Journal of Plant Physiology 229 (2018) 89–99

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Involvement of reactive oxygen species and auxin in serotonin-induced inhibition of primary root elongation

Jinpeng Wan[a,d], Ping Zhang[a,d], Liangliang Sun[a], Shuang Li[a,d], Ruling Wang[a], Huakun Zhou[b], Wenying Wang[c], Jin Xu[a,*]

[a] CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Mengla, Yunnan 666303, China
[b] Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Key Laboratory of Restoration Ecology of Cold Area in Qinghai Province, Xining 810008, China
[c] College of Life Science and Geography, Qinghai Normal University, Xining 810008, China
[d] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Keywords:
Serotonin
auxin
reactive
oxygen
species
primary
root
growth
Arabidopsis thaliana

ABSTRACT

The well-known neurotransmitter 5-hydroxytryptamine (serotonin) not only regulates sleep and mood in humans and animals but may also play important roles in modulating growth, development, and defense responses, such as seed germination, flowering, and abiotic stress tolerance, in plants. Serotonin inhibits primary root (PR) growth; however, the physiological and molecular mechanisms underlying serotonin-mediated PR growth inhibition remain largely unclear. Here, we investigate the effects of serotonin on root growth and development in *Arabidopsis*. Serotonin inhibits PR elongation by affecting both the meristem and elongation zones. In the meristem zone, serotonin represses both meristem cell division potential and stem cell niche activity. Serotonin induces H_2O_2 overaccumulation in the elongation zone and reduces O_2- accumulation in the meristem zone by a *UPB1* pathway, thereby disrupting reactive oxygen species (ROS) equilibrium in root tips, thus resulting in PR growth inhibition. Serotonin also regulates auxin distribution in root tips by decreasing auxin-related gene expression and repressing auxin transport through modulation of AUX1 and PIN2 abundances in root tips. Taken together, our data indicate that high concentrations of serotonin result in stress responses in plants by inhibiting PR elongation through the regulation of H_2O_2 and O_2- distribution in PR tips and through an auxin pathway via the repression of auxin biosynthesis and transport.

1. Introduction

Serotonin (5-hydroxytryptamine) is a well-known neurotransmitter, hormone, and mitogenic factor that mediates a series of physiological activities in humans and animals (Kang et al., 2009; Ramakrishna et al., 2012). Since the first report of the phytoserotonin in cowhage (*Mucuna pruriens*) fruit (Bowden et al., 1954), serotonin has been found in at least 40 plant species (Kang et al., 2009; Pelagio-Flores et al., 2011, 2016). Similar to melatonin, serotonin may play an important role in modulating plant growth, development, morphogenesis, and defense responses (Murch et al., 2009; Kang et al., 2007; Zhang et al., 2013; Pelagio-Flores et al., 2016; Erland et al., 2017). Significant increases in *de novo* shoot formation have been shown to be correlated with increased endogenous serotonin levels (Murch et al., 2009). Both pathogenic infection and senescence induce serotonin accumulation in plants; however, the physiological roles of serotonin in delaying senescence and improving pathogen tolerance are different (Kang et al., 2009). Serotonin was found to accumulate in in senescence-induced tissues of vascular parenchyma cells, and the senescence-retarding activity of serotonin is associated with its high antioxidant activity. Nutrient recycling from senescing leaves to sink tissues was maintained during senescence, while pathogenic infection-induced serotonin accumulation was found to function in the strengthening of the cell wall (Kang et al., 2009).

A sequence of two enzymatic reactions regulates serotonin biosynthesis in plants. The first reaction is the catalytic turnover of tryptophan to tryptamine by tryptophan decarboxylase (TDC), and the second reaction catalytically converts tryptamine into serotonin by tryptamine 5-hydroxylase (T5H). The *T5H* gene is constitutively expressed, whereas *TDC* gene expression is significantly induced by senescence signals; as a result, *TDC* is the bottleneck for serotonin biosynthesis in rice (Kang et al., 2007). The serotonin level is very low in

* Corresponding author.
E-mail address: xujin@xtbg.ac.cn (J. Xu).

https://doi.org/10.1016/j.jplph.2018.07.004
Received 12 April 2018; Received in revised form 16 July 2018; Accepted 16 July 2018
Available online 18 July 2018

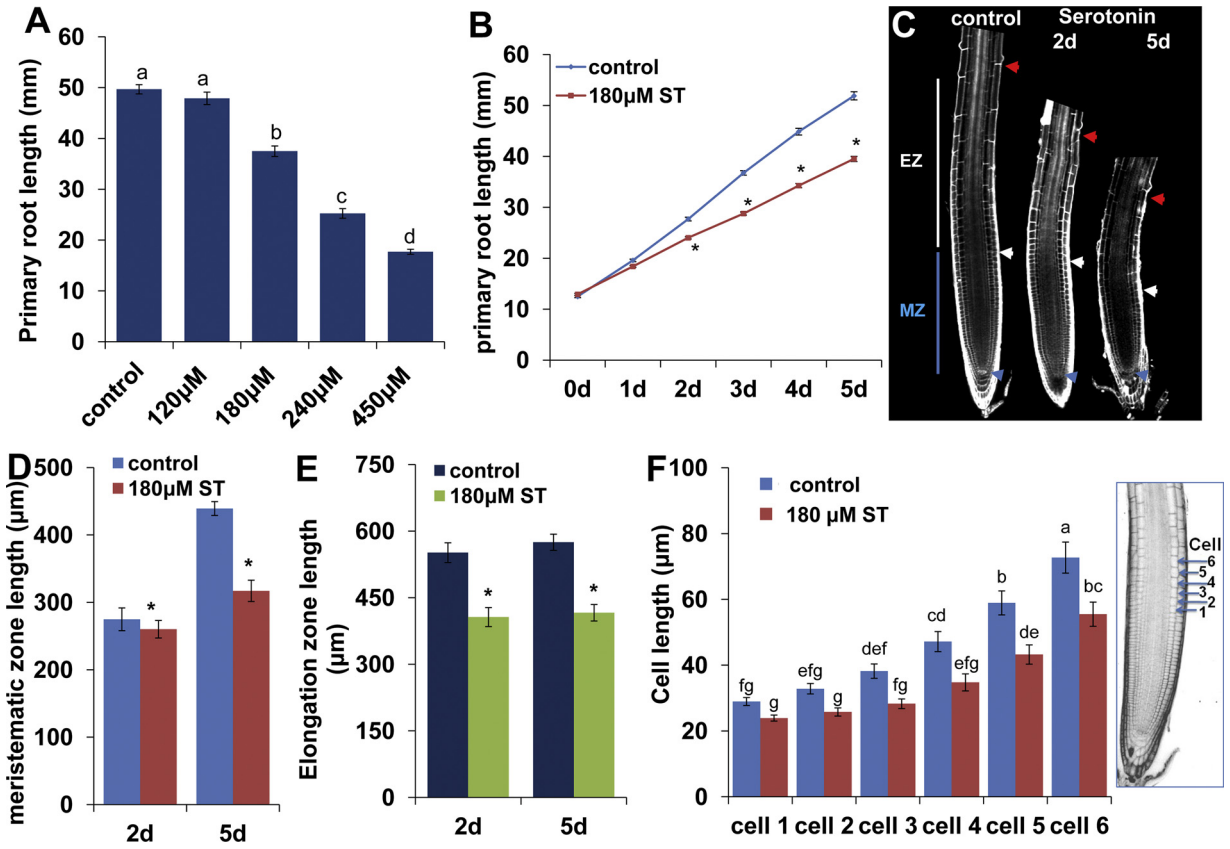

Fig. 1. Serotonin inhibits PR growth by reducing the length of the meristem and elongation zones. (A) Five-day-old seedlings were transferred to 1/4 MS medium containing 0-450 μM serotonin for 5 d. The PR elongation was determined. (B-F) Five-day-old seedlings were transferred to 1/4 MS medium containing 180 μM serotonin for 1-5 d. (B) The length of PRs, (C, D) the length of the meristem zone, (E) the length of the elongation zone, and (F) the cell length of six consecutive cells in the transition zone were determined. ST, serotonin. Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$). Different letters indicate that values were significantly different at $P < 0.05$ according to Tukey's test.

young leaves and seeds; however, nutrient deficiency, leaf detachment, and senescence markedly induce the accumulation of serotonin in plants. Overexpression of two rice *TDC* genes, *OsTDC-1* and *OsTDC-3*, resulted in increased serotonin levels (peaking at approximately 3,500 μg g^{-1} FW) and repressed growth and fertility in transgenic rice and *Arabidopsis* plants (Kanjanaphachoat et al. 2012). However, the physiology and molecular mechanisms underlying serotonin-mediated growth inhibition in plants remain largely unclear. Although *Arabidopsis* has no known functional *TDC* and *T5H* ortholog, many studies have indicated that *Arabidopsis* exhibits T5H activity that produces serotonin to regulate plant growth (Kanjanaphachoat et al. 2012).

