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Melatonin alleviates aluminium toxicity through modulating antioxidative enzymes and enhancing organic acid anion exudation in soybean

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Abstract. Aluminium (Al) toxicity is a major chemical constraint limiting plant growth and production on acidic soils. Melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitous molecule that plays crucial roles in plant growth and stress tolerance. However, there is no knowledge regarding whether melatonin is involved in plant responses to Al stress. Here, we show that optimal concentrations of melatonin could effectively ameliorate Al-induced phytotoxicity in soybean (*Glycine max* L.). The concentration of melatonin in roots was significantly increased by the 50 μM Al treatment. Such an increase in endogenous melatonin coincided with the upregulation of the gene encoding acetyltransferase NSI-like (nuclear shuttle protein-interacting) in soybean roots. Supplementation with low concentrations of melatonin (0.1 and 1 μM) conferred Al resistance as evident in partial alleviation of root growth inhibition and decreased H_2O_2 production: in contrast, high concentrations of melatonin (100 and 200 μM) had an opposite effect and even decreased root growth in Al-exposed seedlings. Mitigation of Al stress by the 1 μM melatonin root treatment was associated with enhanced activities of the antioxidant enzymes and increased exudation of malate and citrate. In conclusion, melatonin might play a critical role in soybean resistance to Al toxicity.

Additional keywords: Al toxicity, citrate, *Glycine max*, H_2O_2, malate, melatonin synthesis.

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Introduction

Aluminium (Al) is the most abundant metal in the earth's crust (Tesfaye *et al.* 2001). Under acidic conditions ($pH_{water} < 5.50$), Al is released into the soil and becomes toxic to plants and limiting crop production (Rengel and Zhang 2003). Overproduction of reactive oxygen species (ROS) in plants is an early product in Al toxicity – ROS formation can then induce oxidative stress, leading to cell membrane peroxidation, structural damage in cells, chromosomal aberration and programmed cell death (Nicoloso *et al.* 2009; Yi *et al.* 2010). Compared with Al-sensitive plant cultivars, Al-resistant cultivars have higher levels of antioxidants, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione-S-transferase (GST), resulting in lower accumulation of ROS in their roots (Darkó *et al.* 2004). For example, the gene expression and enzyme activities of SOD, POD and CAT are higher in Al-resistant than Al-sensitive soybean cultivars (Wu *et al.* 2013). In addition, increased activity of antioxidant enzymes brought about by external application of salicylic acid or nitric oxide was involved in alleviation of Al toxicity in *Cassia tora* (Wang *et al.* 2004; Wang and Yang 2005).

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The Al-activated release of organic acid anions ((OAAs) for example, malate, citrate and oxalate) from roots has been regarded as one of the most efficient Al exclusion mechanisms. The release of OAAs protects the root tips by chelating Al to form stable and nontoxic complexes in the rhizosphere, including the efflux of malate from wheat roots (Ryan *et al.* 1995), oxalate from buckwheat (Ma *et al.* 1997), and citrate from soybean (Silva *et al.* 2001), rice bean (Yang *et al.* 2007) and broad bean (Chen *et al.* 2012). The exudation of OAAs is controlled by the expression of membrane-localised OAA transporters that belong to two families; Al-activated malate transporter (ALMT) and multidrug and toxic compound extrusion (MATE). Additionally, many phytohormones and signal molecules such as IAA, ABA, ethylene, salicylic acid and nitric oxide also play important roles in the regulation of Al-induced OAA exudation (Kopittke 2016; Wang *et al.* 2016*b*).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a tryptophan-derived metabolite that is widespread in bacteria, algae, animals and higher plants (Hardeland 2016). Plant melatonin is synthesised via four sequential enzyme steps, involving tryptophan decarboxylase (TDC), arylalkylamine *N*-acetyltransferase (AANAT)/serotonin *N*-acetyltransferase (SNAT), tryptamine 5-hydroxylase (T5H), *N*-acetylserotonin methyltransferase (ASMT)/hydroxyindole-*O*-methyltransferase (HIOMT) (Zhang *et al.* 2015). Among them, SNAT and ASMT play pivotal roles and are considered the step-limiting enzymes in plant melatonin biosynthesis.

