic marker CYCB1;1::GUS (Cyclin B1;1:: β -glucuronidase) (Ruiz-Herrera and Lopez-Bucio, 2013). However, it is still poorly understood how Al interferes with the cell cycle and the quiescent center cells homeostasis in plant roots.

Nitric oxide (NO) is a ubiquitous signal molecule involved in multiple physiological processes and environmental stresses in plants. Nitrate reductase (NR, EC1.7.1.1) and nitric oxide synthase (NOS, EC1.14.23) are two main sources of NO in plants (Kan et al., 2016). Nitric oxide has been shown to affect several biotic and abiotic stresses (Siddiqui et al., 2011), including Al toxicity (He et al., 2012). For example, previous studies showed that NO alleviates Al toxicity through minimizing oxidative stress in roots of *Cassia tora* (Wang and Yang, 2005) and wheat (Zhang et al., 2008) or through affecting cell wall polysaccharides in rice roots (Zhang et al., 2011). Furthermore, Al resistance was associated with NR-dependent NO production in wheat (Sun et al., 2014), red kidney bean (Wang et al., 2010) and soybean (Wang et al., 2017), and with NOS-dependent NO production in *Hibiscus moscheutos* (Tian et al., 2007). On the other hand, excess accumulation of NO induced cell death and inhibited primary root growth under normal growth and some stress conditions. In particular, higher levels of NO associated with the exogenous application of NO donors (sodium nitroprusside and S-nitrosoglutathione) and the NO overproducer 1 (*cue1/nox1*) mutant lines reduced cell division, affecting the distribution of mitotic cells and the meristem size via decreased PIN1-mediated auxin transport in *Arabidopsis* (Fernandez-Marcos et al., 2011). Similarly, the Cd- and methyl 3-(4-hydroxyphenyl) propionate-induced NO production reduced divisions of meristematic cells and primary root growth in *Arabidopsis* (Liu et al., 2016; Yuan and Huang, 2016). Moreover, exogenous

application of NO donor SNP enhanced Al-induced root growth inhibition in rice bean (Zhou et al., 2012). Additionally, reduction of Al-increased NO production by hydrogen-rich water is involved in alleviation of Al-induced root growth inhibition in alfalfa (Chen et al., 2014). It therefore appears that a positive or negative role of NO in stress responses is dependent on (i) plant species and (ii) NO concentration in various plant tissues/organs.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a tryptophan-derived metabolite that is widespread in bacteria, algae, animals and higher plants (Back et al., 2016). Phytomelatonin is synthesized from tryptophan via similar biosynthetic pathways as in vertebrates (Zhang et al., 2015). The genes encoding melatonin biosynthetic enzymes, namely *serotonin N-acetyltransferase (SNAT)* and *N-acetylserotonin methyltransferase (ASMT)*, have been identified in *Arabidopsis* and rice (Byeon et al., 2016; Lee et al., 2014). Melatonin is involved in various physiological processes, including root growth in rice (Liang et al., 2017) and adventitious root regeneration in shoot tip explants of sweet cherry (Sarropoulou et al., 2012). Additionally, melatonin modulates plant tolerance to several abiotic stresses. For example, exogenous melatonin significantly alleviated growth inhibition caused by salinity (Li et al., 2012) and Cd toxicity (Hasan et al., 2015). In a more recent study, we found that the expression of *SNAT*-like gene and endogenous melatonin biosynthesis in soybean roots were induced by Al (Zhang et al., 2017). Moreover, exogenous melatonin enhanced soybean Al resistance through regulating organic acid anions exudation and antioxidant enzymes activity (Zhang et al., 2017). However, the roles of melatonin in plant responses to Al toxicity are still largely unknown.

In this study, we investigated the roles of NO and melatonin in Al toxicity in *Arabidopsis* roots. The results showed that i) both NR- and NOS-catalyzed NO production contributed to Al toxicity by inhibiting root cell cycle progression and the quiescent center cells homeostasis; ii) exogenous application of melatonin alleviated Al-induced root growth inhibition via interfering with NO signaling; and iii) *SNAT*-mediated melatonin synthesis played a critical role in Al resistance. We propose that applying exogenous melatonin or enhancing the melatonin biosynthesis using transgenic methods might be an effective strategy to enhance plant Al resistance.

