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Serotonin, a Tryptophan-Derived Signal Conserved in Plants and Animals, Regulates Root System Architecture Probably Acting as a Natural Auxin Inhibitor in *Arabidopsis thaliana*

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Regular Paper

Serotonin (5-hydroxytryptamine) is a well-known neurotransmitter in mammals and is widely distributed in plants. This compound is synthesized from tryptophan and shares structural similarity with IAA. To date, little is known about the morphological, physiological and molecular responses of plants to serotonin. In this study, we characterized the effects of serotonin on growth and development in *Arabidopsis thaliana* seedlings. Gas chromatography–mass spectrometry (GC-MS) analysis showed that plants are able to take up serotonin from the growth medium, which coincided with greatly stimulated lateral root development at concentrations from 10 to 160 μM. In contrast, higher doses of serotonin repressed lateral root growth, primary root growth and root hair development, but stimulated adventitious root formation. To investigate the role of serotonin in modulating auxin responses, we performed experiments using transgenic Arabidopsis lines expressing the auxin-responsive marker constructs *DR5:uidA*, *BA3:uidA* and *HS::AXR3NT-GUS*, as well as a variety of Arabidopsis mutants defective at the *AUX1*, *AXR1*, *AXR2* and *AXR4* auxin-related loci. We found that serotonin strongly inhibited both *DR5:uidA* and *BA3:uidA* gene expression in primary and adventitious roots and in lateral root primordia. This compound also abolished the effects of IAA or naphthaleneacetic acid on auxin-regulated developmental and genetic responses, indicating an anti-auxin activity in the plant. Mutant analysis further showed that lateral root induction elicited by serotonin was independent of the *AUX1* and *AXR4* loci but required *AXR1* and *AXR2*. Our results show that serotonin regulates root development probably by acting as a natural auxin inhibitor.

Keywords: Arabidopsis • Auxin signaling • Root architecture • Serotonin.

Abbreviations: ARF, auxin response factor; ER, endoplasmic reticulum; GC-MS, gas chromatography–mass spectrometry; GUS, β-glucuronidase; MS, Murashige and Skoog; NAA, naphthaleneacetic acid; NPA, 1-naphthylphthalamic acid; LR, lateral root; LRP, lateral root primordium; PAT, polar auxin transport; PCIB, *p*-chlorophenoxyisobutyric acid; RNAi, RNA interference; TDC, tryptophan decarboxylase; TIBA, triiodobenzoic acid; WT, wild-type; YFP, yellow fluorescent protein.

Introduction

Plants synthesize and use a variety of signals to adjust growth and development throughout their life cycle. Auxins, including IAA, comprise a group of tryptophan-derived signals, which are involved in most aspects of plant development (Woodward and Bartel 2005). Extensive studies over the past decade have investigated the factors involved in the regulation of plant morphogenesis by auxins. These compounds exert a strong biological activity at very low concentrations in both in vivo and in vitro systems and are essential for maintenance of physiological and morphogenetic processes including gravity and light responses, root hair development, and lateral root (LR), adventitious root and shoot system development (Woodward and Bartel 2005). Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms of auxin, and signal perception through a family of IAA receptors (Ljung et al. 2002, Leyser 2006, Mockaitis and Estelle 2008).

Although IAA is among the most highly characterized metabolites of tryptophan, relatively high levels of IAA-related compounds have been reported in plants such as the mammalian neurotransmitter serotonin (5-hydroxytryptamine). This compound is a ubiquitous signal, which plays multiple roles in neurotransmission, hormone and mitogenic functions as well as acting in immunomodulatory and anti-inflammatory processes in animal cells (Frazer and Hensler 1999). In plants, serotonin has been found in roots, leaves, fruits and seeds from

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at least 42 species (Grose 1982, Engstrom et al. 1992, Roshchina 2001). Serotonin has been implicated in important physiological and developmental functions including flowering, senescence, shoot formation and defense responses (Odjakova and Hadjiivanova 1997, Murch et al. 2001, Roshchina 2001, Ishihara et al. 2008, Kang et al. 2009a).

Serotonin biosynthesis occurs via two enzymatic steps. Tryptophan decarboxylase (TDC) catalyzes the conversion of tryptophan into tryptamine, followed by tryptamine 5-hydroxylase (T5H), which hydroxylates the C-5 position of tryptamine to form serotonin (Kang et al. 2007a, Kang et al. 2007b, Kang et al. 2008, Kang et al, 2009b). In leaves and seeds of rice (*Oryza sativa*) plants, TDC expression is very low or negligible. In leaves, the serotonin concentration was reported to be around 0.3 $\mu g\, g^{-1}$ FW. However, levels greatly increase in response to senescence signals such as nutrient deprivation or leaf detachment, and serotonin synthesis is closely coupled with transcriptional and enzymatic induction of tryptophan biosynthetic genes as well as TDC in senescing leaves (Kang et al. 2009a). Transgenic rice plants overexpressing TDC produced 25-fold higher serotonin levels than wild-type (WT) plants and showed delayed leaf senescence, whereas lines in which expression of TDC was suppressed through an RNA interference (RNAi) system produced less serotonin and senesced faster than the WT line (Kang et al. 2009a). Serotonin accumulation was also reported to occur in rice leaves infected by the fungal pathogen *Bipolaris oryzae* (Ishihara et al. 2008). Serotonin accumulation was preceded by a transient increase in tryptamine content and by marked activation of TDC. Serotonin treatment suppressed the growth of fungal hyphae, indicating that the activation of the tryptophan pathway is involved in the establishment of effective defenses against the pathogen through serotonin production in rice plants. Collectively, this information indicates that serotonin levels in plant tissues may increase by demand, under particular developmental transitions or when challenged by pathogens.

Although serotonin is naturally present in a wide variety of plants, little is known about the molecular mechanisms involved in plant developmental responses to this compound. The Arabidopsis root system is an excellent model to characterize the effects of compounds with novel and interesting activities in plants (López-Bucio et al. 2006, Contreras-Cornejo et al. 2009). Roots perform the essential activities of providing water, nutrients and physical support to the plant. The primary root originates in the embryo and produces many LRs during the lifetime of a plant, and each of these will produce more LRs. The quantity and placement of these structures determine the architecture of the root system, and this in turn plays a major role in determining whether a plant will survive in a particular climate or environment (Malamy and Benfey 1997b, Casimiro et al. 2003, López-Bucio et al. 2005). During the post-embryonic development of plants, new axes of growth emerge from shoot tissues through adventitious organogenesis. This is particularly important in crops such as maize, in which adventitious root formation provides a flexible way for plants to alter their form and resource allocation in response to environmental changes or after injury. While LRs typically form from lateral root primordia (LRPs) initiated on the primary root pericycle, adventitious roots form naturally from stem tissue. LR and adventitious root formation is a complex process affected by multiple endogenous factors, including phytohormones such as auxin, and environmental factors such as light and nutrient deprivation (Casimiro et al. 2003, López-Bucio et al. 2003, Péret et al. 2009).

The control of post-embryonic root growth and LR formation is tightly regulated by auxin (IAA). IAA moves throughout the plant in the phloem or by a more controlled polar transport system [polar auxin transport (PAT)]. PAT is a process regulated by AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX) uptake proteins, PIN-FORMED (PIN) efflux carriers and P-GLYCOPROTEIN (MDR/PGP/ABCB) efflux/conditional transporters (Swarup et al. 2004; Mravec et al. 2008). There are several Arabidopsis mutants defective in the production of auxin transport proteins or in the correct location of these proteins that show auxin-related phenotypes, including *aux1-7* and *axr4-1*. The *aux1-7* mutant is defective at the *AUX1* locus encoding an auxin influx transporter (Swarup et al. 2004), while the *axr4-1* mutant is defective in an accessory protein of the endoplasmic reticulum (ER) that regulates localization of AUX1 proteins. Loss of AXR4 results in abnormal accumulation of AUX1 in the ER of epidermal cells, indicating that the *axr4* agravitropic phenotype is caused by defective AUX1 trafficking in the root epidermis (Dharmasiri et al. 2006).

Auxin is perceived by the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN 1-3 (TIR1/AFB1-3) receptor family. TIR1 is part of the ubiquitin–ligase complex $SCF^{TIR1/AFB}$ that catalyzes the ubiquitination and destruction of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins (Gray et al. 2001, Dharmasiri et al. 2005, Kepinski et al. 2005). These proteins, under low auxin concentrations, form dimers with the auxin response transcription factors (ARFs), thereby blocking their activity. The Arabidopsis *auxin-resistant 1* (*axr1*) mutants were initially isolated in a screen for auxin non-responsive seedlings (Lincoln et al. 1990). Subsequent analysis demonstrated that AXR1 is a subunit in the related to ubiquitin (RUB)-activating enzyme, the first enzyme in the pathway that conjugates the ubiquitin-related protein RUB to members of the ubiquitin protein ligases (del Pozo et al. 2002). Once freed from the AUX/IAAs, ARFs regulate the expression of auxin-responsive genes (Lau et al. 2008, Kieffer et al. 2010). Gain-of-function mutations in *IAA3/SHY2*, *IAA7/AXR2*, *IAA12/BDL*, *IAA14/SLR*, *IAA18/CRANE*, *IAA19/MSG2* and *IAA28* genes lead to plants with altered root development (Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Tatematsu et al. 2004, Uehara et al. 2008). The *slr* and *iaa28* gain-of-function mutants show a strong reduction in LR formation (Rogg et al. 2001, Fukaki et al. 2002). These observations indicate that the AUX/IAA proteins function as negative regulators of LR development, although a detailed direct

comparison of the LR phenotypes of these mutants has not been performed.

Based on its structural similarity to IAA, we hypothesized that serotonin might act through a canonical auxin signaling pathway to modulate developmental responses by either activating or repressing auxin responses. We therefore characterized the effects of serotonin on root system architecture and auxin-mediated responses in *Arabidopsis thaliana*. Interestingly, our results show that the supply of serotonin has a dual effect on LR formation, stimulating it at low (10–160 μM) concentrations, but with repressing effects at higher (150–600 μM) concentrations. Although at high concentrations serotonin also induced adventitious root formation, it repressed typical auxin responses such as primary root growth and root hair formation. Moreover, an analysis of root architecture responses in the *aux1-7*, *axr1-3*, *axr2-1* and *axr4-1* Arabidopsis auxin-related mutants and auxin-inducible gene expression tests revealed that serotonin may act as a natural auxin inhibitor in plants.