Melatonin has been implicated in many physiological processes in plants, including coleoptile growth, root growth, leaf morphology, flowering time and fruit ripening (Arnao and Hernandez-Ruiz 2015; Nawaz *et al.* 2015). Furthermore, it is also associated with plant tolerance to biotic and abiotic stresses. For example, exogenous application of melatonin significantly alleviated growth inhibition caused by elevated salinity (Li *et al.* 2012; Mukherjee *et al.* 2014). In *Solanum lycopersicum* L., Cd stress-induced melatonin biosynthesis and external application of melatonin conferred plant resistance to Cd toxicity (Hasan *et al.* 2015). The application of melatonin also decreased a ROS-related oxidative damage by enhancing the activities of antioxidant enzymes including SOD, POD and CAT (Hasan *et al.* 2015; Wang *et al.* 2016*c*; Arora and Bhatla 2017). However, there is no knowledge of whether melatonin is involved in plant responses to Al stress.

In this study we provide evidence on how melatonin regulates Al resistance in soybean. Our results show that the root concentration of melatonin was increased by Al, which may be due to upregulation of the gene encoding acetyltransferase NSI-like in soybean roots. Furthermore, external application of melatonin enhanced soybean resistance to Al stress through increasing (i) activities of antioxidant enzymes and (ii) Al-induced citrate and malate exudation.

Materials and methods

Plant culture and growth conditions

Seeds of soybean (*Glycine max* L. cv. Dian-6) were obtained from Yunnan Academy of Agricultural Sciences (Kunming, Yunnan province, China). For germination, seeds were soaked in deionised water for 12 h in the dark at 25°C. Then, seeds were placed on a filter paper moistened with half-strength Hoagland solution for germination in the dark at 25°C. Seedlings with roots 1–2 cm long were transferred onto a floating mesh in polypropylene pots with half-strength Hoagland solution (5 L) and grown in a controlled-environment room at 23°C, 12 h light/12 h dark photoperiod and white light intensity of 100 μmol m^{-2} s^{-1}.

Estimation of relative root growth (RRG)

Calcium is an important second messenger in plants that affects all aspects of plant growth and development. It is present in soil solutions at relatively high concentrations, with the median values being around 1–5 mM, generally, concentrations between 0.1 and 1.0 mM of Ca are needed for optimal growth of dicotyledonous species (Rengel 1992). Al decreases accumulation of Ca^{2+} by interfering with the membrane transport and disturbing symplasmic Ca^{2+} homeostasis (Rengel and Zhang 2003). Therefore, to mimic the natural concentrations of Ca and obtain better growth under Al stress, 5-day-old seedlings were pre-grown overnight in a 0.5 mM $CaCl_2$ solution (pH 4.2). Then, roots were transferred into a 0.5 mM $CaCl_2$ solution containing 50 μM $AlCl_3$ with 0, 0.1, 1, 10, 100 or 200 μM melatonin at pH 4.2 for a 24 h treatment. Seedlings grown in a solution of 0.5 mM $CaCl_2$ (pH 4.2) with 0 or 1 μM melatonin were used as controls. Root elongation was measured with a ruler before (0 h) and after the treatments (24 h). The relative root growth (RRG) was calculated as the ratio of the root growth in various treatments to that in the controls.

Measurement of endogenous melatonin

Five-day-old seedlings were pre-treated with 0.5 mM $CaCl_2$ (pH 4.2) for 12 h. The seedlings were then transferred to 0.5 mM $CaCl_2$ solution containing $AlCl_3$ at 0 or 50 μM (pH 4.2) for the 24 h treatment. After treatment, root apices were excised, weighed and immediately frozen in liquid nitrogen. Melatonin was extracted from soybean roots according to the method of Pape and Lüning (2006). The melatonin concentration in soybean roots was determined by a Plant MT Elisa Kit (TSZ, America, catalogue no. PG19022) with the assay range of 0.2–48 ng L^{-1}. A series of melatonin dilutions was made to determine the standard curve and calculate melatonin concentration in soybean roots according to the manufacturer's instructions.