2. Materials and methods

2.1 Plant growth and treatments

The wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and transgenic lines in the Col-0 background CYCB1;1::GUS and QC25::GUS were used in this study. The *Arabidopsis* mutant lines *nia1nia2* and *noa1* in the Col-0 background were also used. The *snat* (SALK032239) mutant line in the Col-0 background was obtained from the Arabidopsis Biological Resource Center (ABRC), and the homozygous plants were screened by PCR as described elsewhere (<http://signal.salk.edu/tdnaprimers.2.html>) using the primers shown in Supplemental Table S1. *Arabidopsis* seeds were surface sterilized with 75% (v/v) ethanol for 5 min and 8% w/v NaClO₃ for 15 min. After washing in distilled water five times, the seeds were sown onto 1/6 Murashige and Skoog (MS) agar medium [supplemented with 0.8% (w/v) agar and 1.0% (w/v) sucrose, pH 5.7], and incubated for 2-3 days at 4°C in the dark to synchronize germination. The seedlings were grown vertically for 6 days in growth conditions at 22°C with a 16 h light/ 8 h dark photoperiod. Six-day-old seedlings were then transferred onto plates supplemented with 0-200 µM Al and various chemicals, such as 10-200 µM SNP (sodium nitroprusside, an NO donor), 100 µM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, an NO scavenger), 100 µM L-NAME (N^G nitro-L-Arg-methylester, an NOS inhibitor), 10 µM tungstate (Na₂WO₄, a nitrate reductase inhibitor), 0-10 µM melatonin, 10 µM *p*-chlorophenylalanine (*p*-CPA, a melatonin synthesis inhibitor that blocks conversion from serotonin to melatonin) (Park, 2011), for additional 3 days of growth. All chemicals were obtained from Sigma-Aldrich.

Supplementary Table S1 List of the primers and their uses

Primer name	Sequence (5'-3')	Use
AtSNATF	CAAGGAGACCGTTAGTGA	RT-PCR analysis
AtSNATR	CTGATGAATCTGAATCTGATGA	RT-PCR analysis
AtASMTF	AAGTGTTGATAGTGAATCG	RT-PCR analysis
AtASMTR	GAGCCATCACCATATC	RT-PCR analysis
Actin11F	GGTGAAGACATTTCAGCCTCTCGTCT	RT-PCR analysis
Actin11R	GCAAGGTCCAAACGCAGA	RT-PCR analysis

SALK_032239LP	TTTTGACCACCACAAAAATTGCAG	Identification of <i>snat</i> homozygous plants
SALK_032239RP	ATTCGAAGATTTGGGTCTTCG	Identification of <i>snat</i> homozygous plants
SALK_032239BP	GCGTGGACCGCTTGCTGCAACT	Identification of <i>snat</i> homozygous plants

2.2 Root elongation measurement

The root elongation was measured as described previously (Yang et al., 2011). Briefly, six-day-old seedlings about 1 cm long were transferred to Petri dishes containing agar-solidified 1/6 MS medium (pH 4.5) with or without the chemicals as described above. After grown vertically for an additional 3 d, the seedlings were photographed using a digital camera. Data were quantified and analyzed by ImageJ.

2.3 Detection of nitric oxide production

Nitric oxide (NO) production was detected using a specific NO fluorescent probe DAF-FM DA (Diamino-fluorescein diacetate, Sigma-Aldrich). *Arabidopsis* roots were incubated with 10 μ M DAF-FM DA at 37°C for 20 min. After washing with distilled water for 3-5 times, NO concentration in *Arabidopsis* roots was observed under a Leica (DMI6000) fluorescent microscope (excitation 450-490 nm, emission 500-530 nm). The quantitative measurement of fluorescence intensity was performed using ImageJ.

2.4 GUS staining

In situ β -glucuronidase (GUS) staining in the roots of QC25::GUS and CYCB1;1::GUS seedlings was performed using the method of Jefferson et al. (Jefferson et al., 1987). The roots were transferred to microfuge tubes containing a solution of 100 mM Na phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% (v/v) Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-b-D-glucuronide. The GUS staining time was dependent on the transgenic marker lines: 12 h for QC25::GUS and 4 h for CYCB1;1::GUS. After staining, the tissues were rinsed in deionized water and photographed.

2.5 Quantitative GUS assay

The frozen roots (0.1 g) were homogenised in 1 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.8) containing 0.2 mM EDTA- Na_2 , 0.1 M ascorbic acid and 1% w/v PVPP using a mortar and pestle. The homogenate was centrifuged at 12,000 g at 4°C for 20 min. The supernatant was immediately used for quantitative GUS assays. The GUS activity was measured using 4-methyl-umbelliferyl- β -D-glucuronide (Sigma-Aldrich) as a substrate as previously described (Jefferson et al., 1987). The released fluorescence was measured with an Infinite M200 Pro plant reader (Tecan, Research Triangle Park, NC). The protein content was measured by the Bradford method (Bradford, 1976).

2.6 Measurement of endogenous melatonin

Melatonin was extracted according to the published method (Pape and Lüning, 2006). The melatonin concentration was determined by a Plant MT Elisa Kit (TSZ, America) according to the manufacturer's instructions.

2.7 RT-PCR analysis

Total RNA was isolated from seedlings (100 mg) using RNAiso Plus (TaKaRa, Da Lian, China) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScriptTM RT Reagent Kit with gDNA Eraser (TaKaRa). The expression levels of *AtSNAT* (At1g32070) and *AtASMT* (At4g35160) were analyzed using SYBR Premix Ex Taq II (TaKaRa). The *Actin11* (At3g12110) gene was used as an internal control. The primers specific for *AtSNAT*, *AtASMT* and *Actin11* were included in Supplemental Table S1. PCR was performed for 40 cycles using Applied Biosystems StepOne (Applied Biosystems) following the manufacturer's instructions.