Results

Serotonin affects *Arabidopsis thaliana* root system architecture

Serotonin and IAA are tryptophan-derived compounds, with similar chemical structures (**Fig. 1**). To evaluate the effects of serotonin on plant growth and development, we used *A. thaliana* as a model system. *Arabidopsis thaliana* (Col-0) seedlings were grown in Petri plates containing solid 0.2× Murashige and Skoog (MS) medium supplemented with the solvent or with increasing concentrations of serotonin from 10 to 160 μM. Twelve days after germination, the primary root length, LR number and LR density were determined for 30 seedlings. We found that serotonin strongly promoted LR development, without affecting primary root growth. This leads to plants with increased LR number and density (LR cm^{-1}) (**Fig. 2A–D**).

An important developmental trait widely used to monitor auxin responses is primary root growth (Woodward and Bartel 2005). To determine whether serotonin treatments of >160 μM could affect primary root growth and other root architectural parameters, Arabidopsis seedlings were supplied with 150–600 μM serotonin. It could be seen that both LR number and density increased at a serotonin concentration of 150 μM but decreased at greater concentrations of this compound (**Fig. 3B, C**). **Supplementary Fig. S1** illustrates the root architectural responses of Arabidopsis seedlings to high serotonin concentrations; it can be seen that this compound dramatically inhibits primary root growth while promoting root branching caused by proliferation of adventitious roots. Our results show that serotonin has a dual effect in modulating root system architecture, promoting LR development at low concentrations (10–160 μM) but inhibiting primary root growth and LR development at higher concentrations.

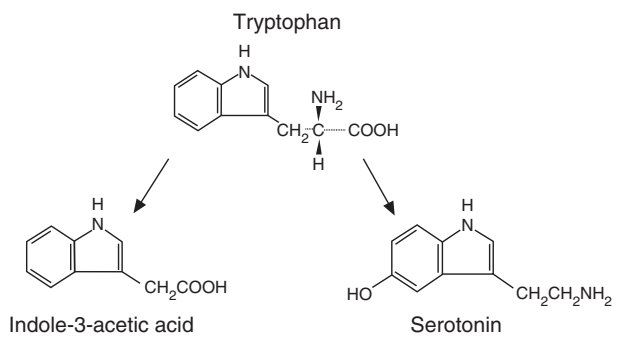

Fig. 1 Comparative chemical structures of serotonin (*N*-acetyl-5-hydroxytryptamine), IAA and their common precursor tryptophan.

Serotonin affects cell division and cell growth in Arabidopsis roots

The post-embryonic root developmental effects of high serotonin concentrations in Arabidopsis seedlings suggested that this compound could play an important role in cell division and/or cell elongation. To study the effects of this compound on cell division and elongation, we measured the length of both fully developed cortical cells from the differentiation region and the primary root meristem from 7 d old WT Arabidopsis (Col-0) seedlings. In addition, we analyzed the expression of *pPRZ1:uidA*, which marks only active meristems (Sieberer et al. 2003), and *CyCB1:uidA*, which is expressed only in cells in the G_2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al. 1999). Strong primary root growth inhibition under concentrations of serotonin ≥300 μM correlated with both decreased cell size of cortical cells and the loss of β-glucuronidase (GUS) expression in the primary root meristem of *pPRZ1:uidA* and *CyCB1:uidA* transgenic seedlings (**Fig. 4A–C**). In addition to these effects, meristem length significantly decreased from 300 μm in solvent-treated seedlings to 220 μm at a concentration of 600 μM serotonin. These results indicate that serotonin inhibits primary root growth by affecting both cell division and elongation.

Serotonin induces lateral root growth but not lateral root primordia initiation

To determine whether serotonin promotes LR development by stimulating LRP growth or inducing de novo formation of LRPs, or modulating both of these processes, we investigated the stages of LRP development affected by serotonin. LRPs were quantified 7 d after germination in plants treated with the solvent or with 150 μM serotonin, which increases LR number and density without affecting primary root growth (**Fig. 2**). Seedling roots were first cleared to enable LRPs at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997a). We found that the stage distribution of LRPs was affected by treatment with serotonin. In particular, LRP stage I, which describes LRPs at the earliest stage of

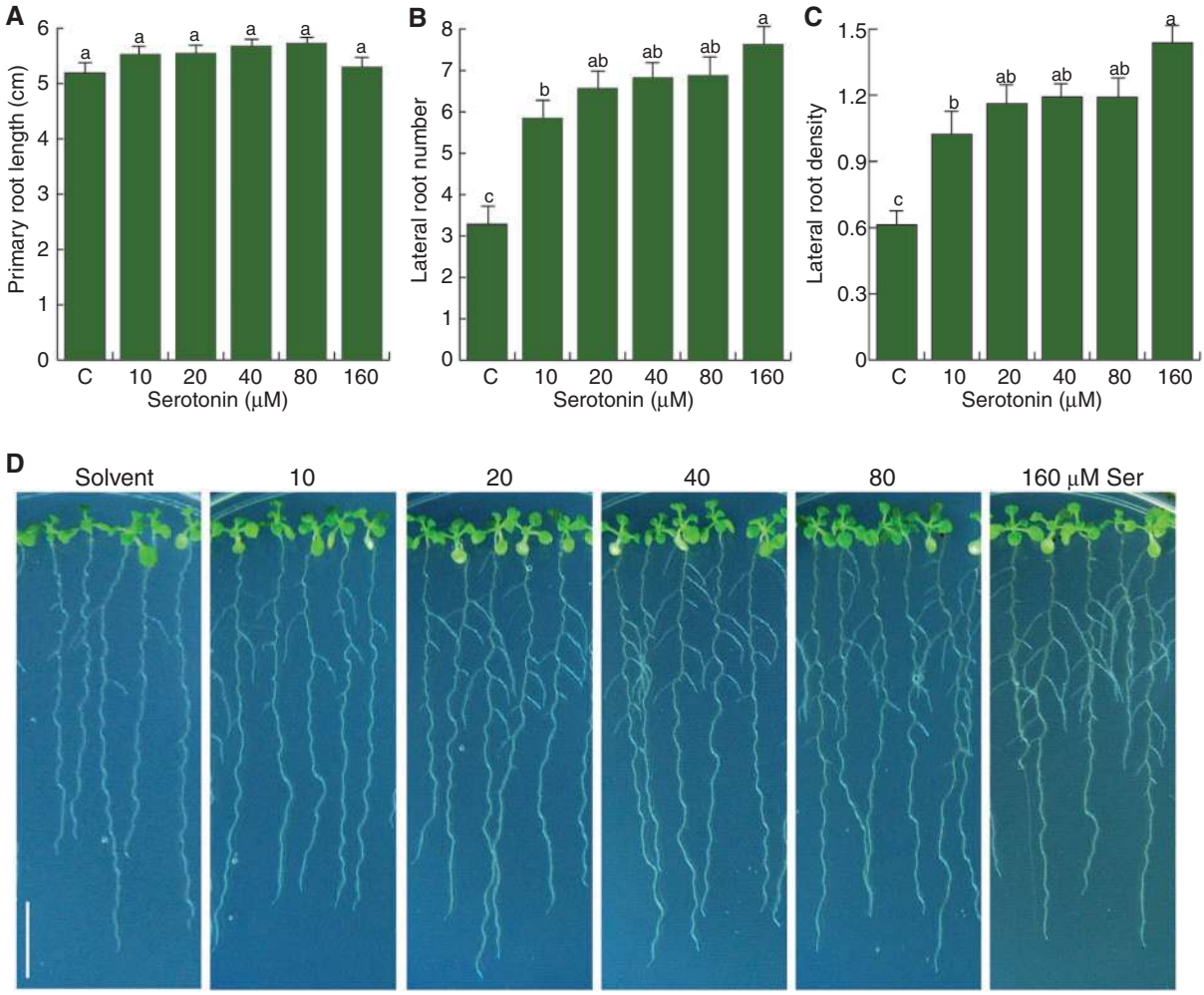

Fig. 2 Effects of low serotonin concentrations on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 12 d under increasing serotonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values shown represent the means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. (D) Representative photographs of Arabidopsis seedlings grown in the indicated serotonin treatments. The experiment was repeated twice with similar results. Scale bar = 1 cm.

development, was significantly decreased in serotonin-treated seedlings (**Fig. 5A**). In marked contrast, LRP stage VII, covering the most developed LRPs giving rise to emerged LRs, was induced 3-fold by serotonin (**Fig. 5A**). The total number of LRPs per seedling did not change in response to serotonin treatments (**Fig. 5B**). These data suggest that serotonin did not induce de novo LRP initiation and probably increases root branching in Arabidopsis by inducing the maturation of pre-formed LRPs from pericycle cells.

Serotonin promotes adventitious root development

To determine whether serotonin is involved in regulation of proliferative events in the shoot system, we assessed its regenerative properties by cultivating stem explants from etiolated Arabidopsis seedlings under increasing concentrations of serotonin and monitoring adventitious root formation as reported by Campos-Cuevas et al. (2008). Arabidopsis explants treated with 150–600 μM serotonin showed a roughly 2-fold increase in adventitious root number compared with solvent-treated explants (**Fig. 6A**). **Fig. 6B and C** shows representative photographs of the effects of serotonin on adventitious root formation. This result illustrates that serotonin is a compound with a strong effect on Arabidopsis adventitious root organogenesis.