Primary root (PR) growth is tightly regulated by the differential accumulation of reactive oxygen species (ROS) in root tips (Tsukagoshi et al., 2010; Silva-Navas et al., 2016). The *UPBEAT1* (*UPB1*) transcription factor regulates the distribution of H_2O_2 and O_2- in elongation and meristem zones of roots by repressing the gene expression of peroxidases in roots independent of the auxin pathway (Tsukagoshi et al., 2010). Recently, Pelagio-Flores et al. (2016) found that serotonin regulates ROS distribution in roots by jasmonic acid/ethylene signaling pathways. However, how serotonin induces ROS accumulation and the elevated ROS levels regulate PR growth in serotonin-treated seedlings requires further investigation.

In addition to the ROS pathway, auxin plays a vital role in modulating root system development (Mähönen et al., 2014; Silva-Navas et al., 2016). Maintaining a steep auxin gradient in the meristem zone and maximal auxin accumulation in the quiescent center (QC) is critical for normal meristem cell activity and root growth (Liu et al., 2016). *PLETHORA* (*PLT*) controls meristem cell activity and root development in a dose-dependent manner (Sabatini et al., 1999; Liu et al., 2016; Silva-Navas et al., 2016). Auxin modulates root stem cell niche activity by affecting PLTs accumulation in root tips (Aida et al., 2004; Mähönen et al., 2014; Silva-Navas et al., 2016).

Plasma membrane-localized auxin carriers, including the auxin influx carriers AUXIN1/LIKE AUX1 (AUX1/LAX) and auxin efflux carriers PIN-FORMEDs (PINs), also play important roles in establishing and maintaining auxin gradients in root tips (De Smet et al., 2007). Different auxin carriers can regulate a common physiological process (Liu et al., 2016). For example, PIN4 is required for root exudate methyl 3-(4-hydroxyphenyl) propionate (MHPP)-mediated root system architecture (RSA) remodeling (Liu et al., 2016). AUX1 and PIN2 are involved in alkaline stress adaptation (Li et al., 2015). Serotonin, derived from the common substrate L-tryptophan with auxin, may act as a competitive inhibitor of auxin-regulated gene expression (Pelagio-Flores et al., 2011). However, the exact role for serotonin-mediated root development by the auxin pathway remains to be elucidated.

In this study, we investigated the involvement of ROS and auxin in serotonin-regulated root system development in *Arabidopsis*. Our results indicate that serotonin disrupts ROS distribution in root tips; serotonin also affects auxin distribution in root tips by decreasing auxin biosynthesis and repressing AUX1 and PIN2 abundances. Potential mechanisms involved in this process are discussed.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of the wild-type *col-0* and transgenic and mutant *Arabidopsis* lines were sterilized with 50 % bleach for 5 min, washed five times with

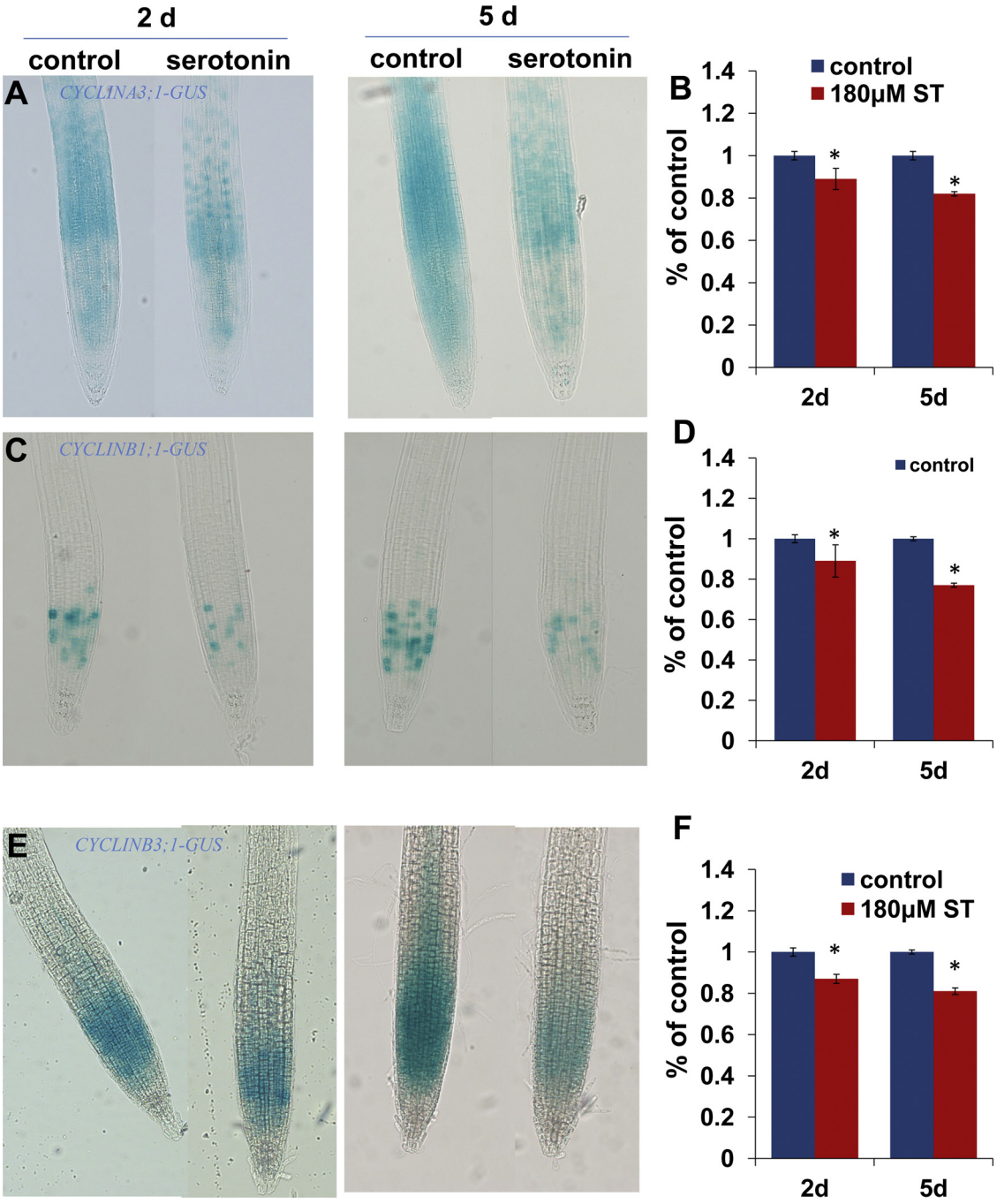

Fig. 2. Serotonin affects cell cycle progression. Images of GUS staining of five-day-old *CYCLINA3;1-GUS* (A), *CYCLINB1;1-GUS* (C), and *CYCLINB3;1-GUS* (E) seedlings exposed to 180 μM serotonin for 2 and 5 d and the relative GUS activity of *CYCLINA3;1-GUS* (B), *CYCLINB1;1-GUS* (D), and *CYCLINB3;1-GUS* (F) seedlings treated as in (A, C, and E). The level of GUS activity in untreated roots was set to 1. ST, serotonin. Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$).

sterile water, and then germinated on vertical 1/4 MS medium (Sigma-Aldrich, St. Louis, MO, USA) containing 1.5 % sucrose and 1 % agar (pH 5.7) with a 16-h light/8-h dark cycle. Five-day-old seedlings were transferred to fresh 1/4 MS medium supplemented with various components and subsequently grown for two to five days. The chemicals 5-hydroxytryptamine (serotonin, 0-450 μM) and catalase (CAT, 1-2 μM) were purchased from TCI (Tokyo, Japan). After 2 or 5 d of treatment, images of them were digitized using a scanner (Epson Perfection V500 Photo, Japan), and the PR growth, length of their meristem and elongation zones, and cell length of their elongation zone were measured using ImageJ software (version 1.51j8).

2.2. GUS staining

The treated seedlings of *pCYCLIN A3;1:CYCLIN A3;1-GUS*, *pCYCLINB1;1:CYCLINB1;1-GUS*, and *pCYCLINB3;1:CYCLINB3;1-GUS* were incubated in GUS buffer containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid cyclohexyl-ammonium, Sigma-Aldrich) at 37 °C for 1-5 h in the dark. At least 30 seedlings were used in each experiment. After the samples were washed, photos were taken using a Carl Zeiss imaging system.