Measurement of H_2O_2

For determination of H_2O_2 concentration, 5-day-old seedlings were grown and treated as described for estimation of relative root growth. After treatment, the root tips were excised (0.5 g for each sample), thoroughly rinsed with deionised water, gently blotted, weighed, and immediately frozen in liquid nitrogen. The frozen roots were homogenised in 2 mL of 0.1% v/v TCA (trichloroacetic acid) for measurement of H_2O_2. The H_2O_2 concentration was measured as described elsewhere (Marta *et al*. 2016).

Measurement of CAT, SOD and POD activities

For determination of CAT, SOD and POD activities, seedlings were grown and treated as described for estimation of RRG.

After treatment, the roots were harvested and immediately frozen in liquid nitrogen for enzyme extraction. To avoid protein degradation, the buffers were pre-chilled and extraction steps were carried out on ice. The frozen roots (0.5 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 mM ascorbic acid and 1% w/v PVPP using a mortar and pestle. The homogenate was centrifuged for 20 min at 12 000*g* at 4°C, and the supernatant was immediately used for enzyme analysis.

SOD activity was measured according to the published method (Giannopolitis and Ries 1977). POD activity was measured according to the method reported by Maehly and Chance (1954) and CAT activity was assayed by monitoring the consumption of H_2O_2 at 240 nm for 2 min (Aebi 1984). The protein content was measured by the Bradford method (Bradford 1976).

Morin staining

Aluminium accumulation was detected by morin staining following the protocol reported by Tice *et al.* (1992). Five-day-old seedlings were pre-treated in 0.5 mM $CaCl_2$ solution (pH 4.2) and then treated by 50 µM $AlCl_3$ with 0 or 1 µM melatonin in 0.5 mM $CaCl_2$ solution (pH 4.2) for 24 h. After treatment, the root apices (0–2 cm) were excised and washed with 0.5 mM $CaCl_2$ solution for 20 min, followed by 1 h incubation in 100 µM morin in 50 mM potassium phosphate buffer and 20 min washing in potassium phosphate buffer. Green fluorescence from the Al-morin complex was observed using the 420 nm excitation and 510 nm emission wavelengths.

Malate and citrate exudation

After 24 h treatments, the soybean root exudates were collected and concentrated as described previously (Chen *et al.* 2012). The estimation of malate and citrate concentrations in the exudates was performed using the published enzymatic methods (Chen *et al.* 2011; Yang *et al.* 2004).

Real-time RT–PCR analysis

The excised root tips were used for isolation of total RNA using Trizol reagent and the synthesis of the first strand cDNA as previously described (Chen *et al.* 2011). Subsequently, 1 µL of 10-fold dilution of cDNA with SYBR Green master mix (Vazyme) was used for gene expression analysis (ABI 7300 real time PCR system) following the manufacturer's instructions. Using *Arabidopsis* serotonin *N*-acetyltransferase (AtSNAT, At1 g32070) as a query, acetyltransferase NSI-like (nuclear shuttle protein-interacting) was identified in soybean (see Fig. S1, available as Supplementary Material to this paper). Two acetyltransferase NSI-like transcript variants X1 (*NSI-X1*, NCBI Sequence ID: XM_006602669.2) and X2 (*NSI-X2*, NCBI sequence ID: XM_014770469.1) were selected for real-time RT–PCR analysis. The primers for *NSI-X1* and *NSI-X2* were designed as follows: 5′-GCTAATCTTCAATGTCAATGCTA T-3′ (sense primer)/5′-AGTGAGAGTGTGGCTACC-3′ (anti-sense primer) and 5′-GCTTACTTTTAGTATTGATTATT-3′ (sense primer)/5′-CCAGAATCCAGCCTTGAG-3′ (anti-sense primer). The β-*tubulin* (CA936138) gene was used as a reference gene with 5′-CTCAGGTGATTTCATCTTTG-3′ (sense primer)/5′-GAATTCAGTCACATCCAC-3′ (antisense primer).

Statistical analysis

Experiments contained at least three replicates, and the data are expressed as means and s.e. SPSS 12.0 for Windows (SPSS Inc.) software packages were used to conduct the least significant difference (l.s.d.) test to determine statistical significance at $P \leq 0.05$.