Serotonin inhibits root hair development and expansin gene expression

The serotonin effects of inhibiting primary root growth and promoting adventitious root formation are reminiscent of those caused by treating plants with auxins (Woodward and Bartel 2005). Auxins have also been found to induce root hair development in several plant species (Parker et al. 2000). To determine whether serotonin could affect root hair development, we performed experiments in which Arabidopsis seedlings were germinated and grown in Petri plates containing 0.2× agar–MS medium supplemented with increased

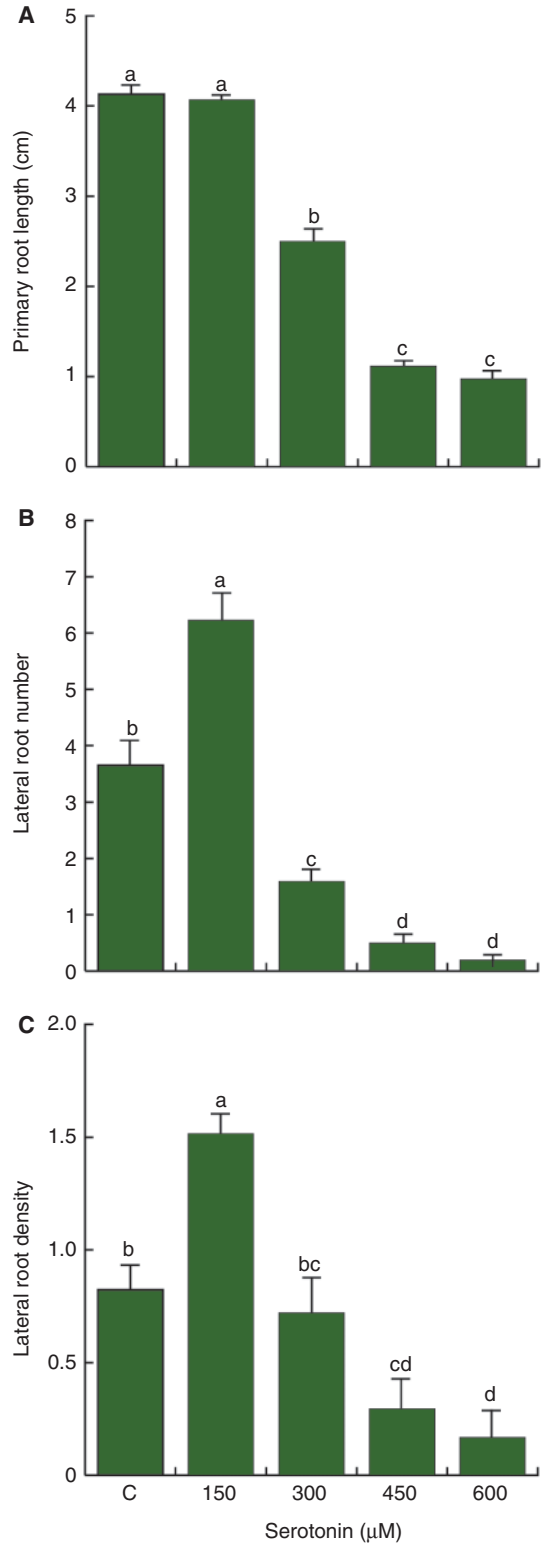

Fig. 3 Effects of high serotonin concentrations on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 10 d under increasing serotonin concentrations. (A) Primary root length. (B) Lateral root number. (C) Lateral root density. Values shown represent the means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

concentrations of the compound, and 5 d after germination root hairs from the differentiation and maturation zones of the primary root were analyzed. In marked contrast to adventitious root development, serotonin treatments dramatically inhibited root hair development both in the differentiation zone and in the maturation zone (**Fig. 7A**). Next, we determined whether the compound altered root hair initiation, root hair elongation or both, by microscopically counting and measuring trichoblast cells present in the maturation zone of the primary root. It was found that 150 μM serotonin significantly inhibited root hair growth, while increased concentrations dramatically blocked hair growth (**Fig. 7B**). The root hair density analysis showed that serotonin also inhibited root hair formation in a dose-dependent way (**Fig. 7C**). To determine whether serotonin affects root hair formation at an early step in development, we used the *pAtEXP7:uidA* transgenic line, which expresses GUS in trichoblast cells and is a marker of root hair initiation (Cho and Cosgrove 2002). Serotonin produced a deficient cell differentiation program in root epidermal cells, evidenced by loss of GUS expression in trichoblast cells in serotonin-treated seedlings (**Fig. 7D**).

GC-MS analysis of serotonin levels in *A. thaliana* seedlings

To determine whether serotonin is naturally produced in *A. thaliana* and if the effects observed on root system architecture correlated with serotonin accumulation in plant tissues, we performed experiments to quantify serotonin from root and shoot tissues from solvent- or serotonin-treated WT (Col-0) seedlings by using gas chromatography–mass spectrometry (GC-MS) analysis. Small yet detectable amounts of serotonin were identified in root and shoot tissue of solvent-treated Arabidopsis seedlings; however, serotonin levels in plant tissues dramatically increased in seedlings treated with this compound (**Fig. 8A**). Serotonin is acetylated in the derivatization process by acetic anhydride, producing *N*-acetylserotonin (**Fig. 8B**). **Fig. 8C and D** shows mass spectra of the *N*-acetylserotonin standard and the extracted sample, respectively. Representative chromatograms of root and shoot samples from solvent- or serotonin-treated seedlings are shown in **Fig. 8E–H**. These findings provide the first evidence that serotonin is produced naturally in Arabidopsis, and that plants are able to take up serotonin from the growth medium.

Serotonin inhibits auxin-inducible gene expression

We next investigated whether serotonin acts in an auxin-related signaling pathway by analyzing the expression of the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers. Since low serotonin concentrations activate LR development by inducing LRP outgrowth (**Figs. 2**, **5**), we first determined histochemical GUS expression during LRP development in 7 d transgenic *DR5:uidA* Arabidopsis seedlings, in response to the solvent or 150 μM serotonin. Interestingly, serotonin clearly

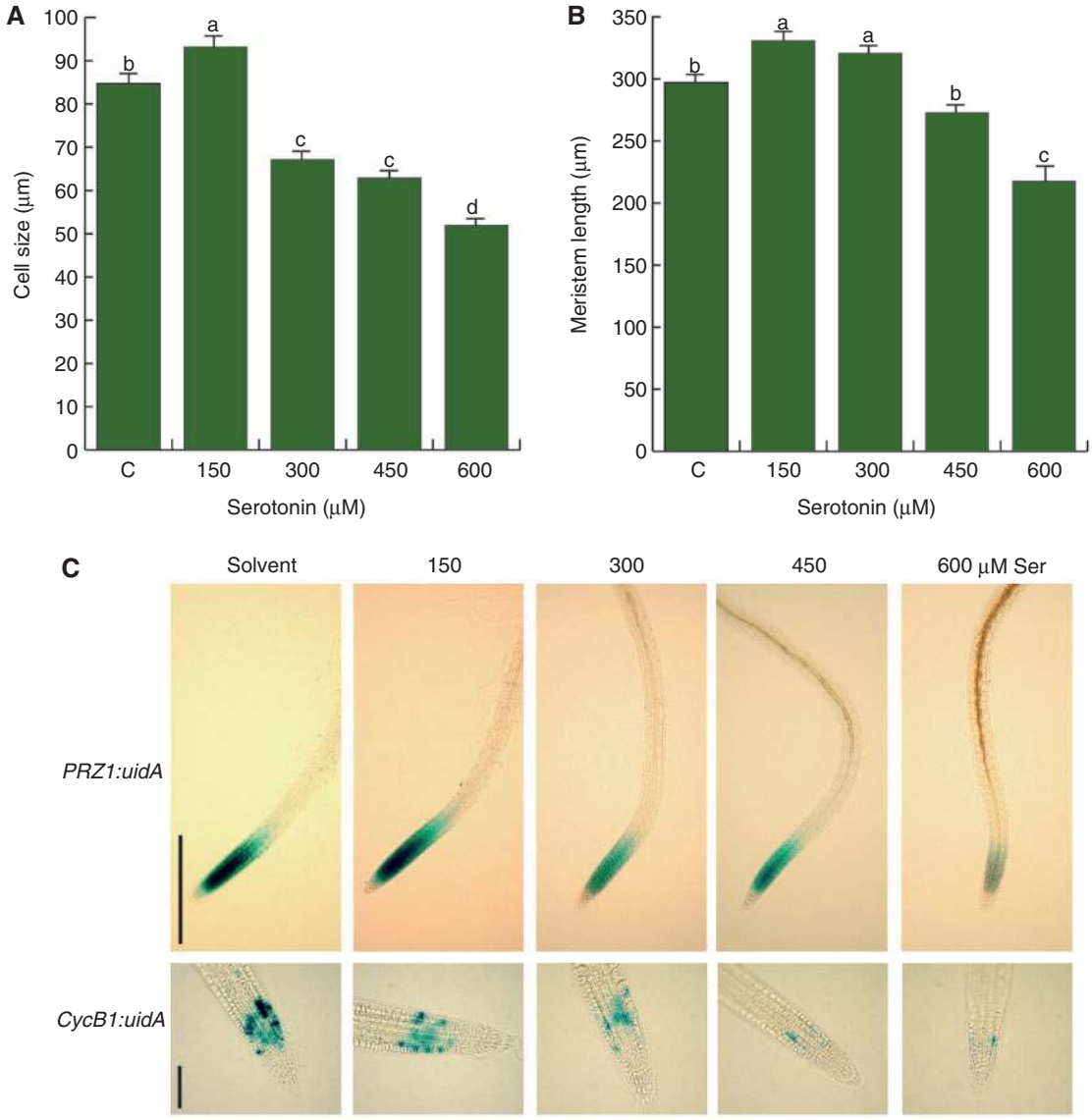

Fig. 4 Effect of serotonin on cell division and elongation. WT (Col-0), *pPRZ:uidA* and *CycB1:uidA A. thaliana* seedlings were grown for 7 d on 0.2× MS medium supplemented with the indicated concentrations of serotonin. (A) Mean epidermal cell length. (B) Meristem length. Data points represent the mean ± SD ($n = 30$). (C) Plants were stained for GUS activity and cleared to show gene expression. Photographs show representative individuals from at least 15 stained plants. The experiment was replicated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Scale bars in *pPRZ:uidA* images = 500 μm, and in *CycB1:uidA* images = 50 μm.

inhibited *DR5:uidA* expression in LRPs from all tested developmental stages (**Fig. 9A**). We also tested the response of the markers to a high serotonin concentration by analyzing histochemical staining of transgenic *DR5:uidA* and *BA3:uidA* Arabidopsis seedlings that were grown for 7 d in 0.2× agar–MS medium and then transferred to liquid 0.2× MS medium supplemented with the solvent, 5 μM IAA or 450 μM serotonin, and incubated for 9 h at 22°C. As previously reported (Ulmasov et al. 1997), in solvent-treated *DR5:uidA* seedlings, GUS expression was absent from cotyledons and leaves and was expressed primarily in the root tip region (**Fig. 9B**). *DR5:uidA* seedlings grown under a concentration of 5 μM IAA showed strong GUS activity throughout the plant (**Fig. 9B**). The pattern of GUS expression in *DR5:uidA* seedlings treated with 450 μM serotonin further decreased when compared with solvent-treated plants (**Fig. 9B**), indicating the lack of auxin activity for this compound. Untreated *BA3:uidA* seedlings did not show detectable levels of GUS activity (**Fig. 9C**), whereas, when treated with 5 μM IAA, they showed GUS expression mainly in the petioles of the cotyledons (**Fig. 9C**) and in the root elongation zone (**Fig. 9C**). GUS expression in seedlings treated with serotonin was undetectable (**Fig. 9C**), indicating that this compound failed to activate *BA3:uidA* expression. We also analyzed *DR5:uidA* expression in developing adventitious roots from