2.8 Statistical analysis

All experiments were repeated at least three times. For the relative root growth, at least 20 roots were analyzed for each treatment. The data were analyzed using ImageJ and SPSS (Statistic Package for Social Science) software. The results were presented as mean $\pm$ SD (standard deviation) of three independent experiments. For the mean pairwise comparisons, we used Tukey's test.

3. Results

3.1 Aluminum induces NO production in *Arabidopsis* root

Primary root growth was inhibited by the application of Al in a dose-dependent manner (Fig. 1A). The relative root growth was inhibited by 30% after exposure to 100 μM AlCl_3 and up to 40% after exposure to 200 μM AlCl_3 (Fig. 1A). Similarly, the fluorescence intensity of NO-specific DAF-FM DA was significantly increased by Al in a dose-dependent manner (Fig. 1B and C), indicating that Al stimulated NO accumulation in the roots of *Arabidopsis*.

Fig. 1 Effect of Al on root growth and NO production in *Arabidopsis* Col-0 roots. A, Effect of 0, 20, 100 or 200 μM AlCl_3 on primary root growth. The NO production in roots exposed to different concentrations of AlCl_3 for 3 days was detected using the NO-specific fluorescent probe DAF-FM DA (Diamino-fluorescein diacetate) (B), and the NO fluorescence intensity was quantified (C). Six-day-old seedlings were transferred to 1/6 MS medium containing 0, 20, 100 or 200 μM AlCl_3 for 3 days. Values are means $\pm$ SD (n=20-25). Different letters indicate significantly different values ($p \leq 0.05$). Bar= 100 μm .

3.2 Involvement of nitric oxide synthase and nitrate reductase in aluminum-induced NO production and root growth inhibition

Nitric oxide synthase (NOS) and nitrate reductase (NR) are the key enzymes in NO synthesis in plants. To further investigate the source and role of Al-induced NO production in *Arabidopsis* roots, we examined endogenous NO content (Fig. 2A, Fig. S1) and relative root growth (Fig. 2B, Fig. S2) after plants were treated by 0 or 100 μ M Al with or without SNP (an NO donor), cPTIO (an NO scavenger), L-NAME (an NOS inhibitor) and tungstate (an NR inhibitor) for 3 days. The exposure to Al resulted in an increase in NO production (Fig. 2A) and a decrease in relative root growth of the wild-type Col-0 *Arabidopsis* (Fig. 2B). Similarly, the application of SNP alone or together with Al significantly promoted NO production (Fig. 2A, Fig. S1) and inhibited primary root elongation (Fig. 2B, Fig S2). The Al-induced NO production was decreased by the addition of cPTIO, L-NAME or tungstate (Fig. 2A), and the relative root growth was increased by 32, 21 and 14%, respectively, compared with the Al-only treatment (Fig. 2B), indicating that NOS and NR were the two main sources of Al-induced NO production and involved in mediating Al-induced root growth inhibition.

The primary root elongation in the wild-type Col-0 and the T-DNA knock-out lines of nitric oxide-associated1 (*noal*) and nitrate reductase (*nia1nia2*) was therefore measured to further evaluate the roles of NOS and NR in the Al toxicity-induced root growth inhibition. In the absence of Al, the root length was shorter in *noal* and *nia1nia2* than Col-0 plants (Fig. 2C); this was due to NOS- and NR-dependent NO production being essential for root growth. However, in the presence of Al, the mutant lines *noal* and *nia1nia2* were insensitive to Al toxicity; the Al-induced inhibition of primary root length was 30% in Col-0 (significant), but only 2 and 12% (non-significant) in *noal* and *nia1nia2*, respectively (Fig. 2C and D). These results indicated that NOS and NR contributed to Al-induced NO production and root growth inhibition.

Fig. 2 Nitric oxide synthase (NOS)- and nitrate reductase (NR)-mediated NO synthesis contributed to Al-induced root growth inhibition in *Arabidopsis*. A, Fluorescence intensity of NO in Col-0 roots exposed to 0 or 100 μ M Al with or without 10 μ M SNP (an NO donor), 100 μ M cPTIO (an NO scavenger), 100 μ M L-NAME (an NOS inhibitor) or 10 μ M tungstate (a nitrate reductase inhibitor) for 3 days using the NO-specific fluorescent probe DAF-FM DA. Values are means $\pm$ SD (n=6). B, Relative root growth of *Arabidopsis* Col-0 seedlings treated by 0 or 100 μ M Al with or without 10 μ M SNP, 100 μ M cPTIO, 100 μ M L-NAME or 10 μ M tungstate. Values are means $\pm$ SD (n=20-25). C and D, Effect of Al on primary root elongation of Col-0, *noa1* and *nia1nia2* plants. Six-day-old seedlings were transferred to 1/6 MS medium containing 0 or 100 μ M AlCl₃ for 3 days. Values are means $\pm$ SD (n=20-25). In a, b and d, different letters indicate significantly different values ($p \leq 0.05$). In c, the white bar represents 10 mm.