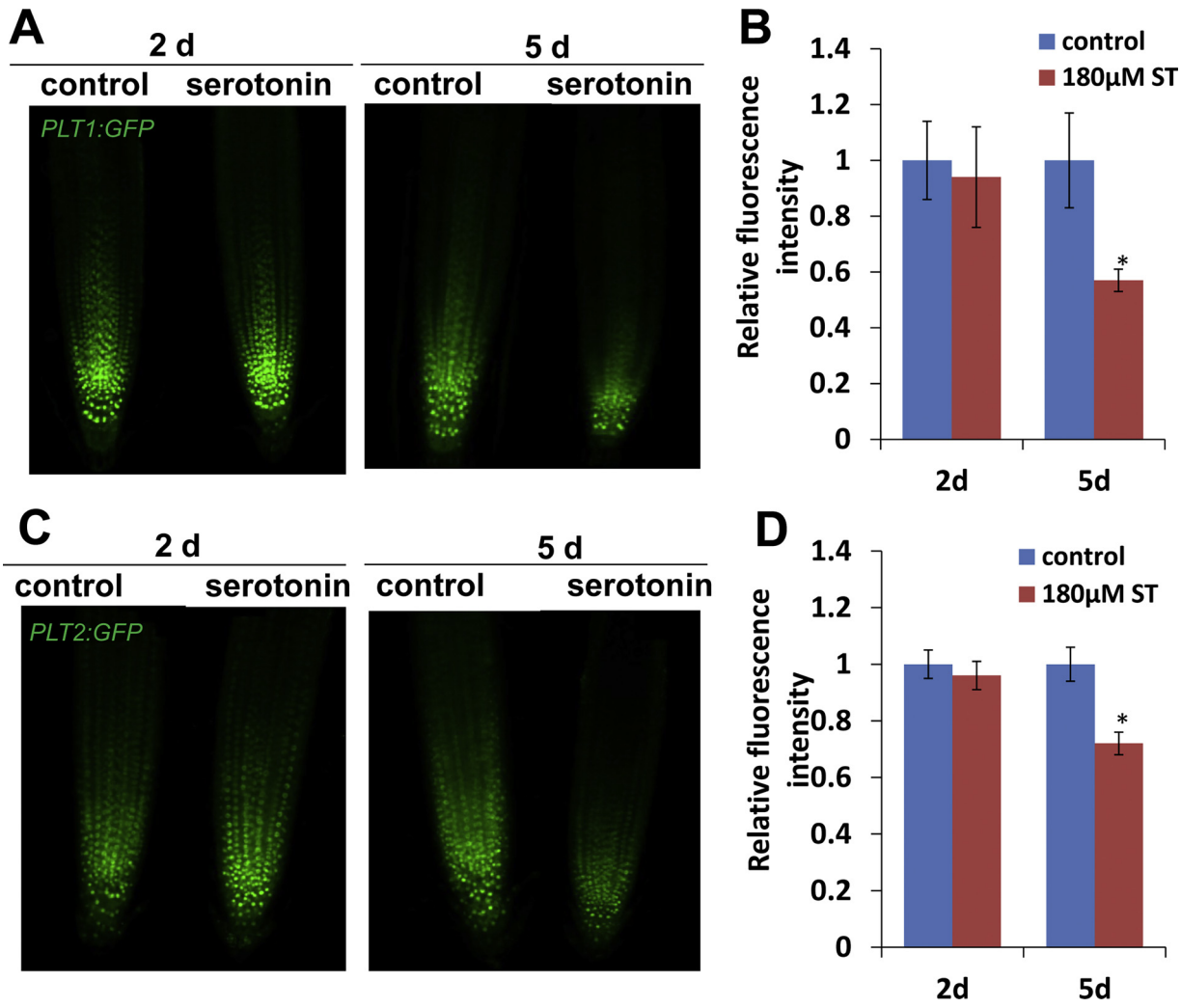

Fig. 3. Serotonin affects stem cell niche activity. (A-D) GFP fluorescence in the roots of five-day-old *pPLT1:PLT1-GFP* (A) and *pPLT2:PLT2-GFP* (C) exposed to 180 μM serotonin for 2 and 5 d and quantification of the *pPLT1:PLT1-GFP* (B) and *pPLT2:PLT2-GFP* (D) fluorescence intensity in plants treated as in (A and C). Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$). ST, serotonin.

2.3. Measurement of ROS levels

Endogenous H_2O_2 and O_2- production in the root tips was detected using the H_2O_2-specific fluorescence probe BES-H_2O_2-AC (WAKO, Japan) and O_2–specific fluorescence probe dihydroethidium (DHE) according to the manufacturer's instructions. Fluorescence was observed using a confocal laser scanning microscope (Zeiss, excitation wavelength of 488 nm and emission wavelength of 520 nm for H_2O_2, excitation wavelength of 514 nm and emission wavelength of 610 nm for O_2-). For localizing H_2O_2 production, roots were immersed in 1 mg ml^{-1} 3-diaminobenzidine (DAB)-HCl (pH 5.5) for 5 h. For localizing O_2- production, the roots were immersed in a solution of 2 mM nitroblue tetrazolium (NBT) in 20 mM phosphate buffer (pH 6.1) for 20 min. After the samples were washed, photos were taken using a Carl Zeiss imaging system. Fresh roots (0.5 g) were ground in potassium phosphate buffer (50 mM, pH 7.0), and the H_2O_2 content was then measured according to the method of Maksymiec and Krupa (2006). The absorbance was recorded at 600 nm. The H_2O_2content was calculated with an absorbance coefficient of 19.8×10^3 M^{-1} cm^{-1}.

2.4. qRT-PCR analysis

After the samples were treated, total RNAs were isolated using an RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). Quantitative reverse transcription (qRT)-PCRs were performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). *ACTIN2* (AT3G18780) was used as an internal control for qRT-PCR normalization (Czechowski et al., 2005; Liu et al., 2016). The specific primers that were used are shown in Supplemental Table S1. All primer pairs exhibited only one peak in DNA melting curves. qRT-PCR analysis was performed on three biological replicates with three technical repetitions.

2.5. Statistical Analysis

At least 30 roots were analyzed in each treatment, and each experiment was repeated at least three times. The data are presented as the means ± SE. For statistical analysis, Student's *t* test,one-way ANOVA and Tukey's test were used to determined statistical significance($P < 0.05$).

3. Results

3.1. Effects of serotonin on root growth and development

Although *Arabidopsis* has no known functional *TDC* and *T5H* ortholog, the overexpression of rice *OsTDC-1* and *OsTDC-3* led to marked