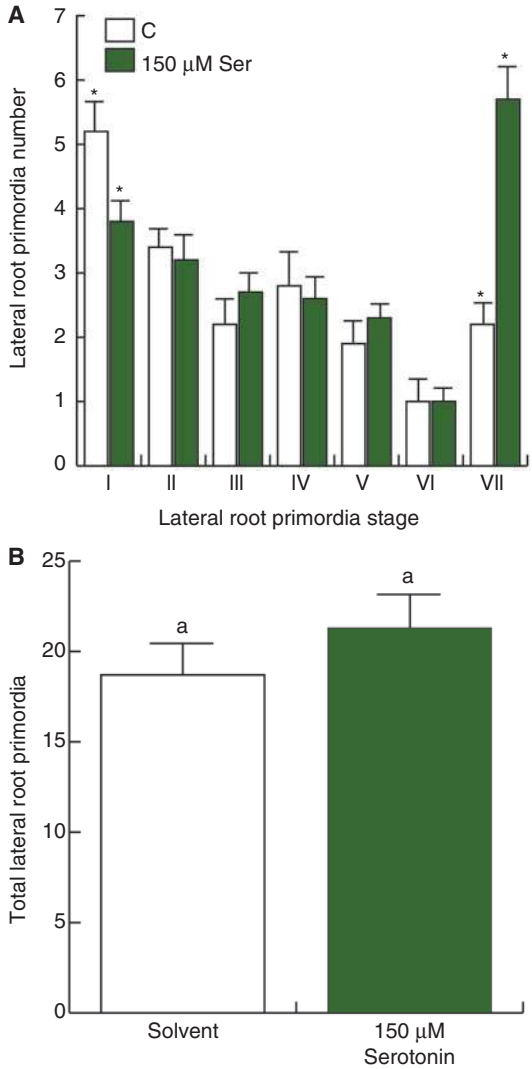

Fig. 5 Effects of serotonin on lateral root primordia development. Arabidopsis Col-0 seedlings were grown for 7 d on agar plates supplemented with the solvent or with 150 μM serotonin. Data are presented for LRP developmental stages (A) and total LRPs per seedling (B). LRP stages were recorded according to Malamy and Benfey (1997a). Values shown represent the mean of 15 seedlings ± SD. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Arabidopsis seedlings treated with 150–600 μM serotonin (**Supplementary Fig. S2**). A dose–response inhibitory effect on GUS expression was clearly observed, indicating that serotonin did not stimulate but rather repressed auxin-inducible gene expression.

To determine in more detail the possible mechanism of action of serotonin, we performed competence assays by using the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers. Transgenic Arabidopsis seedlings expressing each of these markers were grown for 7 d in 0.2× agar–MS medium and then transferred to liquid 0.2× MS medium supplemented with the solvent, 1 μM IAA or naphthaleneacetic acid (NAA), 450 μM serotonin or each auxin in combination with serotonin. When serotonin and IAA were supplied together, a marked reduction of auxin-induced *DR5:uidA* and *BA3:uidA* expression was evident (**Fig. 10A**). Serotonin also antagonized the effects of NAA on auxin-inducible gene expression when supplied at 450 μM (**Fig. 10B**) or under a lower concentration, namely 150 μM (**Supplementary Fig. S3**). These results suggest that serotonin may act as a competitive inhibitor of auxin-regulated gene expression in Arabidopsis.

Serotonin did not antagonize auxin-mediated Aux/IAA protein degradation

Auxin promotes the degradation of Aux/IAA repressor proteins via the ubiquitin–proteasome pathway and thereby induces primary auxin-responsive gene expression (Gray et al. 2001). To address the effect of serotonin on auxin-mediated degradation of Aux/IAA proteins, we examined the effect of IAA and serotonin on Aux/IAA stability using the Arabidopsis *HS::AXR3NT-GUS* line, in which a translational fusion between domains I and II of AXR3 and the GUS reporter protein is expressed under the control of a heat shock promoter (Gray et al. 2001). Seedlings expressing the *HS::AXR3NT-GUS* construct were heat shocked at 37°C for 2 h and further treated with 5 μM IAA, 450 μM serotonin or 5 μM IAA plus 450 μM serotonin for 10, 30 and 60 min. Treatment with IAA showed enhanced degradation of the fusion protein in cotyledons and in the primary root, but serotonin failed to induce degradation of the fusion protein even after 60 min of treatment (**Fig. 11A–L**). Moreover, this compound stabilized the expression of this marker in cotyledons (compare **Fig. 11A–C with I–K**), indicating auxin antagonist activity. When both compounds were supplied together, the effect of IAA predominates, giving rise to a GUS expression pattern similar to that observed in Arabidopsis seedlings treated with IAA alone (**Fig. 11M–P**). Our data indicate that serotonin acts independently of auxin or downstream of auxin receptors, which modulate the degradation of the AXR3 protein.

Effects of serotonin on auxin-induced lateral root formation

Because serotonin strongly inhibited the expression of the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers, it was possible that the repressing effects of this compound on root hair and LR development could be due to serotonin acting as an auxin inhibitor. We next evaluated the LR responses of WT (Col-0) seedlings to serotonin and NAA by growing Arabidopsis WT (Col-0) seedlings on Petri plates containing 0.2× agar–MS medium supplemented with different concentrations of serotonin, NAA or NAA plus serotonin. Seven days after germination, primary root length and LR number were quantified. In these experiments, serotonin concentrations of 150 μM did not affect primary root growth (**Fig. 12A**), but significantly increased LR number (**Fig. 12B**). NAA treatment or NAA plus

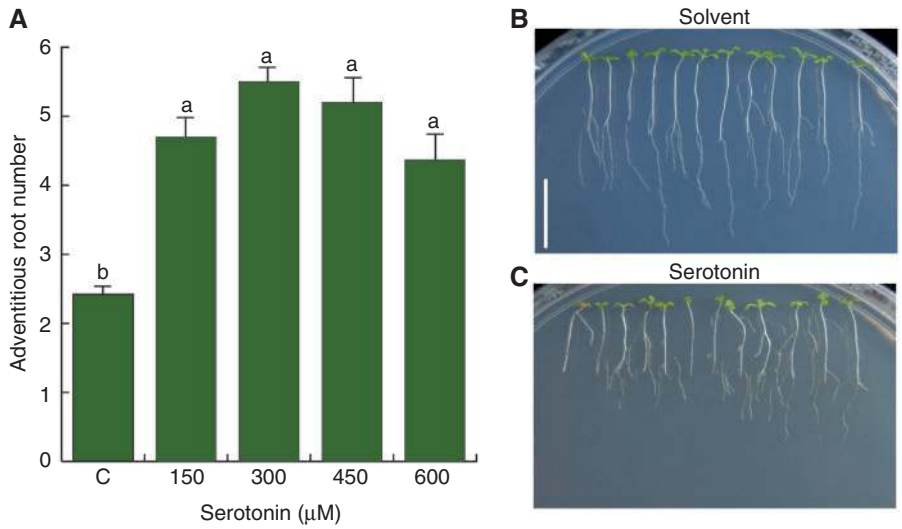

Fig. 6 Effects of serotonin on adventitious root development from Arabidopsis shoot explants. Arabidopsis seedlings were germinated and grown in darkness for 5 d on the surface of agar plates containing 0.2× MS medium and hypocotyl explants were obtained. Hypocotyl explants were transferred to 0.2× MS medium containing the indicated concentrations of the compound and cultivated for a further 9 d period to quantify adventitious root formation. (A) Adventitious root number in response to serotonin treatments. (B) Representative photograph of solvent-treated Arabidopsis (Col-0) explants. (C) Photograph of explants supplied with 450 μM serotonin. Values shown in (A) represent the mean ± SD ($n = 20$). Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated twice with similar results. Scale bar = 1 cm.

150 μM serotonin showed a strong effect of induced root branching. Interestingly, serotonin specifically induced LR growth when supplied together with NAA, indicating that it alleviates the growth-repressing effects of NAA on LR elongation (**Fig. 12C, D**). Supply of 300 and 450 μM serotonin inhibited both primary root growth and LR formation. In contrast, concentrations of 0.1 and 0.2 μM NAA, which inhibit primary root growth, strongly stimulated LR formation (**Supplementary Fig. S4**). Although treatment of seedlings with both serotonin and NAA showed an additive effect on primary root growth inhibition, we found that serotonin had an antagonistic effect on LR response to NAA by decreasing LR formation (**Supplementary Fig. S4**). These results imply that although the effects of serotonin and NAA on primary root growth are similar, they act in an opposite fashion to regulate LR development.

Effect of serotonin on root architectural traits in auxin-related Arabidopsis mutants

To evaluate at the genetic level the role played by selected auxin-related loci in serotonin responses, we compared the primary root growth and adventitious root formation of WT (Col-0) seedlings and the *axr2-1*, *axr4-1*, *aux1-7* and *axr1-3* Arabidopsis mutants in response to 150 μM serotonin treatment. Serotonin significantly induced both LR number and density in WT and in *axr2-1*, *axr4-1* and *aux1-7* seedlings but not in *axr1-3* mutants (**Fig. 13A–C**). Surprisingly, the *axr2-1* mutants showed increased LR numbers when grown in medium without serotonin, indicating that this mutant is inherently potentiated in LR formation (**Fig. 13B, C**). We also tested the effects of a high serotonin concentration on adventitious root development in intact Arabidopsis seedlings. Supply of 450 μM serotonin caused an 80% inhibition in primary root growth in WT seedlings compared with solvent-treated control seedlings. All four auxin-resistant mutants *aux1-7*, *axr1-3*, *axr2-1* and *axr4-1* showed similarly inhibited primary root growth to WT plants (**Supplementary Fig. S5**). When grown in medium without serotonin, WT and mutant seedlings showed an absence of adventitious roots, whereas when treated with the compound the formation of 3–6 adventitious roots was observed. This effect was similar in WT seedlings and in *axr4-1* and *aux1-7* mutants (**Supplementary Fig.S5**). In contrast, *axr2-1* mutants showed exacerbated responses to the compound while *axr1-3* mutants showed decreased adventitious root numbers (**Supplementary Fig. S5**). **Supplementary Fig. S6** illustrates the root architectural responses of Arabidopsis seedlings to IAA treatments; the primary root growth resistance of the lines used in this study with an almost normal adventitious root induction except in *axr1-3* can be seen. Our results indicate that LR and adventitious root induction by serotonin are independent of the *axr2-1*, *axr4-1* and *aux1-7* loci but require an intact *axr1-3* locus.