Results

Optimal concentrations of melatonin alleviated Al-induced root growth inhibition

The root elongation under 50 µM Al stress with 0, 0.1, 1, 10, 100 or 200 µM melatonin was measured to evaluate the effect of melatonin on Al rhizotoxicity in soybean. In the absence of melatonin, the growth of soybean roots was inhibited ~55% by the treatment with 50 µM $AlCl_3$ for 24 h. After application of melatonin to the 50 µM Al treatment solution, the relative root growth was increased by low melatonin concentrations (0.1 and 1 µM) but decreased by high concentrations of melatonin (100 and 200 µM) (Fig. 1*a*, *b*).

Al exposure induced root melatonin accumulation and enhanced expression of genes related to melatonin biosynthesis

Having ascertained that low doses of melatonin could effectively ameliorate Al-induced phytotoxicity in soybean, we then asked whether Al toxicity has an effect on melatonin biosynthesis. Compared with the control treatment, the concentration of melatonin in roots was increased 1.8-fold after the roots were treated by 50 µM Al for 24 h (Fig. 2*a*). Similarly, the expression of *NSI-X1* and *NSI-X2*, homologous to *Arabidopsis* serotonin *N*-acetyltransferase (*SNAT*, At1 g32070), was significantly increased (by 3.1- and 2.6-fold respectively) compared with the control (Fig. 2*b*).

Melatonin decreased Al-induced H_2O_2 production

The H_2O_2 concentration in soybean roots was increased by 32% after the 50 µM Al treatment for 24 h (Fig. 3). Application of 0.1 or 1 µM melatonin significantly decreased Al-induced H_2O_2 production (by 22 and 38% respectively). However, a high concentration of melatonin (10–200 µM) did not significantly change H_2O_2 concentration in soybean roots under Al stress (Fig. 3).

Melatonin upregulated the activities of antioxidant enzymes

The Al treatment significantly increased the activity of CAT, but had little effect on SOD and POD activities (Fig. 4). However, the activities of SOD, POD and CAT were significantly increased by the addition of 1 µM melatonin to the Al treatment solution; this concentration of melatonin also provided the strongest alleviation of Al toxicity (c.f. Fig. 1). High concentrations of melatonin (particularly 100 and 200 µM) decreased activities of SOD, POD and CAT.

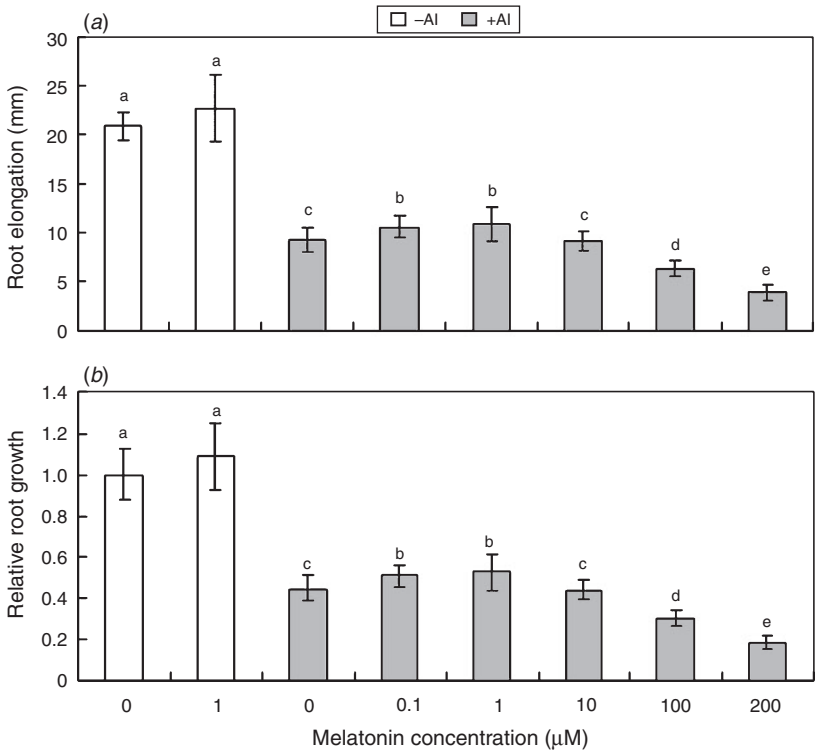

Fig. 1. Effect of increasing melatonin concentrations on root elongation (*a*) and relative root growth (*b*) in soybean seedlings during Al stress. Five-day-old seedlings were treated with 0 or 50 μM Al in 0.5 mM $CaCl_2$ supplemented with 0, 0.1, 1, 10, 100 or 200 μM melatonin for 24 h. Values are means ± s.e. ($n = 15$–20). Different letters indicate significant differences at $P < 0.05$.