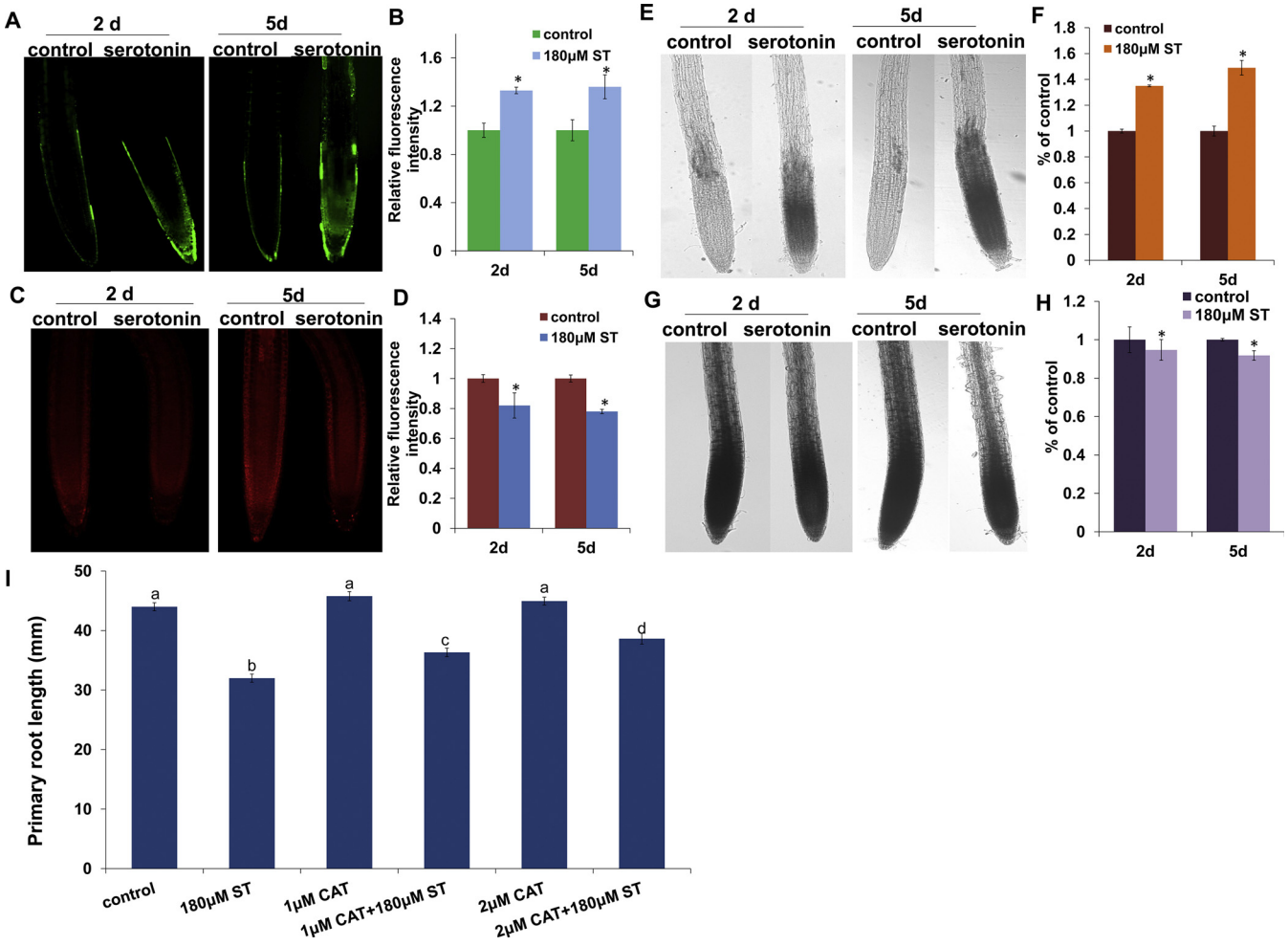

Fig. 4. Serotonin induces ROS imbalance in root tips. (A-D) Detection of endogenous H_2O_2 (A) and O_2- (C) production in the roots of five-day-old wild-type seedlings exposed to 180 μM serotonin for 2 and 5 d using the H_2O_2-specific fluorescence probe BES-H_2O_2-AC and the O_2–specific fluorescence probe DHE, respectively, and quantification of BES-H_2O_2-AC fluorescence intensity (B) and DHE fluorescence intensity (D) in plants treated as in (A and C). (E, F) Image of DAB staining of five-day-old seedlings exposed to 180 μM serotonin for 2 and 5 d (E) and the relative staining intensity of DAB (F) in plants treated as in (E). (G, H) Image of NBT staining of five-day-old seedlings exposed to 180 μM serotonin for 2 and 5 d (G) and the relative staining intensity of NBT (H) in plants treated as in (G). (I) Primary root length of *col-0* seedlings treated with or without 180 μM serotonin in the presence or absence of CAT (1 or 2 μM) for 2 d. ST, serotonin. Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$). Different letters indicate that values were significantly different at $P < 0.05$ according to Tukey's test.

increases in serotonin levels in *Arabidopsis* seedlings (Kanjanaphachoat et al., 2012). Unfortunately, the growth of these transgenic seedlings was severely inhibited and they could not form the siliques and seeds. Therefore, we analyzed the effects of serotonin on root growth and development by using exogenous serotonin.

Five-day-old *Col-0* seedlings were transferred into fresh 1/4 MS medium containing 0, 120, 180, 240, or 450 μM serotonin. After 5 d of treatment, the PR length were measured. The PR growth was unaffected in 120 μM serotonin but was inhibited by 24.5 % in 180 μM serotonin, 49.2 % in 240 μM serotonin, and 64.3 % in 450 μM serotonin (Fig. 1A), implying that high concentrations of serotonin result in stress response in seedlings. To further examine serotonin-inhibited PR growth in detail, we measured the length of the meristem and elongation zones in 180 μM serotonin-treated roots (Fig. 1B-1E). With the extension of treatment time, both PR length and meristem zones were shorter in serotonin-treated seedlings than in the control. We also analyzed the cell length in the transition zone and found that the cells in the transition zone of 180 μM serotonin-treated roots elongated more slowly than those in the transition zone of control roots after 5 d of treatment (Fig. 1F).

3.2. Serotonin represses meristem size by decreasing root meristematic cell division potential and stem cell niche activity

We next analyzed whether the serotonin-repressed meristem sizes are due to decreased meristematic cell division potential or stem cell niche activity in roots. For this purpose, we used the *pCYCLINA3;1:CYCLINA3;1-GUS*, *pCYCLINB1;1:CYCLINB1;1-GUS*, and *pCYCLINB1;1:CYCLINB3;1-GUS* transgenic lines to monitor the cell cycle (Colón-Carmona et al., 1999) and the *pPLT1:PLT1-GFP* and *pPLT2:PLT2-GFP* transgenic lines to monitor the stem cell niche activity (Sabatini et al. 2003) in roots. Our results showed that GUS staining of all three cell cycle marker lines was significantly reduced in the roots of seedlings that were treated with 180 μM serotonin for 2 or 5 d (Fig. 2A-2 F). These results indicated that serotonin inhibits PR growth by repressing the meristematic cell division potential. The *PLT* pathway controls meristem homeostasis in roots (Sabatini et al., 1999). The expression abundances of both PLT1 and PLT2 proteins in the roots of seedlings that were treated with 180 μM serotonin for 2 d were the same as those in control roots; however, they were significantly lower in the roots of seedlings that had been treated with 180 μM serotonin for 5 d, as indicated by pPLT1:PLT1-GFP and pPLT2:PLT2-GFP fluorescence

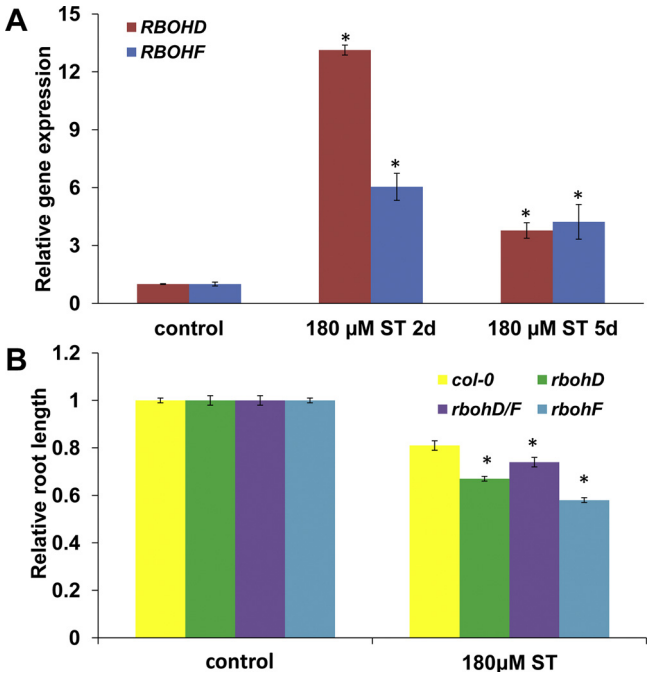

Fig. 5. *RBOHD* and *RBOHF* are involved in serotonin-induced ROS accumulation in roots. (A) qRT-PCR analysis of *RBOHD* and *RBOHF* gene expression in *col-0* seedlings treated with 180 μM serotonin for 2 and 5 d. The expression levels of the indicated genes in untreated roots were set to 1. (B) The relative root growth of *col-0*, *rbohD*, *rbohF*, and *rbohD/F* seedlings treated with 180 μM serotonin for 5 d relative to that of untreated seedlings. ST, serotonin. Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$).

(Fig. 3A-3D).

3.3. Serotonin represses PR growth by modulating ROS accumulation in root tips

ROS play important roles in modulating root system development (Xu et al., 2010; Tsukagoshi et al., 2010). Pelagio-Flores et al. (2016) found that high concentrations of serotonin induced ROS over-accumulation in roots, thereby inhibiting PR growth. To further study the function of ROS in serotonin-mediated root growth, we first examined ROS levels in serotonin-treated roots. We analyzed the accumulation of O_2- and H_2O_2 in root tips using the O_2–specific fluorescence probe dihydroethidium (DHE) and the H_2O_2-specific fluorescence probe BES-H_2O_2-AC. Interestingly, we found that serotonin markedly induced H_2O_2 accumulation in both the meristem and elongation zones (Fig. 4A and 4B), whereas it reduced O_2- accumulation in the root meristem zone (Fig. 4C and 4D). Similarly, DAB staining also indicated that serotonin induced H_2O_2 accumulation in the roots of seedlings (Fig. 4E and 4 F), while NBT staining indicated that serotonin reduced O_2- accumulation in root tips (Fig. 4G and 4 H). Quantification of H_2O_2 content in roots further confirmed that serotonin markedly induced H_2O_2 accumulation (Fig. S1). We then examined the effects of serotonin-induced H_2O_2 accumulation on PR growth by applying the H_2O_2 scavenger catalase (CAT) (Chen and Schopfer, 1999). Supplementation with CAT alleviated serotonin-induced PR growth inhibition (Fig. 4I).

To further investigate the source of ROS in root tips, we also investigated the gene expression of *RBOHD* and *RBOHF*, the two NADPH oxidase subunits that are expressed throughout the plant for O_2- and ROS accumulation (Sagi and Fluhr, 2006), using qRT-PCR. Supplementation with serotonin markedly induced the expression of *RBOHD* and *RBOHF* genes (Fig. 5A). To test the biological functions of the two NADPH oxidase subunits, we examined the *rbohD*, *rbohF*, and *rbohD/F* mutants (Torres et al., 2002). All three mutants were inhibited more by exogenous serotonin than wild type (Fig. 5B). The result is discussed below.

3.4. UPB1 is involved in serotonin-mediated ROS accumulation in root tips

The above results showed that serotonin increases H_2O_2 accumulation, whereas it reduces O_2- accumulation in root tips. Tsukagoshi et al. (2010) found that *UPBEAT1* (*UPB1*) regulates the differential distribution of O_2- and H_2O_2 in root tips to control the transition from proliferation to differentiation by directly repressing a set of peroxidases and thereby affecting root elongation. We thus wondered whether *UPB1* is involved in serotonin-mediated ROS accumulation in roots. For this purpose, we used *pUPB1:GFP* transgenic line (Tsukagoshi et al., 2010) to examine the effect of serotonin on *UPB1* expression in root tips. GFP fluorescence showed that exposing roots to serotonin markedly improved the expression levels of pUPB1:GFP (Fig. 6A and 6B). We then investigated the roles of *UPB1* in the serotonin-induced inhibition of PR growth using a *upb1-1* (SALK_115536) mutant (Tsukagoshi et al., 2010) and found that the PR growth in the presence of serotonin is less inhibited in the *upb1-1* mutant than the wild type (Fig. 6C). These data indicate that *UPB1* is involved in serotonin-mediated PR growth inhibition.