Discussion

Serotonin is a highly conserved indolic compound occurring in evolutionarily distinct organisms from humans to plants. The results of research with serotonin have uncovered several facts:

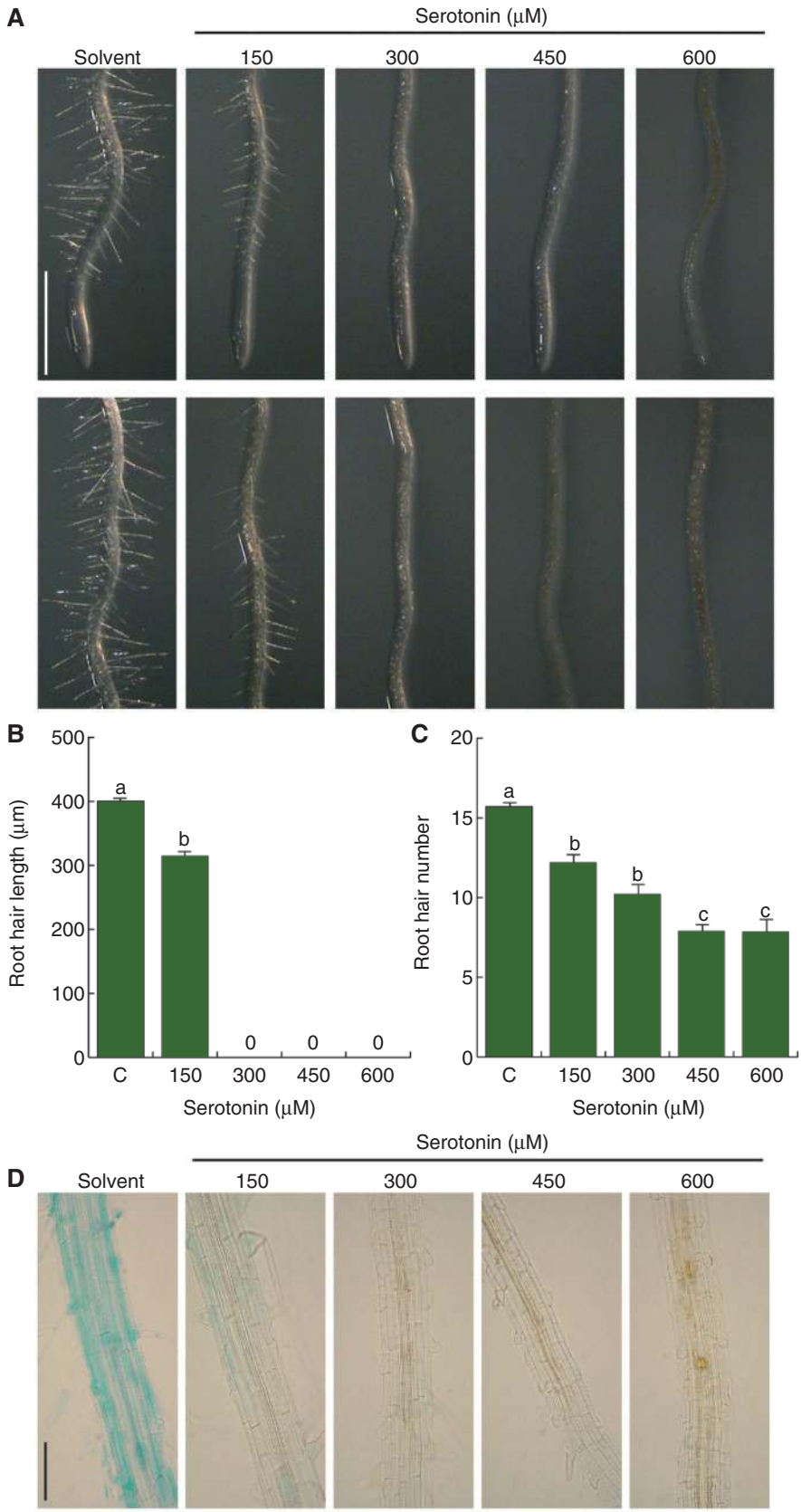

Fig. 7 Effects of serotonin on root hair development. *Arabidopsis thaliana* seedlings were grown for 5 d on 0.2× MS medium supplemented with the indicated concentrations of serotonin. (A) Representative photographs of root hairs formed at the differentiation and maturation regions of

Continued

(i) it is present in a wide number of plant species; (ii) it is produced from tryptophan; and (iii) its concentration may vary in plant tissues or in response to environmental conditions, suggesting important developmental and adaptive functions (Ishihara et al. 2008, Kang et al. 2009a). However, little is known about its signaling role in the plant. In a previous study by Kang et al. (2009a), it was found that serotonin is greatly accumulated in rice (*O. sativa*) leaves undergoing senescence induced by either nutrient deprivation or detachment, and its synthesis is closely coupled with transcriptional and enzymatic induction of the tryptophan biosynthetic genes as well as TDC. Transgenic rice plants that overexpressed TDC accumulated higher levels of serotonin than the WT and showed delayed senescence of rice leaves. In contrast, transgenic rice plants, in which expression of TDC was suppressed through an RNAi system, produced less serotonin and senesced faster than the WT, suggesting that serotonin is involved in attenuating leaf senescence.

Since the role of serotonin in plant development is not well understood, in this work we tested the hypothesis that it could act as a plant growth regulator by probably acting as an auxin or interfering with auxin action. Pharmacological tools that have increased our understanding of auxin signaling are auxin response inhibitors, which belong to two main classes: those that alter auxin transport and those that perturb auxin signaling. Most studies have employed synthetic inhibitors, such as 1-naphthylphthalamic acid (NPA), *p*-chlorophenoxyisobutyric acid (PCIB) and triiodobenzoic acid (TIBA), or small molecule antagonists of the TIR1 receptor function produced by introducing different alkyl chains to the α-position of IAA (Fujita and Syono 1996, Casimiro et al. 2001, Oono et al. 2003, Hayashi et al. 2008). While synthetic auxin inhibitors have provided important information about the molecular mechanisms involved in auxin action, the in planta role of these unnatural compounds is questionable. More recently, naturally occurring tryptophan derivatives such as tryptophan conjugates of jasmonic acid and IAA have been found to interfere with a broad range of auxin-mediated processes (Staswick 2009). Our results show that serotonin may also act as an endogenous auxin inhibitor. We used the Arabidopsis root system to test the effects of exogenously supplied serotonin on several morphogenetic processes including primary root growth, LR formation, adventitious root formation and root hair development, most of which are auxin-regulated processes. We found that serotonin stimulated LR development at concentrations of 10–160 μM by inducing LRP maturation (**Figs. 2**, **5**). This effect correlated with decreased expression of the auxin response marker *DR5:uidA* in LRPs (**Fig. 9A**). These results suggest that under normal growth conditions, auxin synthesis/response in developing LRPs is supraoptimal for LR growth. Serotonin might thus increase LRP maturation by antagonizing auxin signaling in LRPs. However, it also repressed LR formation and root hair growth at higher concentrations but increased adventitious root formation from intact plants and from shoot explants (**Fig. 6**, **7**; **Supplementary Fig. S1**). The activity of serotonin in modulating root growth was lower than that observed for auxins. **Fig. 2** shows that this compound only modestly slowed root growth. Even at 300 μM, primary root growth inhibition was <50% of the control value. By comparison, under similar growth conditions and using the same growth medium, IAA or auxin-related signals produced 50% inhibition at concentrations of at least two orders of magnitude lower than this (Contreras-Cornejo et al. 2009). Intriguingly, another animal neurotransmitter, glutamate, exerted a 60% primary root growth inhibition in WT Arabidopsis (Col-0) seedlings at a 500 μM concentration (Walch-Liu et al. 2006). Similarly to glutamate, serotonin did not interfere with LR initiation but potentiated LR outgrowth. An important difference in the mode of action of serotonin compared with glutamate is the role played by auxin signaling in plant responses to these signals. Two loss-of-function mutants at the *AXR1* locus (*axr1-3* and *axr1-12*) were hypersensitive to glutamate in primary root growth inhibition, whereas *aux1-7* was resistant, indicating that auxin transport and signaling might be important for root responses to glutamate (Walch-Liu et al. 2006). In contrast, primary root growth in both *axr1-3* and *aux1-7* was inhibited similarly to that in WT seedlings when treated with 450 μM serotonin (**Supplementary Fig. S5**).

The effects of serotonin on inhibiting primary root growth and repressing LR formation are similar to those caused by auxin influx or efflux inhibitors such as TIBA and NPA (Fujita and Syono 1996, Casimiro et al. 2001). Serotonin effects also resemble those caused by application of yokonolide B, an inhibitor of auxin action isolated from *Streptomyces diastatochromogenes*, which stimulated LR formation at low concentrations, whereas at higher concentrations it promoted adventitious root development (Hayashi et al. 2003). Although serotonin was detected at low levels in roots and shoots of solvent-treated Arabidopsis seedlings (**Fig. 8**), it may still play a significant role since it increases in concentration under particular developmental transitions and in response to pathogen attack (Ishihara et al. 2008, Kang et al. 2009a). Auxin is very important for root architecture remodeling and it is highly regulated by a complex network of interacting mechanisms; therefore, serotonin as an endogenous auxin inhibitor might be expected to remain low in most tissues under conditions of normal growth.