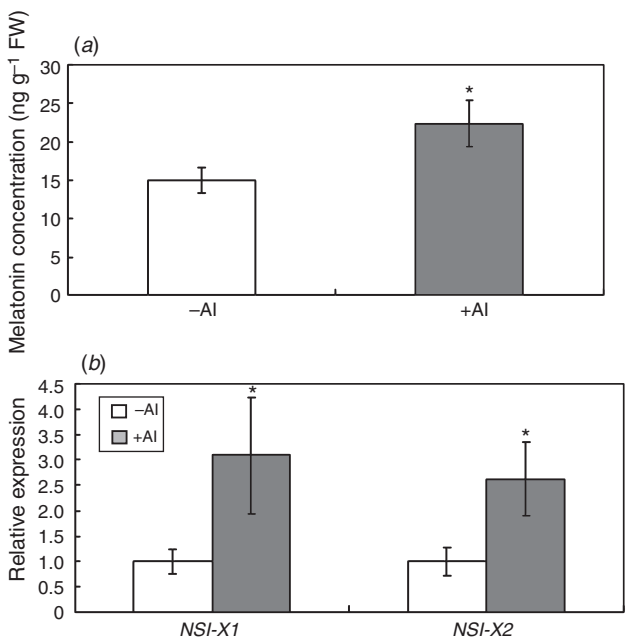

Fig. 2. Effect of Al on melatonin accumulation (*a*) and the relative expression levels of *NSI-X1* and *NSI-X2* (*b*) in soybean roots. Five-day-old seedlings were treated with 0 or 50 μM Al in 0.5 mM $CaCl_2$ for 24 h. Values are means ± s.e. ($n = 3$). Significant differences between control and Al treatments are indicated: *, $P < 0.05$.

Melatonin enhanced Al-induced citrate and malate exudation

Compared with the control, the 24 h treatment with 50 μM Al increased malate exudation by 42% and that of citrate by 47% (Fig. 5*a*, *b*). The treatment with 1 μM melatonin did not change malate and citrate exudation compared with the control; however, 1 μM melatonin together with 50 μM Al significantly increased malate (by 14%) and citrate exudation (by 20%) compared with the Al treatment (Fig. 5*a*, *b*).

Morin is a fluorescent Al-sensitive dye. In soybean roots, morin staining was barely detectable in root tips not exposed to Al (regardless of the presence of 1 μM melatonin), but was quite vivid in roots exposed to 50 μM Al for 24 h (Fig. 5*c*). Inclusion of 1 μM melatonin in the Al treatment solutions decreased morin staining (i.e. Al concentration) by 70% in root tips (Fig. 5*c*, *d*).

Discussion

Melatonin is a ubiquitous molecule that serves multiple biological functions in plant growth, development and responses to abiotic stresses. In the present work we elucidated amelioration of Al toxicity in soybean roots by melatonin. Our results showed that Al toxicity induced endogenous melatonin accumulation in soybean roots (Fig. 2). Moreover, exogenous application of low concentrations of melatonin significantly improved soybean resistance to Al

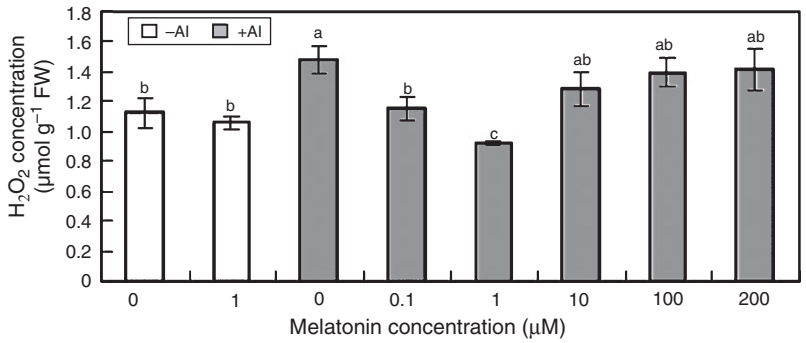

Fig. 3. Effect of external application of melatonin on Al-induced H_2O_2 production in soybean roots. Five-day-old seedlings were treated with 0 or 50 μM Al in 0.5 mM $CaCl_2$ supplemented with 0, 0.1, 1, 10, 100 or 200 μM melatonin for 24 h. Values are means ± s.e. ($n = 6$). Means with different letters are significantly different at $P < 0.05$.