Supplemental Fig. S1 The NO production in *Arabidopsis* Col-0 roots exposed to 100 μ M Al with or without 10 μ M SNP (an NO donor), 100 μ M cPTIO (an NO scavenger), 100 μ M L-NAME (an NOS inhibitor) or 10 μ M tungstate (an NR inhibitor) for 3 days detected using the NO-specific fluorescent probe DAF-FM DA (Diamino-fluorescein diacetate).

Supplemental Fig. S2 Effect of different concentrations of SNP on *Arabidopsis* Col-0 root growth under control (0 Al) or 100 μ M Al stress.

3.3 Aluminum inhibits root growth by NO-mediated reduction of cell division progress and the quiescent center cellular activity

The reduction in root growth could be caused by disturbing a division potential of meristematic cells and the quiescent center activity in plant roots. To investigate the mechanisms of how the NO signaling influenced Al-induced root growth inhibition, we analyzed the cell cycle progression and the quiescent center (QC) activity using transgenic lines expressing CYCB1;1::GUS or QC25::GUS that allow the visualization of cells, respectively, in the G2-M phase of the cell cycle, or cells in the quiescent center. The application of SNP accelerated Al-induced root growth inhibition in both transgenic lines (Fig. 3A), whereas the GUS activity was obviously decreased in CYCB1;1::GUS (Fig. 3B and D) and QC25::GUS (Fig. 3C and D) seedlings. Conversely, the application of cPTIO significantly alleviated Al-induced primary root growth in both transgenic lines (Fig. 3A) and increased GUS staining intensity in roots of CYCB1;1::GUS (Fig. 3B and D) and QC25::GUS (Fig. 3C and D) seedlings. These results indicated that an alteration in the cell division cycle and a reduction in the quiescent center cellular activity were involved in the NO-mediated Al-induced root growth inhibition.

Fig. 3 NO mediated the Al-induced root growth inhibition by interfering with the cell division cycle and the quiescent center activity. *Arabidopsis* transgenic lines CYCB1;1::GUS and QC25::GUS were treated with 0 (CK) or 100 μ M Al with or without 10 μ M SNP or 100 μ M cPTIO for 3 days. A, Relative root growth (n=20-25). Different letters indicate significantly different values ($p \leq 0.05$). Images of GUS staining of CYCB1;1::GUS (B) and QC25::GUS roots (C). The white bar represents 100 μ m. D, Relative quantitative GUS activity (n=3). Values are means $\pm$ SD.

3.4 External application of melatonin alleviates aluminum-induced root growth inhibition and decreases aluminum-induced NO production

Melatonin is associated with plant tolerance to biotic and abiotic stresses. To evaluate the effect of melatonin on Al rhizotoxicity in *Arabidopsis*, the relative root growth under 100 μ M Al stress with increasing concentrations of melatonin was measured. Compared with the Al-only treatment, the application of 0.1, 1 and 10 μ M melatonin increased the primary root growth by 7, 35 and 37%, respectively (Fig. 4A). Coinciding with these observations, the application of melatonin at concentrations greater than 0.1 μ M totally abolished the Al-induced NO production (Fig. 4B). Furthermore, the application of 1 μ M melatonin significantly alleviated root growth inhibition induced by Al or Al+SNP exposure (Fig. 4C) as well as the cell cycle arrests (Fig. 4D and F) and the decreased quiescent center activity (Fig. 4E and F).

Fig. 4 Exogenous application of melatonin (MT) alleviated Al-induced root growth inhibition, NO production, and a decrease in the meristematic cell division potential and the quiescent center activity. A, Effect of different concentrations of melatonin on root growth in *Arabidopsis* Col-0 exposed to 0 or 100 μM Al stress for 3 days. B, Relative fluorescence intensity of NO [detected using the NO-specific fluorescent probe (DAF-FM DA)] in *Arabidopsis* Col-0 roots exposed to 0 or 100 μM Al stress with or without various concentrations of melatonin (0.1, 1 or 10 μM) for 3 days. C, Effect of 1 μM melatonin on relative root growth of *Arabidopsis* Col-0 treated with 0 (CK) or 100 μM Al in the presence or absence of 10 μM SNP. D and E, Images of GUS staining of CYCB1;1::GUS (D) and QC25::GUS roots (E) exposed to 0 (CK) or 100 μM Al with or without 1 μM melatonin or 10 μM SNP. F, Relative quantitative GUS activity. Bar=100 μm. For A (n=20-25), B (n=6), C (n=20-25) and F (n=3), the values are means ± SD. Different letters indicate significantly different values ($p \leq 0.05$).

3.5 The melatonin synthesis inhibitor, *p*-chlorophenylalanine (*p*-CPA), enhances NO production and exacerbates aluminum sensitivity

The NO concentration and the relative root growth were further examined after the plants were treated with Al in the presence or absence of 1 μM melatonin (MT) and/or 10 μM *p*-chlorophenylalanine

(*p*-CPA). Compared with the Al-only treatment, the application of *p*-CPA further increased, and the melatonin treatment decreased, the Al-induced NO production in *Arabidopsis* roots (Fig. 5A and B). Accordingly, compared with the Al-only treatment, the application of *p*-CPA further decreased (by 46%) and that of melatonin increased (by 35%) the relative root growth (Fig. 5C). Therefore, melatonin synthesis might be involved in *Arabidopsis* resistance to Al stress.