UPB1 inhibits POD activity and thereby induces H_2O_2 accumulation in root tips (Tsukagoshi et al., 2010). We thus examined the POD activity in serotonin-treated roots and found that exogenous serotonin inhibited POD activity (Fig. 6D). Taken together, these results indicate that serotonin modulates ROS distribution in root tips through the UPB1 pathway.

3.5. Auxin is involved in serotonin-induced PR growth inhibition

Auxin is another important regulator of root system development (Liu et al., 2016). A previous study has indicated that serotonin reduces auxin accumulation in root tips, as indicated by DR5:GUS and BA3:GUS expression, but it does not affect auxin perception in roots, as indicated by HS:AXR3NT-GUS expression (Pelagio-Flores et al., 2011). However, how serotonin reduces auxin accumulation in roots remains unclear. To address this question, we first examined auxin accumulation in root tips using the auxin-sensitive *Aux/IAA-auxin interaction domain II* (*DII-VENUS*) (Brunoud et al., 2012) and *DR5:GFP* transgenic marker lines. Treatment with 180 μM serotonin reduced auxin accumulation in root tips as indicated by DII-VENUS and DR5:GFP fluorescence (Fig. 7A and 7B). Then, we determined the transcript levels of auxin-related genes in serotonin-treated seedlings using qRT-PCR. We found that serotonin significantly decreased the transcript levels of many indole-3-acetic acid (IAA)-related genes, including those of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) (Stepanova et al., 2008), *ABSCISIC ALDEHYDE OXIDASE3* (*AAO1*), *AAO3* (Seo et al., 1998), *ATP SULFURYLASE ARABIDOPSIS1* (*ASA1*) (Logan et al., 1996), *AMIDASE-LIKE PROTEIN 1* (*AMI1*) (Pollmann et al., 2003), *SUPERROOT 1* (*SUR1*) (Boerjan et al., 1995), *PHOSPHORIBOSYLANTHRANILATE TRANSFERASE 1* (*PAT1*) (Rose and Last, 1997), *YUCCA2* (*YUC2*), *YUC3*, and *YUC9* (Cheng et al., 2006), in *Arabidopsis* seedlings (Fig. 7C). These results suggest that serotonin depresses the expression of auxin biosynthesis-related genes, which influences auxin content in seedlings.

We next tested whether the reduced auxin accumulation is responsible for serotonin-induced PR growth inhibition. Five-day-old seedlings were transferred to 1/4 MS medium containing 180 μM serotonin and 0.5 or 1 nM IAA, and the PR length and LR number were measured 2 d after treatment. Treatment with 0.5 or 1 nM IAA alone did not affect either PR growth or LR number; however, supplementation with 0.5 or 1 nM IAA markedly alleviated serotonin-induced PR growth inhibition and further promoted LR formation (Fig. 8A and 8B). These results indicate that the reduced auxin accumulation in root tips is responsible for reduced PR growth.

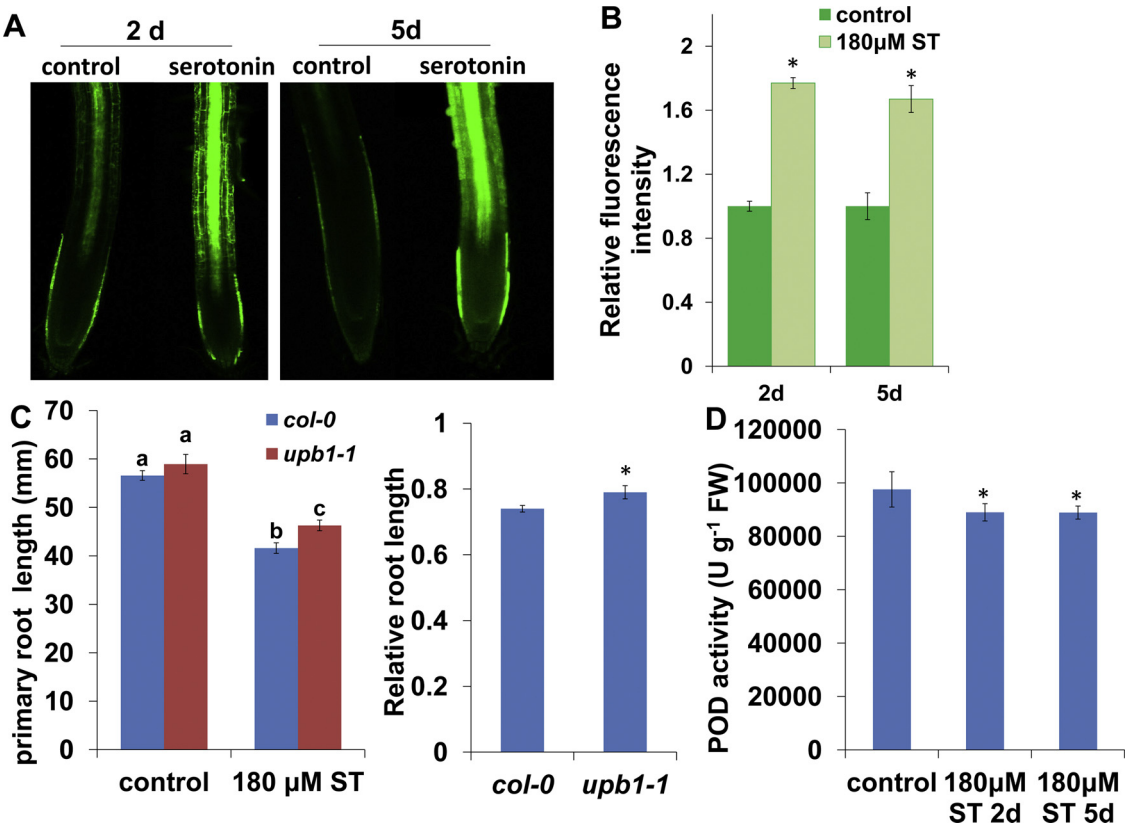

Fig. 6. *UPB1* is involved in serotonin-mediated PR growth. (A, B) Serotonin induces the expression of *pUPB1:GFP*. GFP fluorescence in the roots of 5-d-old *pUPB1:GFP* (A) exposed to 180 μM serotonin for 2 and 5 d and quantification of *pUPB1:GFP* fluorescence intensity (B) in plants treated as in (A). (C) PR length and the relative root growth of *col-0* and *upb1* seedlings treated with 180 μM serotonin for 5 d compared with that of untreated seedlings. (D) The peroxidase (POD) activity in *col-0* seedlings treated with 180 μM serotonin for 2 and 5 d. ST, serotonin. Error bars represent the SE. $^{*}P < 0.01$. Different letters indicate that values were significantly different at $P < 0.01$ according to Tukey's test.

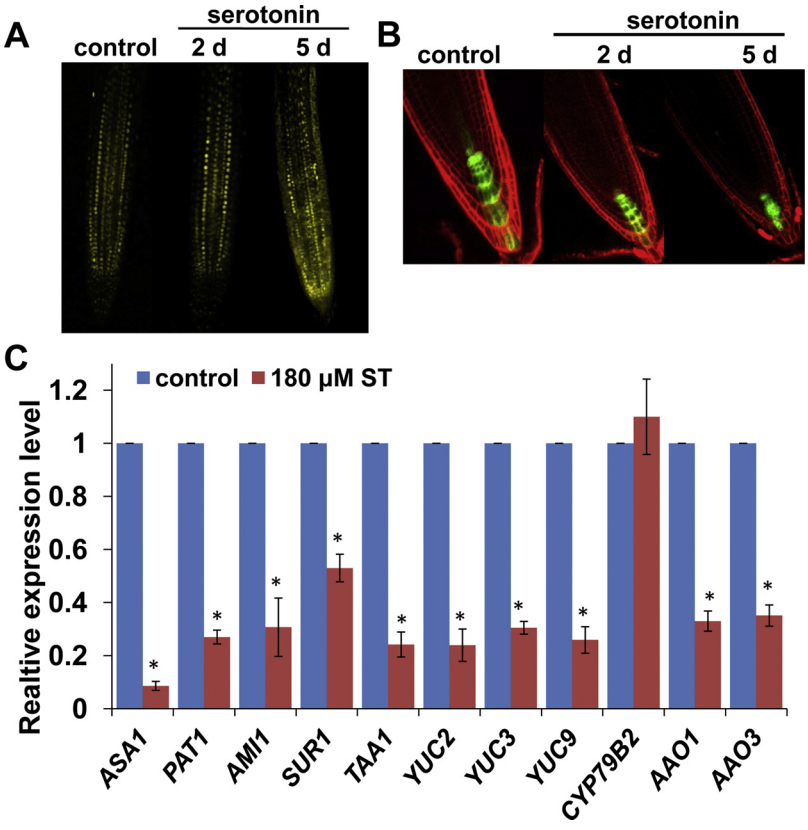

Fig. 7. Serotonin reduces auxin accumulation in root tips. (A, B) YFP/GFP fluorescence in the roots of five-day-old *DII-VENUS* (A) and DR5:GFP (B) seedlings exposed to 180 μM serotonin for 2 and 5 d. (C) qRT-PCR analysis of auxin biosynthesis-related gene expression in *col-0* seedlings treated with 180 μM serotonin for 2 d. The expression levels of the indicated genes in untreated roots were set to 1. ST, serotonin. Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$).