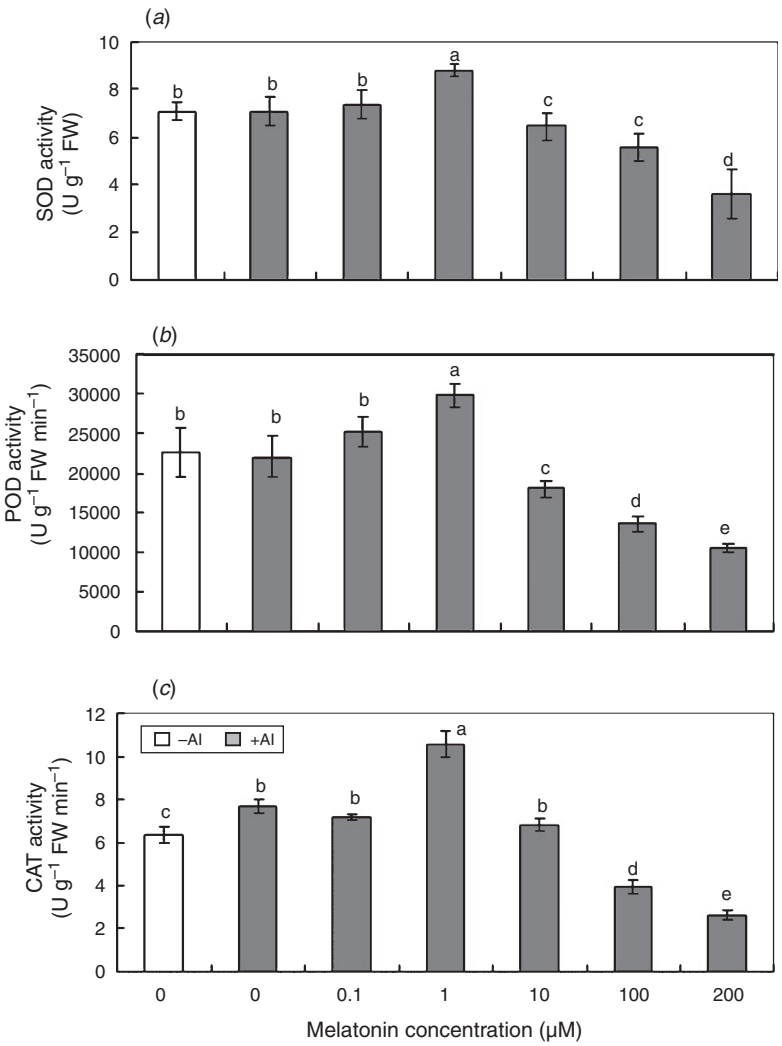

Fig. 4. Effect of external application of melatonin on the activities of superoxide dismutase (SOD) (*a*), peroxidase (POD) (*b*) and catalase (CAT) (*c*) in soybean roots under Al stress. Five-day-old soybean seedlings were treated with 0 or 50 μM Al in 0.5 mM $CaCl_2$ supplemented with 0, 0.1, 1, 10, 100 or 200 μM melatonin for 24 h. Values are means ± s.e. ($n = 6$). Means with different letters are significantly different at $P < 0.05$.