Fig. 7 Continued
the primary root of 5-day-old Arabidopsis seedlings grown on the surface of agar plates supplemented with the indicated concentrations of serotonin. (B and C) Data points indicate the mean ± SD for root hair length (B) or root hair number (C) of 10 epidermal cells located in a fully differentiated zone of the primary root from 20 seedlings analyzed. (D) *AtExp7:uidA* expression in response to serotonin treatments. These experiments were repeated twice with similar results. Different letters indicate statistical differences at $P < 0.05$. Scale bars in (A) = 500 μm and in (D) = 100 μm.

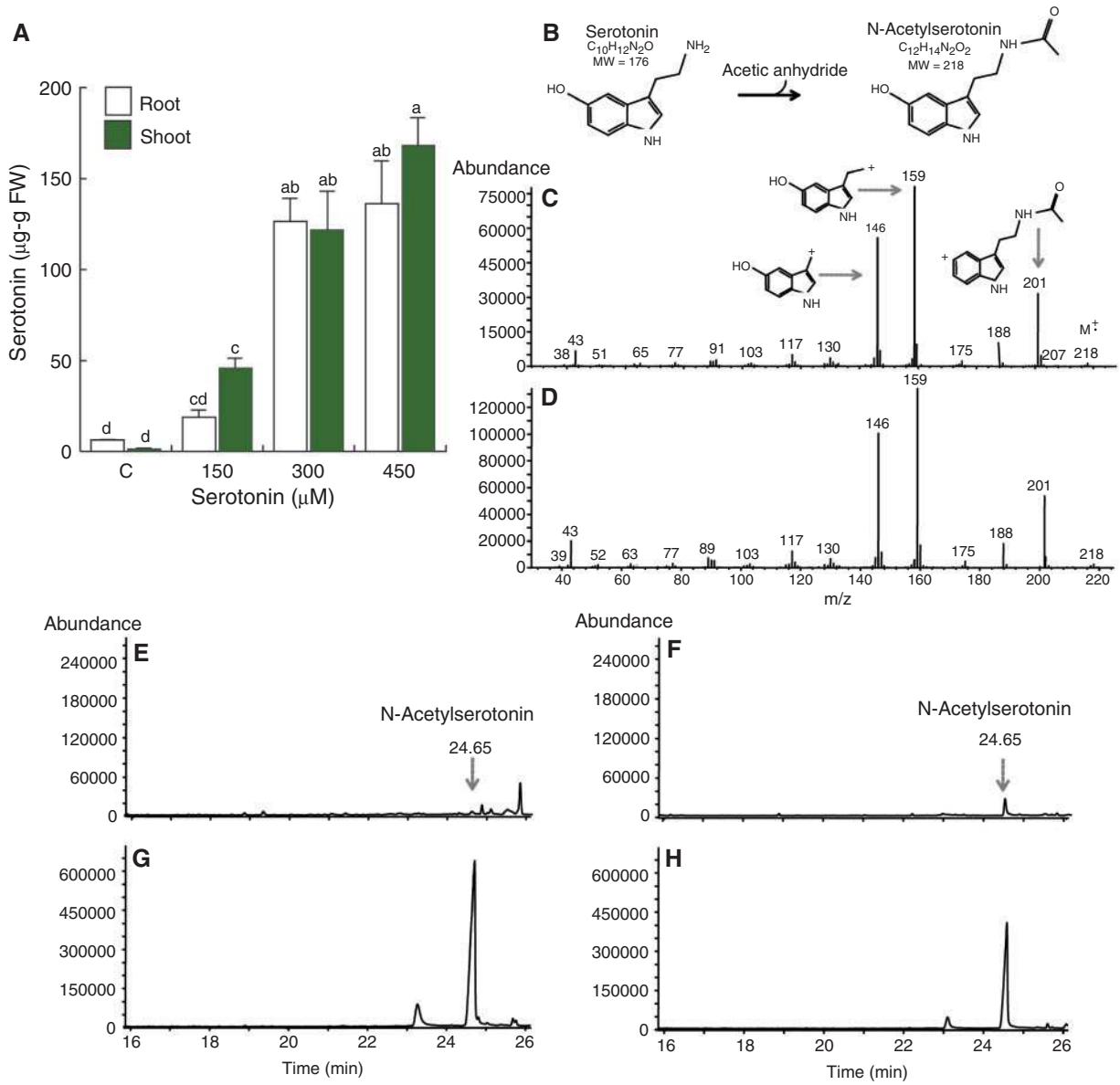

Fig. 8 Determination of serotonin from control or serotonin-treated Arabidopsis seedlings by GC-MS. Arabidopsis WT seedlings were germinated and grown for 22 d in 0.2× MS medium supplemented with the solvent or with increased concentrations of serotonin. (A) Serotonin quantification in root and shoot. (B) Acetylation reaction of serotonin with acetic anhydride. (C) The 70 eV electron impact full scan mass spectra from *m*/*z* 50 to 500 of the *N*-acetylserotonin standard. (D) *N*-Acetylserotonin in a plant extract. (E) Total ion chromatogram of *N*-acetylserotonin from solvent-treated shoots or roots (F), or from shoot and roots of plants treated with 450 μM serotonin, respectively (G–H).

Interestingly, supplementation of serotonin in the growth medium dramatically increased serotonin concentrations in both root and shoot tissues, indicating that Arabidopsis seedlings are able to take up serotonin from the medium and transport it within different plant tissues (**Fig. 8**).

To test whether serotonin may act on auxin transport or signaling, we examined the effect of serotonin on *DR5:uidA* and *BA3:uidA* gene expression induced by auxins with different transport properties. IAA is a substrate for auxin influx carriers, while NAA freely diffuses through membranes (Delbarre et al. 1996, Marchant et al. 1999). Serotonin similarly antagonized auxin-inducible gene expression revealed by both marker lines in response to both IAA and NAA (**Figs. 9**, **10**), suggesting that serotonin does not perturb auxin transport.

Auxin alters the stability of AUX/IAA repressors, and therefore serotonin may act by blocking AUX/IAA protein degradation, thus explaining the inhibitory effects of this compound on *DR5:uida* and *BA3:uidA* gene expression. To test this possibility, we analyzed the effects of serotonin on auxin-induced degradation of an AUX/IAA protein. The Arabidopsis *HS::AXR3NT-GUS* transgenic line strongly expresses an IAA17/AXR3 translational fusion protein under control of a heat shock

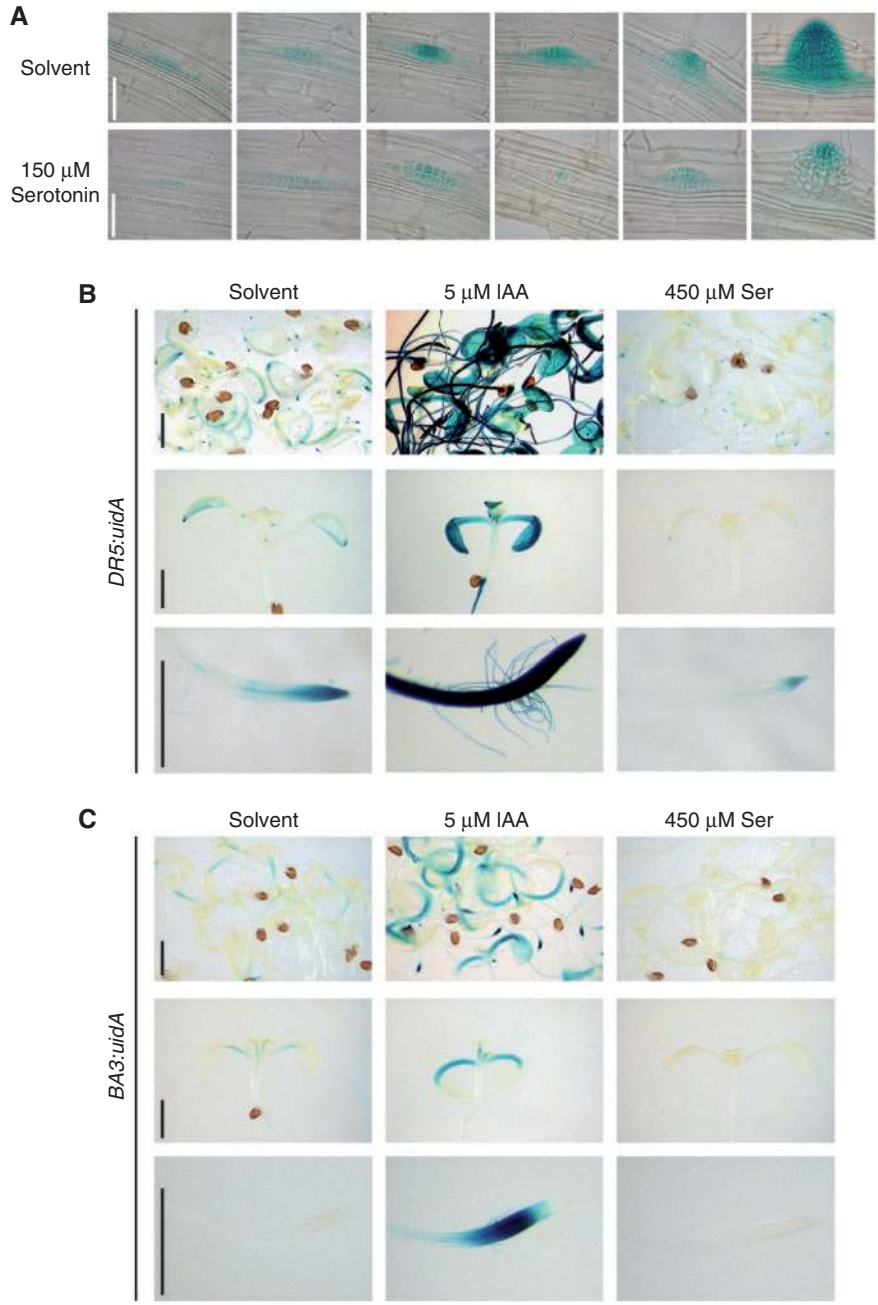

Fig. 9 Effect of serotonin on auxin-regulated gene expression. Twelve hour GUS staining of *DR5:uidA* or *BA3:uidA* Arabidopsis seedlings grown for 7 d on agar plates containing 0.2× MS medium or medium supplemented with IAA or serotonin. (A) *DR5:uidA* expression in lateral root primordia. Comparative effect of IAA and serotonin on *DR5:uidA* (B) and *BA3:uidA* expression (C) Notice the decrease in GUS expression in LRPs, shoots and roots in the treatments with serotonin. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Scale bars in (A) = 50 µm and in (B, C) = 500 µm.

promoter (Gray et al. 2001). The degradation rate of the AUX/IAA fusion protein is rapid and was enhanced by IAA treatment (**Fig. 11**). In contrast, serotonin failed to induce degradation of the fusion protein, indicative of the lack of an auxin activity. When IAA and serotonin are supplemented together, the degradation rate of the AUX/IAA fusion protein is rapid and resembles the effects of applying IAA alone (**Fig. 11**). These data suggest that serotonin may not compete for auxin binding to its receptors or that it acts downstream of auxin receptors, which modulate the degradation of the AXR3 protein.