Fig. 5 *p*-chlorophenylalanine (*p*-PCA), a melatonin (MT) synthesis inhibitor, accelerated Al-induced root growth inhibition and NO production. A, Effect of melatonin and *p*-CPA on relative root growth. The NO production in roots exposed to 0 (CK) or 100 μ M Al stress with melatonin and/or *p*-PCA for 3 days was detected using the NO-specific fluorescent probe DAF-FM DA (B), and the NO fluorescence intensity was quantified (C). Six-day-old seedlings were transferred to 1/6 MS medium containing 0 or 100 μ M Al supplemented with 1 μ M melatonin and/or 10 μ M *p*-CPA. In B and C, the values are means $\pm$ SD (n=20-25). Different letters indicate significantly different values ($p \leq 0.05$). The white bar represents 100 μ m.

3.6 Aluminum exposure decreases the expression of *AtSNAT* and melatonin accumulation in *Arabidopsis*

We analyzed the expression of *AtSNAT* and *AtASMT* as well as endogenous melatonin concentration in *Arabidopsis* under Al stress. Compared with the control treatment (-Al), the expression of *AtSNAT* was significantly decreased (about 72%) and that of *AtASMT* was unchanged after the Col-0 roots were treated by Al (Fig. 6A). Similarly, the concentration of melatonin in the Al-stressed wild-type Col-0 seedlings was decreased by 49% compared with the control (Fig. 6B). In the absence of Al, the concentration of melatonin in the *snat* seedlings was decreased by 37% in comparison with the wild-type Col-0 seedlings. Interestingly, there was no difference in the concentration of endogenous melatonin between the Al-treated wild-type Col-0 seedlings and the *snat* seedlings regardless of the Al treatment (Fig. 6B), suggesting that the Al treatment chemically decreased *AtSNAT* activity in the wild-type seedlings to a similar level achieved by the genetic knock-out of *AtSNAT* activity in the *snat* mutant. Hence, down-regulation of *AtSNAT* activity is involved in the Al inhibition of melatonin synthesis in *Arabidopsis*.

Fig. 6 Al toxicity decreased the expression of *AtSNAT* as well as melatonin concentration in *Arabidopsis*. Effect of Al on the expression of *AtSNAT* and *AtASMT* (A) and melatonin concentration (B) in *Arabidopsis*. Six-day-old *Arabidopsis* Col-0 seedlings were transferred to 1/6 MS medium containing 0 or 100 μ M Al. Values are means $\pm$ SD (n=3). Different letters or asterisk (*) indicate

significantly different values ($p \leq 0.05$).

3.7 The *snat* mutant plants with decreased melatonin concentration exhibit NO overproduction and aluminum sensitivity

Having ascertained that melatonin accumulation was reduced by Al via down-regulation of the *AtSNAT* expression, we then tested the role of this gene in *Arabidopsis* response to Al toxicity. In the absence of Al, the root elongation of *snat* mutant seedlings, lacking the *AtSNAT1* expression (Fig. S3), was decreased significantly (by 19%) in comparison with the wild-type Col-0, indicating that melatonin is essential for root growth (Fig. 7A). Application of Al caused further decreases in root growth in both wild-type and mutant seedlings (Fig. 7A), but to a slightly greater degree in the latter (Fig. 7B). Coinciding with these results, the NO production in roots was significantly increased in the *snat* mutants after the Al treatment, this increase being greater than in the Col-0 plants (Fig. 7C and D).

Fig. 7 The *Arabidopsis snat* mutant seedlings showed higher Al sensitivity and NO accumulation than Col-0. Root elongation (A) and relative root growth (B) of 6-day-old *Arabidopsis* seedlings transferred to 1/6 MS medium containing 0 or 100 μ M Al for 3 days ($n=50$). The NO production was detected by using the NO-specific fluorescent probe DAF-FM DA (C) and quantified (D, $n=6$) in roots exposed to 0 or 100 μ M $AlCl_3$ for 3 days. Values are means $\pm$ SD. Different letters indicate significantly different values ($p \leq 0.05$ by Tukey's test). The white bar in C represents 100 μ m.

Supplemental Fig. S3 Expression of *AtSNAT* in wild-type (Col-0) and the homozygous mutant *snat*.

4. Discussion

4.1 NO signaling contributes to aluminum-induced root growth inhibition via disturbing cell division progression and the quiescent center activity