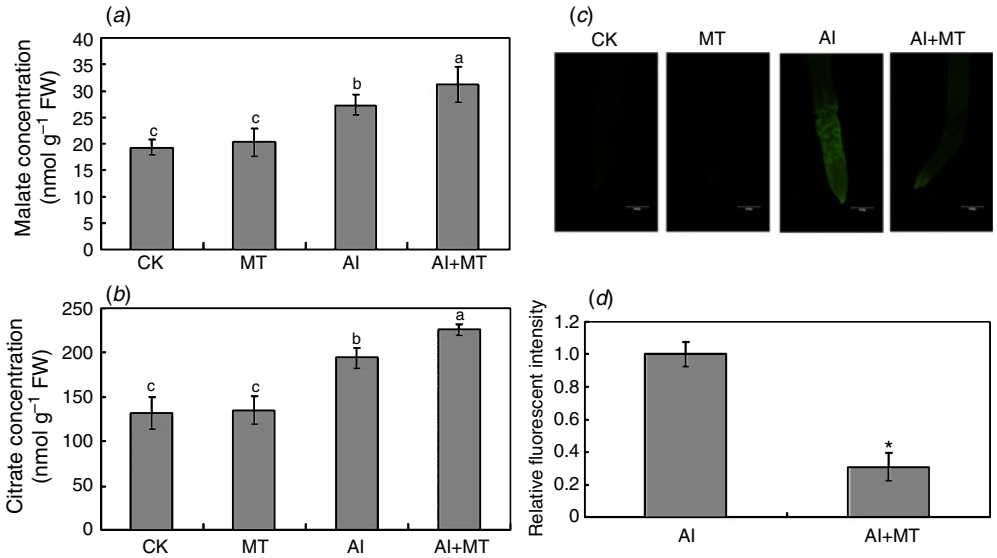

Fig. 5. Effect of external application of melatonin on Al-induced malate (*a*) and citrate (*b*) exudation and morin staining to indicate Al accumulation (*c*, *d*) in soybean roots under Al stress. Five-day-old soybean seedlings were treated with 50 µM Al in presence of 0 (Al) or 1 µM melatonin (Al + MT) and 0.5 mM $CaCl_2$ for 24 h. The seedlings grown in 0.5 mM $CaCl_2$ (pH 4.2) with 0 (CK) or 1 µM melatonin (MT) for 24 h were used as controls. (*c*) White bar represents 500 µm; (*d*) fluorescence intensity was determined using ImageJ. (*a*, *b*, *d*) values are means ± s.e. ($n = 6$); means with different letters are significantly different at $P < 0.05$.

(Fig. 1) by enhancing the antioxidant capacity (Fig. 4) and malate and citrate exudation (Fig. 5).

Melatonin is a major animal hormone involved in modulating circadian rhythms, seasonal reproductive function and immunology. In the past two decades, melatonin has been reported in numerous plant species as being involved in various physiological processes (Hardeland 2016). Similar to IAA, melatonin acts as a growth promoter regulating the growth of coleoptiles and lateral and adventitious roots in several plant species (Hernández-Ruiz *et al.* 2004, 2005). In addition to regulating plant growth and development, melatonin is also involved in a wide range of stress responses (Kaur *et al.* 2015). For example, abiotic stresses induced a significant rise in melatonin concentration in sunflower exposed to salinity (Mukherjee *et al.* 2014), Cd-stressed *S. lycopersicum* (Hasan *et al*. 2015) and barely roots under H_2O_2 and Zn toxicity (Arnao and Hernandez-Ruiz 2009). In sunflower seedlings exposed to salt stress, exogenous serotonin and melatonin restored root growth and hypocotyl elongation (Mukherjee *et al.* 2014; Kaur and Bhatla 2016).

It has been suggested that plant melatonin is synthesised via similar biosynthetic pathways as in vertebrates (Zhang *et al.* 2015). Recently, genes encoding melatonin biosynthetic enzymes have been identified. For example, genes encoding serotonin *N*-acetyltransferase (SNAT) and *N*-acetylserotonin methyltransferase (ASMT) were shown to be involved in melatonin synthesis in *Arabidopsis* and rice (Kang *et al.* 2013; Lee *et al.* 2014; Byeon *et al.* 2016). Furthermore, upregulation of *AtASMT* expression was closely associated with Cd-induced melatonin synthesis in *Arabidopsis* (Byeon *et al.* 2016). Similarly, we found that the expression of acetyltransferase NSI-like transcript variants *NSI-X1* and *NSI-X2*, homologous to *Arabidopsis* serotonin N-acetyltransferase (*AtSNAT*), was significantly increased by Al stress, coinciding with Al-stress-induced melatonin accumulation in soybean roots (Fig. 2*a*, *b*). These results indicated that upregulation of *NSI-X1* and *NSI-X2* could be involved in Al-induced melatonin biosynthesis in soybean roots.