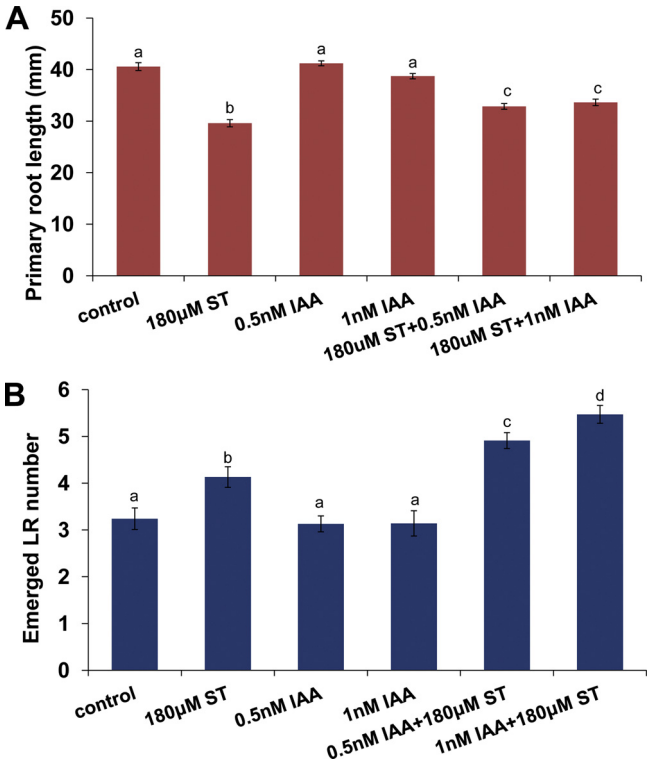

Fig. 8. Auxin is involved in serotonin-mediated PR growth inhibition. (A) The PR length and (B) LR number of *col-0* seedlings treated with or without 180 μM serotonin in the presence or absence of IAA (0.5 or 1 nM) for 5 d. ST, serotonin. Error bars represent the SE. Different letters indicate that values were significantly different at $P < 0.05$ according to Tukey's test.

3.6. Serotonin affects auxin transport by regulating AUX1 and PIN2 levels in root tips

The above results showed that serotonin reduces auxin accumulation in root tips. Auxin distribution is mediated by the auxin influx carriers AUX/LAX and the PIN2 auxin efflux carrier proteins (Péret et al., 2012). We thus investigated the effects of serotonin on the abundance of auxin carriers using *AUX1:YFP* and *PIN1/2/4/7:GFP* transgenic marker lines. The expression of AUX1 (Fig. 9A and 9B) and PIN2 (Fig. 9C and 9D) was markedly reduced after 5 d of serotonin treatment; however, the abundances of PIN1, PIN4, and PIN7 were unaffected (Fig. S2).

We then investigated the roles of AUX1 and PIN2 in serotonin-induced PR growth inhibition using *aux1-21* and *pin2* mutants. PR growth was more inhibited in the *aux1-21* mutant (Fig. 10A and 10B) and less inhibited in the *pin2* mutant (Fig. 10C and 10D) than in the controls. The result is discussed below.

4. Discussion

Pelagio-Flores et al. (2011, 2016) found that exogenous serotonin inhibits PR growth and induces LR formation by reducing auxin accumulation in root tips and inducing ROS overaccumulation in roots. These studies indicate that serotonin regulates root morphogenesis and growth by auxin-dependent and auxin-independent mechanisms (Murch et al., 2009; Pelagio-Flores et al., 2011, 2016). However, how serotonin modulates the accumulation of auxin and ROS in root tips is still largely unclear. In this study, we found that low concentrations of serotonin have no effect on the PR growth, whereas high concentrations of serotonin result in PR growth inhibition by reducing the length of the meristem and elongation zones, suggesting that high concentrations of serotonin result in stress responses in plants. Further study indicates that high concentrations of serotonin inhibit PR elongation by modulating the differential distribution of O_2- and H_2O_2 via the *UPB1* pathway, repressing auxin biosynthesis and affecting auxin transport via modulation of AUX1 and PIN2 abundances in root tips.

4.1. UPB1-mediated H_2O_2 accumulation in root tips is responsible for serotonin-induced PR growth inhibition

ROS are important signal molecules that modulate plant growth and development (Liu et al., 2016). However, high levels of ROS inhibit root growth. Serotonin induces H_2O_2 overaccumulation in root tips, thereby inhibiting PR growth. Pharmacological analysis provided further evidence for the involvement of H_2O_2 in serotonin-mediated root growth. Supplementation with H_2O_2 scavenger CAT partially recovered PR growth from serotonin-induced inhibition, indicating that H_2O_2 overaccumulation is involved in serotonin-induced PR growth inhibition.

H_2O_2 induces quiescence in mouse cells, whereas O_2- regulates proliferation (Sarsour et al., 2008). Similarly, Tsukagoshi et al. (2010) found that ROS levels control the transition from proliferation to differentiation in root tips. The authors proposed that O_2- accumulation in the meristematic zone is required for maintaining meristem zone activity, whereas H_2O_2 accumulation in the elongation zone induces differentiation. *UPB1* regulates the differential distribution of O_2- and H_2O_2 in roots by repressing the expression of peroxidase genes. Interestingly, we found that serotonin reduces O_2- accumulation in the meristematic zone but increases H_2O_2 accumulation in both the meristem and elongation zones. These results imply that *UPB1* is involved in serotonin-mediated root growth. Indeed, we found that serotonin markedly induced *UPB1* expression in root tips. The loss-of-function *upb1-1* mutant showed less PR growth inhibition than the *Col-0* seedlings. These data indicate serotonin modulates root growth by regulating the accumulation of O_2- and H_2O_2 by the *UPB1* pathway.

In this study, we found that exogenous serotonin significantly induced the gene expression of *RBOHD* and *RBOHF* in roots, thereby increasing ROS accumulation. The PR growth of loss of function *rbohD*, *rbohF*, and *rbohD/F* mutants was inhibited more than that of *Col-0* plants, implying that reduced O_2- accumulation inhibited PR growth more in serotonin-treated plants than in controls and that maintaining the balance between O_2- and H_2O_2 is important in modulating root growth. These results support Tsukagoshi et al.'s opinion (2010) and indicate that serotonin inhibits PR growth by inducing an ROS imbalance in root tips through the induction of *UPB1* expression. However, how serotonin reduces O_2- accumulation still needs to be elucidated in future studies.

4.2. Decreased expression of auxin biosynthesis-related genes and auxin transport are responsible for serotonin-mediated PR growth inhibition

Pelagio-Flores et al. (2011) found that serotonin can act as a competitive inhibitor of auxin-regulated gene expression; however, it could not affect auxin perception. In this study, we found that serotonin markedly repressed the expression of auxin biosynthesis-related genes and that exogenous IAA alleviated serotonin-mediated PR growth inhibition. These results indicate that the reduced auxin accumulation in root tips that resulted from the decreased expression of auxin biosynthesis-related genes is responsible for reduced PR growth.

Auxin transport plays vital roles in modulating auxin accumulation in root tips. Auxin levels could also affect the abundance of auxin carriers. High concentrations of serotonin reduced auxin accumulation in roots by repressing the expression of auxin biosynthesis-related genes, thereby maybe leading to reduced abundance of auxin carriers in roots. In this study, we indeed found that serotonin also repressed the abundance of AUX1 and PIN2 in root tips. However, we thought that the reduced abundance of AUX1 and PIN2 was a direct effect of serotonin, because only AUX1 and PIN2 abundance were reduced in roots, while other auxin carriers, including PIN1, PIN4, and PIN7, were not