Serotonin did not suppress the inhibition of primary root growth caused by IAA or NAA treatment (**Fig. 12**, **Supplementary Fig. S4**). However, it stimulates LR growth at low concentrations in the absence of exogenous auxin and

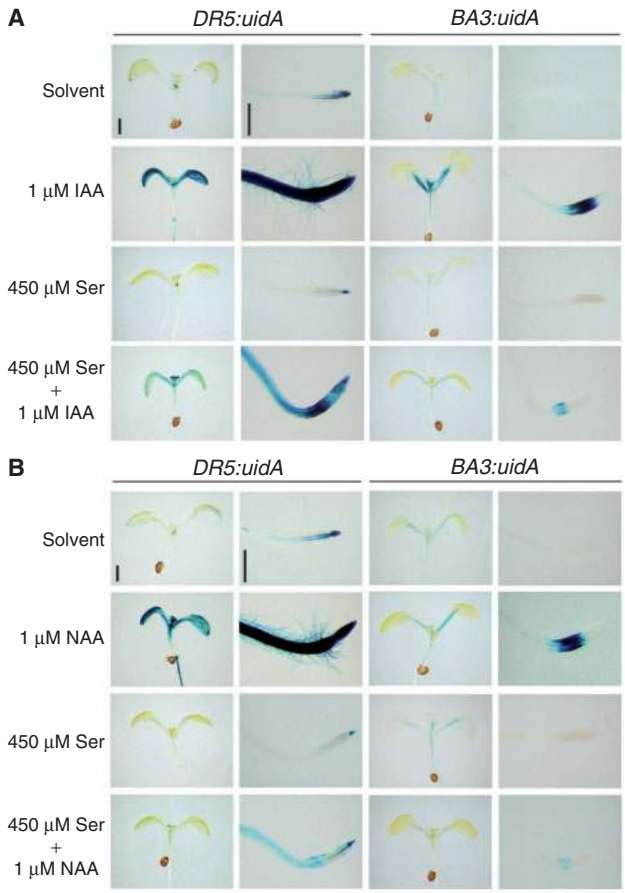

Fig. 10 Serotonin antagonizes the auxin-inducible expression modulated by IAA or NAA treatment. Twelve hour GUS staining of *DR5:uidA* and *BA3:uidA* Arabidopsis seedlings grown for 7 d on agar plates containing 0.2× agar–MS medium and then transferred to 0.2× MS liquid medium supplemented with auxins, with serotonin or both. Note the decrease in GUS expression in shoots and roots in both IAA (A) and NAA (B) treatments by serotonin. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Scale bars = 250 μm.

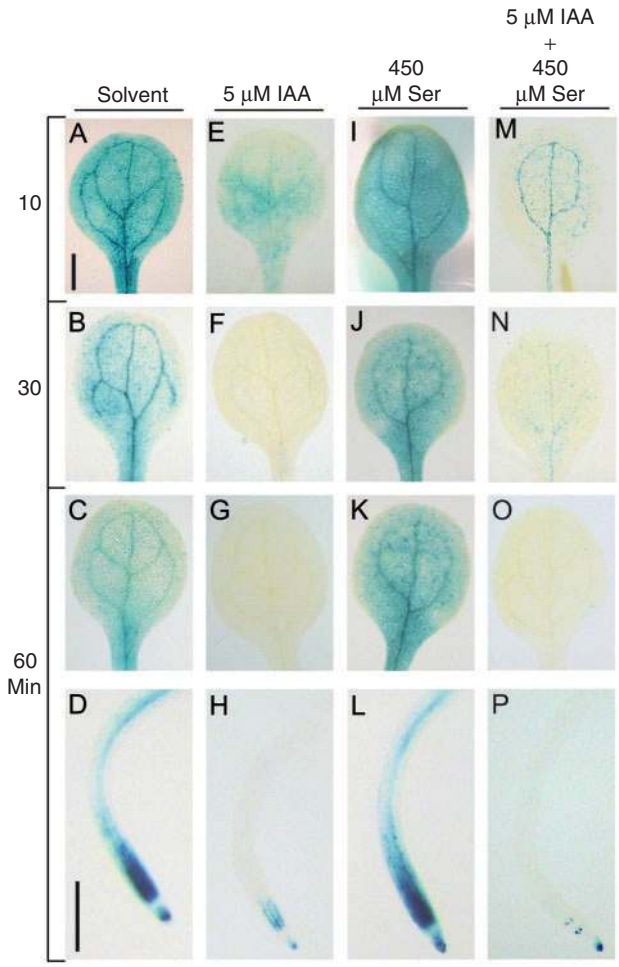

Fig. 11 Analysis of AUX/IAA stability with a *HS::AXR3NT-GUS* fusion. Transgenic seedlings expressing the *HS::AXR3NT-GUS* constructs were heat shocked at 37°C for 2 h. After heat induction, the seedlings were treated with IAA, serotonin or IAA plus serotonin for different times at the indicated concentrations, and stained overnight for GUS activity. Representative photographs of cotyledons from at least 20 stained seedlings are shown. Similar results were obtained in two independent experiments. Scale bars = 250 μm.

exacerbates the effects of NAA by inducing growth of LRs (**Fig. 12C, D**). High serotonin concentrations (i.e. 450 μM) blocked the stimulation of lateral rooting in response to exogenous auxin (**Supplementary Fig. S4**). Auxin antagonists vary significantly in how they affect root architecture. For example, terfestatin A and a synthetic auxin inhibitor having an alkyl substitution at the α-position of IAA stimulated primary root growth, which was attributed to the inhibition of endogenous auxin (Yamazoe et al. 2005, Hayashi et al. 2008). In contrast, both PCIB and yokonolide B suppressed primary root growth, and this was dependent on TIR1 and AUX/IAA7, suggesting it was not a toxic effect (Hayashi et al. 2003, Oono et al. 2003). By using transgenic Arabidopsis seedlings expressing *AtHistH2B:YFP* (yellow fluorescent protein) and vital staining with propidium iodide, we determined that the primary root growth inhibitory effect of serotonin was not due to toxicity, as serotonin-treated seedlings did not show meristem cell damage (**Supplementary Fig. S7**). Instead, it could be due to serotonin modulating cell division and elongation (**Fig. 4**).

Several auxin-resistant Arabidopsis mutants such as *axr1* and *aux1-7*, which are defective in auxin signaling and transport, respectively, exhibit fewer LRs and reduced root hair formation. Since similar phenotypes were observed in WT Arabidopsis (Col-0) roots treated with serotonin, it is possible that high levels of serotonin in the plant impair the cellular auxin response and thereby inhibit the initiation of LRs and root hairs. To determine whether auxin-related mutants were also resistant to serotonin, we tested the effects of this compound on LR development, primary root growth inhibition and adventitious root formation in WT Arabidopsis seedlings and in *axr2-1*, *axr4-1*, *aux1-7* and *axr1-3* auxin-related mutants. We