The balance between generation and scavenging of ROS (such as superoxide anion and H_2O_2) is disturbed under a range of environmental stresses, including Al toxicity (Richards *et al.* 1998; Mittler 2002). Excess concentration of ROS induced by Al toxicity could have detrimental effects, causing lipid peroxidation in cellular membranes, protein denaturation and DNA damage. Exogenous application of melatonin was found to be effective in protecting plant cells from oxidative damage induced by several stresses via directly scavenging H_2O_2 and/or enhancing the activities of antioxidant enzymes (Hasan *et al.* 2015; Marta *et al.* 2016). However, the effect of exogenously applied melatonin at different concentrations ranged from significant amelioration to being ineffective or even toxic (Zhang *et al.* 2015). For example, melatonin promoted root growth at low concentrations, but inhibited it at high concentrations in several plant species (Chen *et al.* 2009; Sarropoulou *et al.* 2012; Wang *et al.* 2016*a*). Additionally, high melatonin concentrations aggravated cold temperature-mediated oxidative damage to cucumber (Marta *et al.* 2016) and Cu-mediated oxidative damage to red cabbage (Posmyk *et al.* 2008). Similar results were also obtained in the present study with soybean. Application of low concentrations of melatonin (0.1 and 1 µM) under Al toxicity significantly increased root growth (Fig. 1) and decreased H_2O_2 production (Fig. 3), which coincided with the activation of antioxidant enzymes (Fig. 4). In contrast, melatonin at 100 and

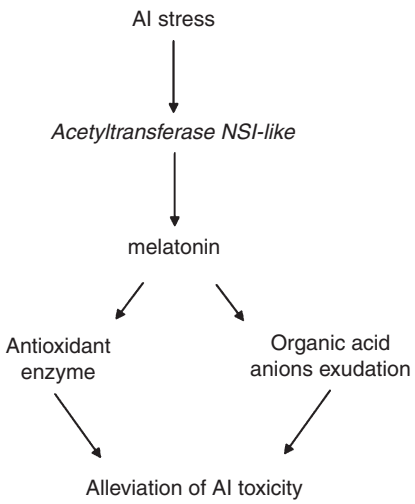

Fig. 6. A proposed mechanism of melatonin-mediated alleviation of Al toxicity in soybean. Aluminium induced melatonin biosynthesis through upregulating expression of two acetyltransferase NSI-like (nuclear shuttle protein-interacting) variants in soybean roots. Optimal concentration of melatonin enhanced the activities of antioxidant enzymes and malate and citrate exudation under Al stress, thereby conferring Al resistance.

200 μM exacerbated Al-induced reduction in root growth (Fig. 1) and decreased the activities of antioxidant enzymes (Fig. 4).

Organic acid anions (OAAs) can contribute to internal and external detoxification of heavy metals and Al. Exuded organic acid anions chelate Al to form non-toxic complexes in the rhizosphere. The main organic acid anions associated with Al detoxification are citrate, malate and oxalate depending on the plant species and the genotype (Ryan *et al.* 1995; Silva *et al.* 2001; Liang *et al.* 2013). Additionally, external application of Mg, salicylic acid or IAA can alleviate Al toxicity by enhancing Al-induced citrate and malate exudation in some plant species (Yang *et al.* 2003; Chen *et al.* 2015; Wang *et al.* 2016*b*). In the present study, exposing soybean roots to 1 μM melatonin for 24 h caused an increase in malate and citrate exudation under Al stress (but no change was measured in the absence of Al) (Fig. 5*a*, *b*), indicating that melatonin signalling is involved in the regulation of Al-induced organic anion exudation in soybean roots.

A possible mechanism of Al-induced increase in melatonin biosynthesis (or low concentrations of externally supplied melatonin) enhancing Al resistance in soybean is presented in Fig. 6. Aluminium toxicity triggers melatonin accumulation by upregulating two NSI-like variants of acetyltransferase that may be involved in melatonin biosynthesis. Optimal concentrations of melatonin enhance soybean resistance to Al stress by increasing activity of the antioxidant enzymes and increasing exudation of malate and citrate. However, the present understanding of melatonin signalling in abiotic stresses in plants is still poor, and further work is needed to better characterise the melatonin signalling pathways in Al resistance regulation.

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