The growth and development of root architecture is critical for water and nutrient acquisition from soil. Root growth is determined by the balance between cell division and cell elongation (Beemster and Baskin, 1998). It is generally accepted that NO is involved in root signaling and development under normal and stress situations. NO shared a similar pathway with auxin in root elongation and development, promoting at low and inhibiting at high concentrations (Stohr and Stremlau, 2006). For example, overproduction of NO contributes to root growth inhibition caused by Cd and methyl 3-(4-hydroxyphenyl) propionate (Liu et al., 2016; Yuan and Huang, 2016). Under Al stress, a decrease in NO production in roots of red kidney bean (Wang et al., 2010) or *Hibiscus moscheutos* (Tian et al., 2007) was associated with decreased activities of NR and NOS. Thereby, external application of SNP significantly alleviated Al-induced root growth inhibition in these two plant species (Tian et al., 2007; Wang et al., 2010). In contrast, application of exogenous NO aggravated Al-induced root growth inhibition in alfalfa (Chen et al., 2014) and rice bean (Zhou et al., 2012), maybe due to Al-induced accumulation of NO in roots. Comparable results were obtained in the present study. Both NR and NOS were involved in Al-induced NO accumulation (Fig. 2A); an increase in NO concentration by SNP significantly inhibited root growth (Fig. 2B and Fig. S2), whereas a decrease in the concentration of NO by application of cPTIO, tungstate, L-NAME or melatonin significantly promoted root growth under Al stress (Fig. 2B; Fig. 4A-C). In accordance with these pharmacological results, genetic analysis using *nia1nia2* and *noa1* further confirmed that both NR and NOS were involved in NO production, with decreased NO concentrations alleviating Al-induced root growth inhibition (Fig. 2C and D). These

results indicated that the alleviating or exacerbating effect of NO on root elongation under Al stress is dependent on NO concentrations and the sensitivity of various plant species to Al-induced root NO accumulation.

The root apex is the primary target of Al toxicity. Al-reduced root cell elongation is quick, and may occur in minutes to hours (Barcelo and Poschenrieder, 2002). In contrast, a long-term (days) exposure to Al inhibited primary root growth probably via a decrease in root cell divisions (Kochian, 1995). In *Arabidopsis*, arresting of cell cycle and the loss of the quiescent center activity were involved in Al-induced root growth inhibition (Rounds and Larsen, 2008; Ruiz-Herrera and Lopez-Bucio, 2013). Here, we also found that root growth inhibition after the 3-day treatment with Al or Al+SNP was positively related to a decrease in GUS staining and activity in roots of *Arabidopsis* transgenic lines CYCB1;1::GUS and QC25::GUS, whereas this decrease caused by Al was significantly alleviated by the addition of cPTIO and melatonin (Fig. 3 B-D and Fig. 4 D-F). These results indicated that Al inhibited root growth, at least partly, by NO-mediated disturbance of cell division and a decrease in the quiescent center activity.

4.2 Melatonin protects against aluminum toxicity by interfering with NO production

The balance between generation and scavenging of reactive oxygen (ROS, such as H₂O₂) and nitrogen species (RNS, including NO), both common signaling agents, was disturbed under a range of environmental stresses, including Al toxicity (Mittler, 2002; Richards et al., 1998). Melatonin is a highly-conserved ancient molecule widespread in living organisms. Melatonin has both receptor-dependent and -independent actions (Reiter et al., 2007). The receptor CAND2/PMTR1-mediated phytomelatonin signaling regulates stomatal closure through activation of a H₂O₂ and Ca²⁺ signaling transduction cascade in *Arabidopsis* (Wei et al., 2018). The primary function of melatonin as a scavenger and/or a broad-spectrum antioxidant protectant against ROS and RNS in a receptor-independent manner has been widely investigated in plants and animals (Schipper and Nichols, 2014). For example, exogenous application of melatonin was found to be effective in protecting plant cells from oxidative damage induced by Cd (Hasan et al., 2015) and Al (Zhang et al., 2017) stresses via directly scavenging H₂O₂ and/or enhancing the activities of antioxidant enzymes.

Melatonin has a variable function in NO signaling. For instance, exogenous melatonin induced NO production in *Arabidopsis* under iron deficiency stress (Zhou et al., 2016); conversely, NO reduced melatonin synthesis in rice under Cd stress (Lee et al., 2017). In animals, melatonin can efficiently scavenge NO, decreasing its concentration (du Plessis et al., 2010; Noda et al., 1999). In *Medicago sativa* plants, melatonin alleviated drought stress via interfering with NO signaling (Antoniou et al., 2017). Similar results were also obtained in the present study. Under Al stress, the application of melatonin significantly decreased Al-induced NO production and alleviated root growth inhibition in *Arabidopsis* (Fig. 4A-C). In support of these findings, melatonin synthesis inhibitor *p*-CPA increased NO production and further exacerbated Al-induced root growth inhibition (Fig. 5); moreover, the *Arabidopsis snat* mutant with lower melatonin concentration showed higher NO production and Al sensitivity in comparison with the wild type Col-0 seedlings (Fig. 7). These pharmacological and genetic results suggested that melatonin alleviated Al-induced root growth inhibition by interfering with an Al-induced increase in NO production.

The synthesis of melatonin plays important roles in the plant stress responses. For example, upregulation of *AtASMT* expression was closely associated with Cd-induced melatonin synthesis in *Arabidopsis* (Byeon et al., 2016). In apple plants, drought induced melatonin synthesis in mitochondria through induction of *MzSNAT5*, and overexpression of *MzSNAT5* in *Arabidopsis* enhanced melatonin synthesis in mitochondria and conferred drought tolerance (Wang et al., 2017). In soybean, upregulation of a *SNAT*-like gene and melatonin accumulation conferred Al tolerance by regulating organic acid anion exudation and antioxidant enzymes activity (Zhang et al., 2017). In contrast, Lee and Back (2017) recently showed that Al did not influence the synthesis of melatonin in rice roots. In the study presented here, we found that Al reduced the expression of *AtSNAT* (Fig. 6A) and the endogenous concentration of melatonin in *Arabidopsis* (Fig. 6B). Therefore, the dynamics of melatonin synthesis might be related to a degree of Al stress and Al sensitivity of plant species and genotypes.