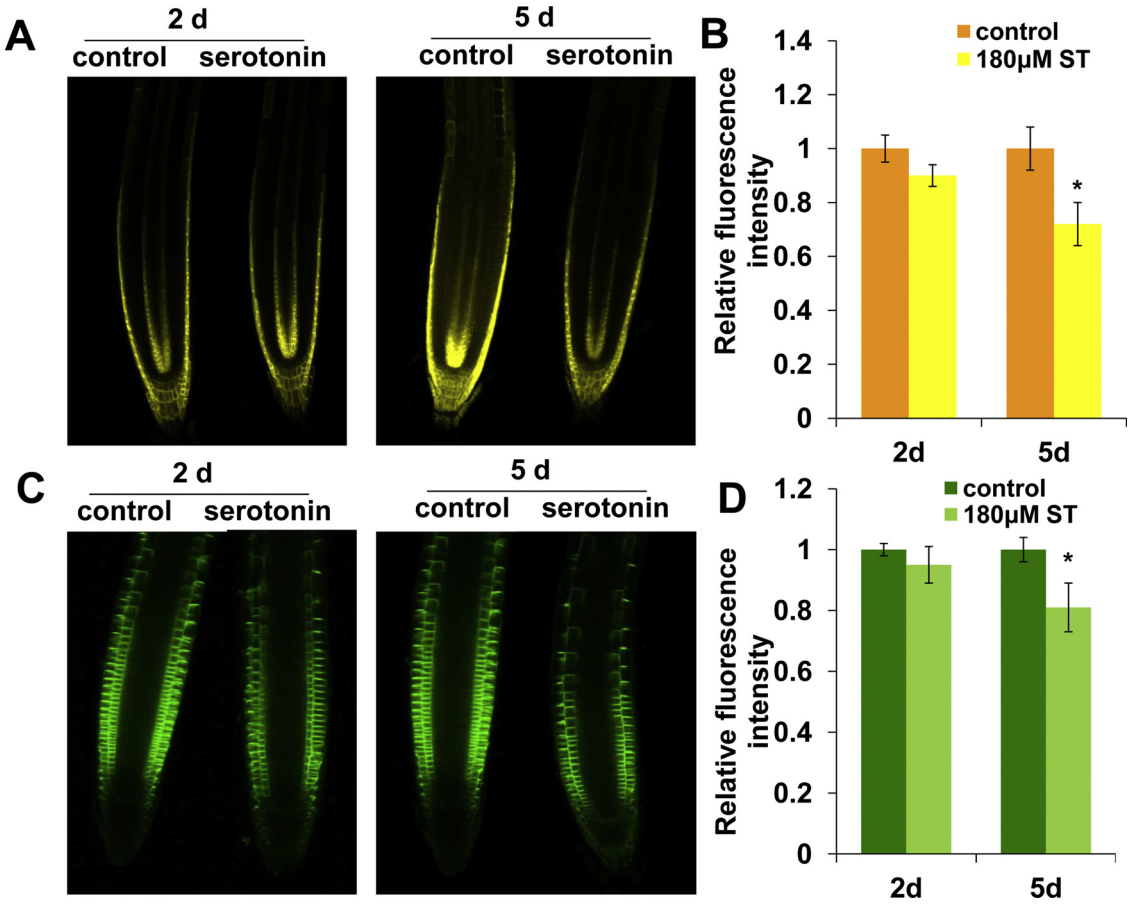

Fig. 9. Serotonin represses the expression abundances of auxin carriers AUX1 and PIN2. (A-D) GFP/YFP fluorescences in the roots of five-day-old *AUX1:YFP* (A) and *PIN2:GFP* (C) seedlings exposed to 180 μM serotonin for 2 and 5 d and quantification of the *AUX1:YFP* (B) and *PIN2:GFP* (D) fluorescence intensity in plants treated as in (A and C). ST, serotonin. Error bars represent the SE. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$).

affected. A genetic analysis found that although serotonin inhibits both AUX1 and PIN2 protein production in root tips, PR growth of the *pin2* mutant showed reduced sensitivity to serotonin and that of the *aux1-21* mutant showed increased sensitivity. These results suggest that PIN2 and AUX1 are at least partly involved in serotonin-mediated auxin distribution to regulate PR growth. Both auxin efflux carrier PIN2 and auxin influx carrier AUX1 regulate auxin levels and gradients in root tips; a *pin2* mutant accumulates more auxin, whereas an *aux1* mutant accumulates less auxin in root tips (Yuan et al., 2013). These results partly explain why *pin2* and *aux1* mutants exhibit contrary phenotypes: serotonin represses AUX1 abundance, thereby leading to reduced auxin accumulation in root tips; meanwhile, serotonin also represses PIN2 abundance, and the reduced PIN2 abundance disrupts auxin transport, thereby resulting in increased auxin accumulation in root tips. Compared with wild-type *col-0* seedlings, the *aux1* mutant therefore showed less PR growth in response to serotonin, whereas the *pin2* mutant showed more PR growth. These results indicate that serotonin-repressed AUX1 abundance resulted in reduced auxin accumulation in root tips, thereby inhibiting PR growth; however, serotonin-repressed PIN2 abundance might be an adaptive response to serotonin for maintaining auxin levels in root tips and PR growth. However, the manner by which AUX1 and PIN2 are involved in serotonin-mediated PR growth inhibition remains to be explored.

4.3. Exogenous serotonin inhibits PR growth by reducing stem cell niche activity in root tips through an auxin-dependent PLT pathway

Maintaining the meristematic cell division potential is an important factor for root meristem size and root growth. Our study showed that serotonin markedly represses root meristematic cell division potential by using three cell-cycle marker lines, *pCYCLINA3;1:CYCLIN A3;1-GUS*, *pCYCLINB1;1:CYCLINB1;1-GUS*, and *pCYCLINB3;1:CYCLINB3;1-GUS* (Colón-Carmona et al., 1999). The *PLT* pathway modulates auxin-dependent maintenance of stem cell niche activity (Sabatini et al., 1999; Sabatini et al., 2003; Aida et al., 2004; Mähönen et al., 2014; Liu et al., 2016; Silva-Navas et al., 2016). Consistent with the reduced auxin accumulation in roots, the abundances of both the PLT1 and PLT2 proteins were reduced in serotonin-treated roots, indicating that serotonin reduces root stem cell niche activity by an auxin-dependent *PLT* pathway.

In conclusion, based on previous studies (Pelagio-Flores et al., 2011, 2016) and our results, high concentrations of serotonin result in stress responses by inhibiting PR growth through regulation of the accumulation and distribution of O_2- and H_2O_2 via the *UPB1* pathway and auxin biosynthesis and transport, consequently reducing root stem cell niche activity and meristematic cell division potential in root tips. Serotonin induces H_2O_2 accumulation in roots and represses O_2- accumulation in the meristematic zone, thereby reducing elongation zone growth and meristem zone activity. Serotonin also reduces auxin accumulation in root tips by decreasing the expression of auxin biosynthesis-related genes and disrupting auxin transport in root tips, thereby reducing PLTs abundances and subsequently reducing stem cell niche activity, finally resulting in PR growth inhibition (Fig. S3). These findings provide new insight into how serotonin regulates root growth through modulation of ROS signaling and auxin signaling.

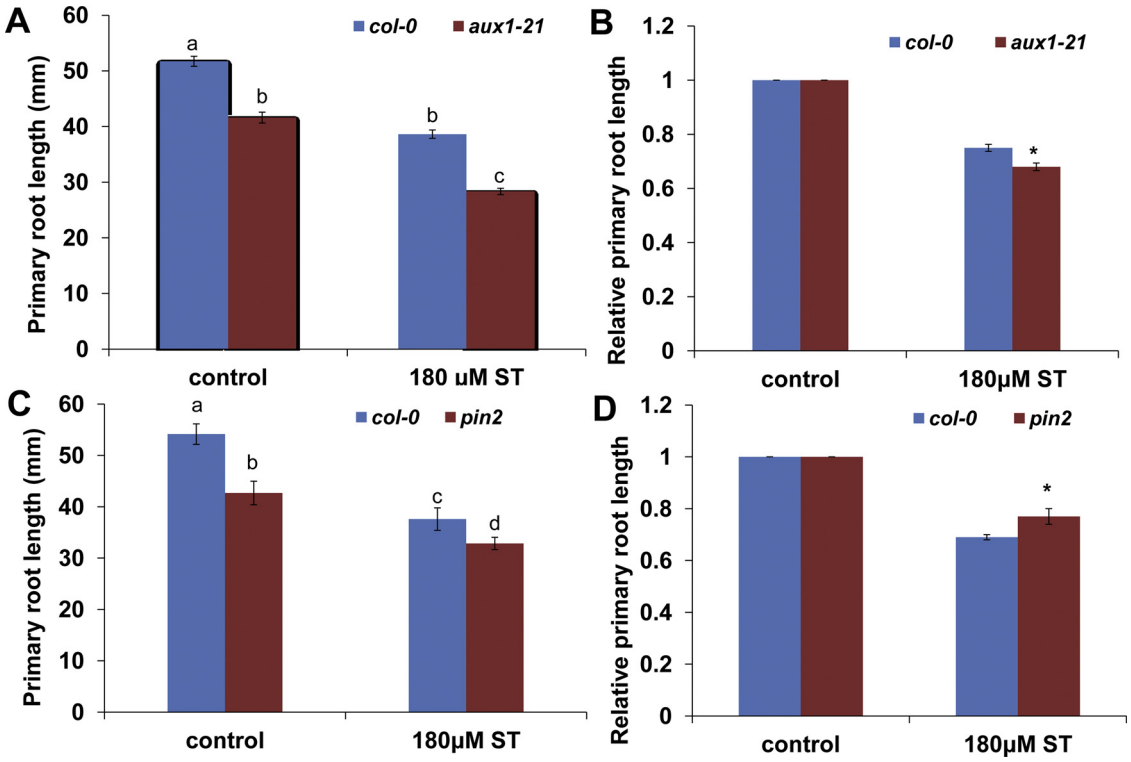

Fig. 10. (A, B) PR growth of *col-0* and *aux1-21* seedlings treated with or without 180 μM serotonin for 5 d (A) and relative root growth of the two genotypes treated with 180 μM serotonin compared with that of untreated seedlings (B). (C, D) PR growth of *col-0* and *pin2* seedlings treated with or without 180 μM serotonin for 5 d (C) and relative root growth of seedlings of the two genotypes treated with 180 μM serotonin relative that of untreated seedlings (D). ST, serotonin. Error bars represent the SE. Different letters indicate that values were significantly different at $P < 0.05$ according to Tukey's test. Asterisks indicate a significant difference from the control (Student's t test, $P < 0.05$).

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Prof. Philip N. Benfey (Duke University) for providing *pUPB1:GFP* and *upb1-1* seeds. The authors gratefully acknowledge the Public Technology Service Center of the Xishuangbanna Tropical Botanical Garden of CAS for providing research facilities. This research was supported by the China National Natural Sciences Foundation (31772383, 31272239), the National Key Research and Development Program of China (2016YFC0501901), Qinghai innovation platform construction project (2017-ZJ-Y20), and the Yunnan Province Foundation for academic leader (2014HB043).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2018.07.004.

References

Aida, M., Beis, D., Heidstra, R., Willemsen, V., Blilou, I., Galinha, C., Nussaume, L., Noh, Y., Amasino, R., Scheres, B., 2004. The PLETHORA genes mediate patterning of the *Arabidopsis* root stem cell niche. Cell 119, 109–120.

Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., ... Inzé, D., 1995. Superroot, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell 7, 1405–1419.

Brunoud, G., Wells, D.M., Oliva, M., Larrieu, A., Mirabet, V., Burrow, A.H., Beeckman, T., Kepinski, S., Traas, J., Bennett, M.J., Vernoux, T., 2012. A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature 482, 103.

Bowden, K., Brown, B.G., Batty, J.E., 1954. 5-hydroxytryptamine: its occurrence in cowhage. Nature 174, 925–926.

Chen, S.X., Schopfer, P., 1999. Hydroxyl-radical production in physiological reactions. Europ. J. Biochem. 260, 726–735.

Cheng, Y., Dai, X., Zhao, Y., 2006. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes Dev. 20, 1790–1799.

Colón-Carmona, A., You, R., Haimovitch-Gal, T., Doerner, P., 1999. Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant J. 20, 503–508.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., Scheible, W.R., 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 139, 5–17.

De Smet, I., Tetsumura, T., De Rybel, B., Frei dit Frey, N., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inzé, D., Bennett, M.J., Beeckman, T., 2007. Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development 134, 681–690.

Erland, L.A., Shukla, M.R., Singh, A.S., Murch, S.J., Saxena, P.K., 2017. Melatonin and Serotonin: Mediators in the Symphony of Plant Morphogenesis. J. Pineal Res. https://doi.org/10.1111/jpi.12452.

Kang, K., Kim, Y.S., Park, S., Back, K., 2009. Senescence-induced serotonin biosynthesis and its role in delaying senescence in rice leaves. Plant Physiol. 150, 1380–1393.

Kang, S., Kang, K., Lee, K., Back, K., 2007. Characterization of rice tryptophan decarboxylases and their direct involvement in serotonin biosynthesis in transgenic rice. Planta 227, 263–272.

Kanjanaphachoat, P., Wei, B.Y., Lo, S.F., Wang, I.W., Wang, C.S., Yu, S.M., Yen, M.L., Chiu, S.H., Lai, C.C., Chen, L.J., 2012. Serotonin accumulation in transgenic rice by over-expressing tryptophan decarboxylase results in a dark brown phenotype and stunted growth. Plant Mol. Biol. 78, 525–543.

Li, J., Xu, H.H., Liu, W.C., Zhang, X.W., Lu, Y.T., 2015. Ethylene Inhibits Root Elongation during Alkaline Stress through AUXIN1 and Associated Changes in Auxin Accumulation. Plant Physiol. 168, 1777–1791.

Liu, Y.Y., Wang, R.L., Zhang, P., Chen, Q., Luo, Q., Zhu, Y.Y., Xu, J., 2016. The nitrification inhibitor methyl 3-(4-hydroxyphenyl) propionate modulates root development by interfering with auxin signaling via the NO/ROS pathway. Plant Physiol. 171, 1686–1703.

Logan, H.M., Cathala, N., Grignon, C., Davidian, J.C., 1996. Cloning of a cDNA Encoded by a Member of the *Arabidopsis thaliana* ATP Sulfurylase Multigene Family EXPRESSION STUDIES IN YEAST AND IN RELATION TO PLANT SULFUR NUTRITION. J. Biol. Chem. 271, 12227–12233.

Mähönen, A.P., Tusscher, K.T., Siligato, R., Smetana, O., Triviño, S.D., Díaztriviño, S., Wachsman, G., Prasad, K., Heidstra, R., Scheres, B., 2014. PLETHORA gradient formation mechanism separates auxin responses. Nature 515, 125–129.

Maksymiec, W., Krupa, Z., 2006. The effects of short-term exposition to Cd, excess Cuions and jasmonate on oxidative stress appearing in Arabidopsis thaliana. Environ. Exp.

Bot. 57, 187–194.
Murch, S.J., Alan, A.R., Cao, J., Saxena, P.K., 2009. Melatonin and serotonin in flowers and fruits of *Datura metel* L. J. Pineal Res. 47, 277–283.
Pelagio-Flores, R., Ortíz-Castro, R., Méndez-Bravo, A., Macías-Rodríguez, L., López-Bucio, J., 2011. Serotonin, a tryptophan-derived signal conserved in plants and animals, regulates root system architecture probably acting as a natural auxin inhibitor in Arabidopsis thaliana. Plant Cell Physiol. 52, 490–508.
Pelagio-Flores, R., Ruiz-Herrera, L.F., López-Bucio, J., 2016. Serotonin modulates Arabidopsis root growth via changes in reactive oxygen species and jasmonic acid–ethylene signaling. Physiol. Plantarum 158, 92–105.
Péret, B., Swarup, K., Ferguson, A., Seth, M., Yang, Y., Dhondt, S., James, N., Casimiro, I., Perry, P., Syed, A., Yang, H., Reemmer, J., Venison, E., Howells, C., Perez-Amador, M.A., Yun, J., Alonso, J., Beemster, G.T., Laplaze, L., Murphy, A., Bennett, M.J., Nielsen, E., Swarup, R., 2012. AUX/LAX genes encode a family of auxin influx transporters that perform distinct functions during Arabidopsis development. Plant Cell 24, 2874–2885.
Pollmann, S., Neu, D., Weiler, E.W., 2003. Molecular cloning and characterization of an amidase from *Arabidopsis thaliana* capable of converting indole-3-acetamide into the plant growth hormone, indole-3-acetic acid. Phytochemistry 62, 293–300.
Ramakrishna, A., Giridhar, P., Sankar, K.U., et al., 2012. Melatonin and serotonin profiles in beans of *Coffea* species. J. Pineal Res. 52, 470–476.
Rose, A.B., Last, R.L., 1997. Introns act post-transcriptionally to increase expression of the *Arabidopsis thaliana* tryptophan pathway gene PAT1. Plant J. 11, 455–464.
Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, B., Weisbeek, P., Scheres, B., 1999. An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell 99, 463–472.
Sabatini, S., Heidstra, R., Wildwater, M., Scheres, B., 2003. SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. Genes Dev. 17, 354–358.
Sagi, M., Fluhr, R., 2006. Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol. 141, 336–340.
Sarsour, E.H., Venkataraman, S., Kalen, A.L., Oberley, L.W., Goswami, P.C., 2008. Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. Aging Cell 7, 405–417.
Seo, M., Akaba, S., Oritani, T., Delarue, M., Bellini, C., Caboche, M., Koshiba, T., 1998. Higher Activity of an Aldehyde Oxidase in the Auxin-Overproducing superroot1 Mutant *of Arabidopsis thaliana*. Plant Physiol. 116, 687–693.
Silva-Navas, J., Moreno-Risueno, M.A., Manzano, C., Téllez-Robledo, B., Navarro-Neila, S., Carrasco, V., Pollmann, S., Gallego, F.J., Del Pozo, J.C., 2016. Flavonols mediate root phototropism and growth through regulation of proliferation-to-differentiation transition. Plant Cell 28, 1372–1387.
Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.Y., Dole_zal, K., Schlereth, A., Jürgens, G., Alonso, J.M., 2008. TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 133, 177–191.
Torres, M.A., Dangl, J.L., Jones, J.D.G., 2002. *Arabidopsis gp91*(*phox*) homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. 99, 517–522.
Tsukagoshi, H., Busch, W., Benfey, P.N., 2010. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 143, 606–616.
Xu, J., Yin, H.X., Li, Y.L., Liu, X.J., 2010. Nitric oxide is associated with long-term zinc tolerance in Solanum nigrum. Plant Physiol. 154, 1319–1334.
Yuan, H.M., Xu, H.H., Liu, W.C., Lu, Y.T., 2013. Copper regulates primary root elongation through PIN1-mediated auxin redistribution. Plant Cell Physiol. 54, 766–778.
Zhang, N., Zhang, H.J., Zhao, B., et al., 2013. The RNA-seq approach to discriminate gene expression profiles in response to melatonin on cucumber lateral root formation. J. Pineal Res. 56, 39–50.