Fig. 8 A proposed mechanism of melatonin-mediated alleviation of Al toxicity in *Arabidopsis*. Al toxicity induced NO production through the NR- and NOS-dependent pathways (1). The overproduction of NO induced by Al arrested the cells in the G2-M phase of the cell cycle (CC) and decreased the quiescent center cell (QC) activity, which resulted in root growth inhibition (2). Exogenous application of melatonin abolished Al-induced NO production (3) and alleviated Al toxicity (4). However, downregulation of the *AtSNAT* gene (5) by Al caused a decrease in endogenous melatonin concentration (6 and 7), resulting in a lack of effect on the Al-induced NO production (8) and therefore Al toxicity was not alleviated. Red color shows Al-induced processes leading to root growth inhibition. Green color signifies exogenous melatonin alleviation of Al toxicity. Blue color denotes a lack of endogenous melatonin alleviation of Al toxicity.

5. Conclusions

Aluminum (Al) toxicity in acidic soils is the global agronomic problem affecting plant growth and crop production. Inhibition of root elongation is the primary symptom of Al toxicity. In this study, we found that the NOS- and NR-dependent NO production contributed to Al-induced root growth inhibition via interference with the cell division cycle and a decrease in the quiescent center activity in *Arabidopsis* (Fig. 8). Pharmacological and genetic evidence suggested that exogenously applied and endogenously synthesized melatonin was involved in alleviation of Al-induced root growth inhibition, cell cycle arrest and a decrease in the quiescent center activity by interfering with Al-induced NO signaling.

Expression of *AtSNAT*, encoding a key enzyme involved in melatonin synthesis, was down-regulated by AI, resulting in decreased melatonin concentration and thus enhanced AI-induced NO production and exacerbated root growth inhibition (Fig. 8). Therefore, melatonin plays the vital roles in *Arabidopsis* AI resistance by preventing AI-induced NO accumulation and thus alleviating AI-caused root growth limitation.

Author Contributions

Q.C. conceived and designed the study, analyzed the data, interpreted the results, wrote and revised the manuscript. Z.R. analyzed the data, interpreted the results and revised the manuscript. J.R.Z. performed most of the experiments; D.X.L. and J.W. provided technical assistance. J.R.Z. and D.X.L. processed data and performed the data analysis. All authors discussed the results and commented on the manuscript.

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Figure captions

Fig. 1. Effect of Al on root growth and NO production in *Arabidopsis* Col-0 roots. A, Effect of 0, 20, 100 and 200 μM AlCl_3 on primary root growth. The NO production in roots exposed to different concentrations of AlCl_3 for 3 days was detected using the NO-specific fluorescent probe DAF-FM DA (Diamino-fluorescein diacetate) (B), and the NO fluorescence intensity was quantified (C). Six-day-old seedlings were transferred to 1/6 MS medium containing 0, 20, 100 or 200 μM AlCl_3 for 3 days. Values are means $\pm$ SD (n=20-25). Different letters indicate significantly different values ($p \leq 0.05$). Bar= 100 μm .

Fig. 2. Nitric oxide synthase (NOS)- and nitrate reductase (NR)-mediated NO synthesis contributed to Al-induced root growth inhibition in *Arabidopsis*. A, Fluorescence intensity of NO in Col-0 roots exposed to 100 μM Al with or without 10 μM SNP (an NO donor), 100 μM cPTIO (an NO scavenger), 100 μM L-NAME (an NOS inhibitor) or 10 μM tungstate (a nitrate reductase inhibitor) for 3 days using the NO-specific fluorescent probe DAF-FM DA. Values are means $\pm$ SD (n=6). B, Relative root growth of *Arabidopsis* Col-0 seedlings treated by 0 or 100 μM Al with or without 10 μM SNP, 100 μM cPTIO, 100 μM L-NAME or 10 μM tungstate. Values are means $\pm$ SD (n=20-25). C and D, Effect of Al on primary root elongation of Col-0, *noa1* and *nia1nia2* plants. Six-day-old seedlings were transferred to 1/6 MS medium containing 0 or 100 μM AlCl_3 for 3 days. Values are means $\pm$ SD (n=20-25). In a, b and d, different letters indicate significantly different values ($p \leq 0.05$). In c, the white bar represents 10 mm.

Fig. 3 NO mediated the Al-induced root growth inhibition by interfering with the cell division cycle and the quiescent center activity. *Arabidopsis* transgenic lines CYCB1;1::GUS and QC25::GUS were treated with 0 (CK) or 100 μM Al with or without 10 μM SNP or 100 μM cPTIO for 3 days. A, Relative root growth (n=20-25). Different letters indicate significantly different values ($p \leq 0.05$). Images of GUS staining of CYCB1;1::GUS (B) and QC25::GUS roots (C). The white bar represents 100 μm . D, Relative quantitative GUS activity (n=3). Values are means $\pm$ SD.

Fig. 4 Exogenous application of melatonin (MT) alleviated Al-induced root growth inhibition, NO production, and a decrease in the meristematic cell division potential and the quiescent center activity. A, Effect of different concentrations of melatonin on root growth in *Arabidopsis* Col-0 exposed to 0 or 100 μM Al stress for 3 days. B, Relative fluorescence intensity of NO [detected using the NO-specific

fluorescent probe (DAF-FM DA)] in *Arabidopsis* Col-0 roots exposed to 0 or 100 μ M Al stress with or without various concentrations of melatonin (0.1, 1 or 10 μ M) for 3 days. Cc, Effect of 1 μ M melatonin on relative root growth of *Arabidopsis* Col-0 treated with 0 (CK) or 100 μ M Al in the presence or absence of 10 μ M SNP. D and E, Images of GUS staining of CYCB1;1::GUS (d) and QC25::GUS roots (e) exposed to 0 (CK) or 100 μ M Al with or without 1 μ M melatonin or 10 μ M SNP. F, Relative quantitative GUS activity. Bar=100 μ m. For a (n=20-25), b (n=6), c (n=20-25) and F (n=3), the values are means $\pm$ SD. Different letters indicate significantly different values ($p \leq 0.05$).

Fig. 5 P-chlorophenylalanine (p-PCA), a melatonin (MT) synthesis inhibitor, accelerated Al-induced root growth inhibition and NO production. A, Effect of melatonin and p-CPA on relative root growth. The NO production in roots exposed to 0 (CK) or 100 μ M Al stress with melatonin and/or p-PCA for 3 days was detected using the NO-specific fluorescent probe DAF-FM DA (B), and the NO fluorescence intensity was quantified (C). Six-day-old seedlings were transferred to 1/6 MS medium containing 0 or 100 μ M Al supplemented with 1 μ M melatonin and/or 10 μ M p-CPA. In B and C, the values are means $\pm$ SD (n=20-25). Different letters indicate significantly different values ($p \leq 0.05$). The white bar represents 100 μ m.

Fig. 6 Al toxicity decreased the expression of *AtSNAT* as well as melatonin concentration in *Arabidopsis*. Effect of Al on the expression of *AtSNAT* and *AtASMT* (A) and melatonin concentration (B) in *Arabidopsis*. Six-day-old *Arabidopsis* Col-0 seedlings were transferred to 1/6 MS medium containing 0 or 100 μ M Al. Values are means $\pm$ SD (n=3). Different letters or asterisk (*) indicate significantly different values ($p \leq 0.05$).

Fig. 7 The *Arabidopsis snat* mutant seedlings showed higher Al sensitivity and NO accumulation than Col-0. Root elongation (A) and relative root growth (B) of 6-day-old *Arabidopsis* seedlings transferred to 1/6 MS medium containing 0 or 100 μ M Al for 3 days (n=50). The NO production was detected by using the NO-specific fluorescent probe DAF-FM DA (C) and quantified (d, n=6) in roots exposed to 0 or 100 μ M $AlCl_3$ for 3 days. Values are means $\pm$ SD. Different letters indicate significantly different values ($p \leq 0.05$ by Tukey's test). The white bar in C represents 100 μ m.

Fig. 8 A proposed mechanism of melatonin-mediated alleviation of Al toxicity in *Arabidopsis*. Al toxicity induced NO production through NR- and NOS-dependent pathways (1). The overproduction of

NO induced by Al arrested the cells in the G2-M phase of the cell cycle (CC) and decreased the quiescent center cell (QC) activity, which resulted in root growth inhibition (2). Exogenous application of melatonin abolished Al-induced NO production (3) and alleviated Al toxicity (4). However, downregulation of the *AtSNAT* gene (5) by Al caused a decrease in endogenous melatonin concentration (6 and 7), resulting in a lack of effect on the Al-induced NO production (8) and therefore Al toxicity was not alleviated. Red color shows Al-induced processes leading to root growth inhibition. Green color signifies exogenous melatonin alleviation of Al toxicity. Blue color denotes a lack of endogenous melatonin alleviation of Al toxicity.

Supplemental Materials

Supplementary Table S1 List of the primers and their uses

Supplemental Fig. S1 The NO production in *Arabidopsis* Col-0 roots exposed to 100 μ M Al with or without 10 μ M SNP (an NO donor), 100 μ M cPTIO (an NO scavenger), 100 μ M L-NAME (an NOS inhibitor) or 10 μ M tungstate (an NR inhibitor) for 3 days detected using the NO-specific fluorescent probe DAF-FM DA (Diamino-fluorescein diacetate).

Supplemental Fig. S2 Effect of different concentrations of SNP on *Arabidopsis* Col-0 root growth under control (0 Al) or 100 μ M Al stress.

Supplemental Fig. S3 Expression of *AtSNAT* in wild-type (Col-0) and the homozygous mutant *snat*.