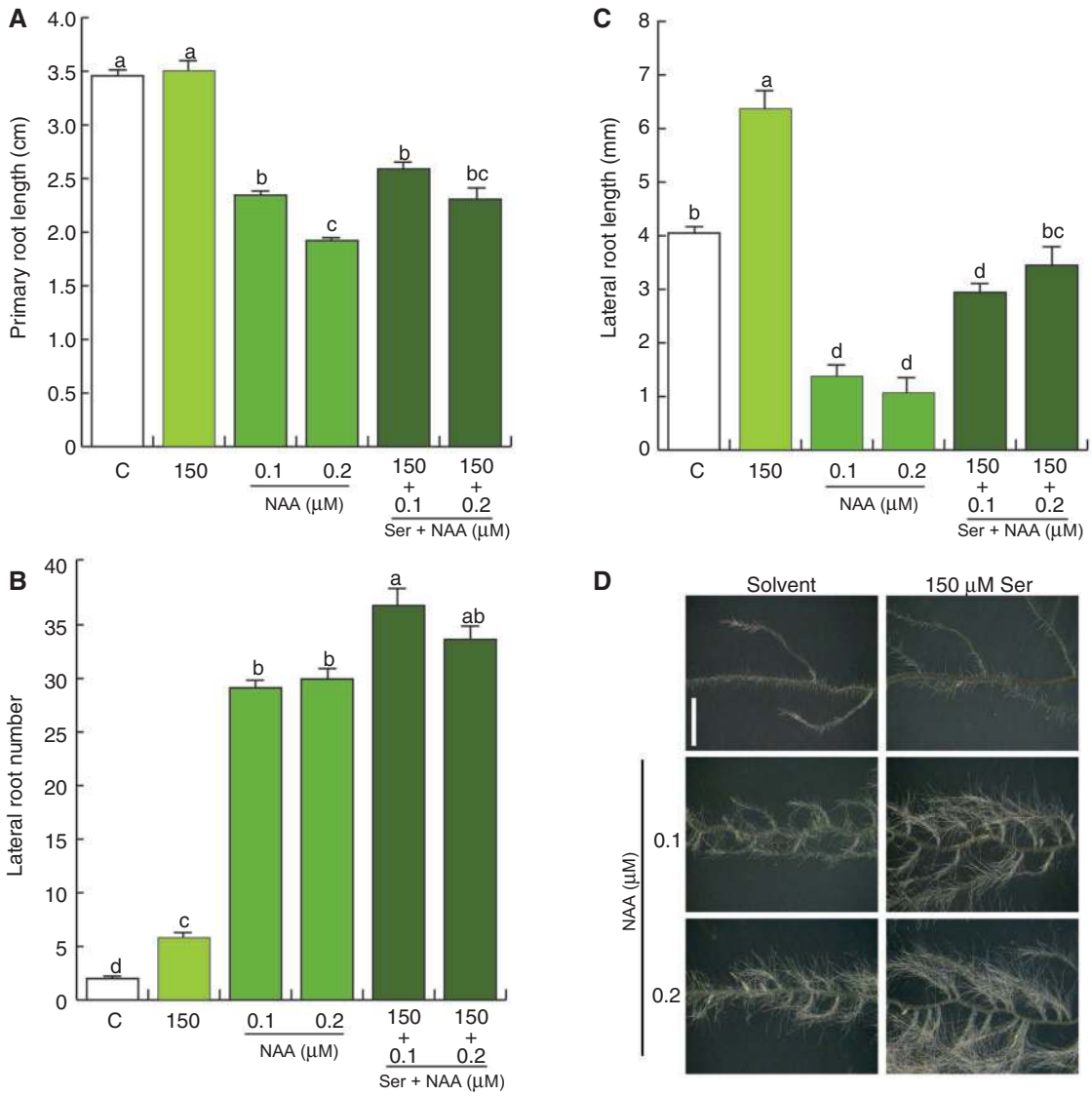

Fig. 12 Serotonin induces lateral growth in combination with auxin. Arabidopsis (Col-0) seedlings were germinated and grown for 7 d on agar solidified 0.2× MS medium supplemented with NAA, 150 μM serotonin or both compounds in combination. (A) Primary root length. (B) Lateral root number per plant. (C) Lateral root length. (D) Representative photographs of lateral roots grown in the indicated treatments. Data points indicate the mean ± SD from 20 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiments were repeated twice with similar results. Scale bar = 1 mm.

found that 150 μM serotonin significantly increased LR number and density in WT, *axr2-1*, *axr4-1* and *aux1-7*, but not in *axr1-3* (**Fig. 13B, C**). Surprisingly, the dominant *axr2-1* mutant, with a gain of function in *IAA7/AXR2*, caused increased LR formation both under normal growth conditions and in response to serotonin. This result suggests that *IAA7/AXR2* plays a positive role in LR development, in agreement with previously published information (Nagpal et al. 2000). The lack of response of *axr1-3* to serotonin indicates that this compound requires an intact AXR1 protein to activate LR development. Treatment with 450 μM serotonin also showed exacerbated adventitious root production in *axr2-1* and decreased adventitious root response in *axr1-3* when compared with WT plants (**Supplementary Fig. S6**). Although the exact mechanism of action of serotonin is still unclear, several lines of evidence indicate that serotonin probably acts as a natural auxin inhibitor. (i) Serotonin is present in Arabidopsis tissues at low concentrations. (ii) Serotonin treatment stimulates LRP maturation by decreasing auxin responses during LRP development. (iii) Exogenous application of serotonin inhibited root developmental processes which are under auxin control, such as primary root growth, LR formation and root hair development. (iv) Serotonin blocked auxin-responsive *DR5:uidA* and *BA3:uidA* gene expression and auxin-regulated LR formation. (v) Mutant analyses indicate that serotonin inhibits primary root growth and promotes adventitious root formation

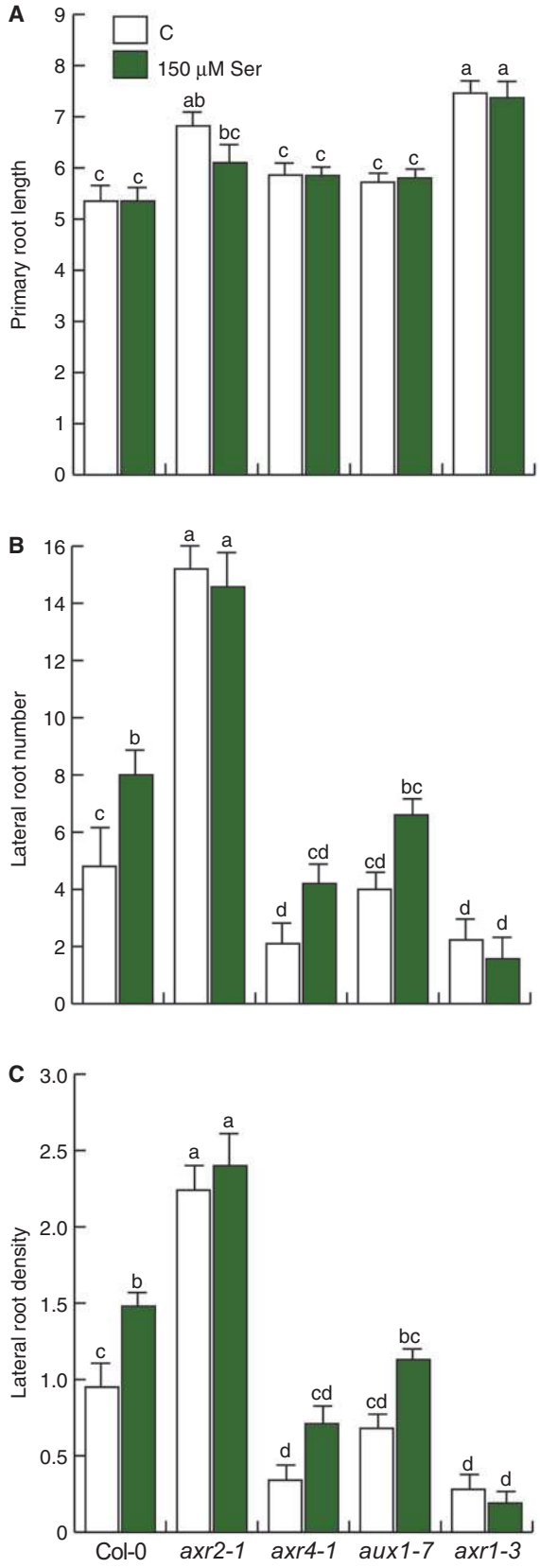

Fig. 13 Effects of serotonin on primary root growth and lateral root formation in WT Arabidopsis (Col-0) seedlings and auxin-related mutants. Arabidopsis WT and mutant seedlings were germinated and grown for 12 d in 0.2× MS medium supplemented with the solvent or 150 μM serotonin. (A) Primary root length. (B) Lateral root number per seedling. (C) Lateral root density. Values shown represent the means of 30 seedlings ± SD. The experiment was repeated three times with similar results.

independently of at least three auxin-related loci, namely *axr2-1*, *axr4-1* and *aux1-7*.

Plant neurobiology has recently emerged as an integrated view of plant signaling. Plants process the information from a changing environment to develop and reproduce. Communication between cells and tissues is essential for plant fitness, which involves an integrated signaling system that includes long-distance electrical signals, vesicle-mediated transport of IAA and production of chemicals known to be neuronal in animals (Baluska et al. 2005, Brenner et al. 2006). Among the animal neurotransmitters, acetylcholine, catecholamines, histamine, serotonin, dopamine, melatonin and glutamate are the most common in the animal nervous system, playing roles in information processing and development. It is of interest that each of these compounds is present in plants. Similarly to IAA, serotonin and melatonin are tryptophan derivatives. Interestingly, IAA, which is transported from cell to cell has some characteristics reminiscent of neurotransmitters, such as a poorly understood vesicle-based process that involves IAA transporters and recycling between the plasma membrane and endosomes (Geldner et al. 2003, Schlicht et al. 2006). Our results showing that serotonin regulates root architecture in a similar way to another neurotransmitter, glutamate, and that these compound can also affect auxin-mediated responses in Arabidopsis are in agreement with the proposed role of IAA in the plant neurobiological perspective. The possible roles played by other neurotransmitter signals in plant processes may be further clarified by using the molecular tools available in *A. thaliana* and other model plants.

Materials and Methods

Plant material and growth conditions

Arabidopsis (*A. thaliana* Col-0), the transgenic Arabidopsis lines *HS::AXR3NT-GUS* (Gray et al. 2001), *DR5:uidA* (Ulmasov et al. 1997), *BA3:uidA* (Oono et al. 1998), *pPRZ1:uidA* (Sieberer et al. 2003) and *CyCB1:uidA* (Colón-Carmona et al. 1999), histone *H2B:YFP* (Biosnard-Lorig et al. 2001), and the mutant lines *axr1-3* (Lincoln et al. 1990), *aux1-7* (Picket et al. 1990), *axr2-1* (Timpte et al. 1994) and *axr4-1* (Hobbie and Estelle 1995) were used for the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and with 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar

(commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h light/8 h darkness, light intensity of 100 μmol m^2 s^{-1} and temperature of 22°C.

Analysis of growth

Growth of primary roots was registered using a ruler. LR numbers were determined by counting the LRs present in the primary root, from the tip to the root/stem transition. LR densities were determined by dividing the LR number by the primary root length and expressed as LRP cm^{-1}. Root hairs were measured in a 500 μm region from the primary root tip. The average length of root hairs was determined by measuring 100 hairs for each root, taking as a reference the root protoxylematic plane to locate the radical hair base in the epidermal cell.

Determination of developmental stages of LRP

LRPs were quantified 7 d after germination. Seedling roots were first cleared to enable LRPs at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as folllows, Stage I: LRP initiation. In the longitudinal plane, approximately 8–10 'short' pericycle cells are formed. Stage II: the LRP is divided into two layers by a periclinal division. Stage III: the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV: an LRP with four cell layers. Stage V: the LRP is midway through the parent cortex. Stage VI: the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appears to be just about to emerge from the parent root.

Histochemical analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were stained and incubated overnight at 37°C in a GUS reaction buffer (0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained plants were cleared and fixed with 0.24 N HCl in 20% methanol (v/v) and incubated for 60 min at 62°C. The solution was substituted by 7% NaOH (w/v) in 60% ethanol (v/v) for 20 min at room temperature. Plants were dehydrated with ethanol treatments at 40, 20 and 10% (v/v) for a 24 h period each, and fixed in 50% glycerol (v/v). The processed roots were placed on glass slides and sealed with commercial nail varnish. For each marker line and for each treatment, at least 20 transgenic plants were analyzed.

Aux/IAA protein degradation assay

Six-day-old *HS::AXR3NT-GUS* transgenic Arabidopsis seedlings were incubated on 0.2× liquid MS medium for 2 h at 37°C, followed by transfer of the seedlings into 0.2× liquid MS medium supplemented with IAA or serotonin or both for 10, 30 or 60 min at 22°C. The seedlings were washed with fresh 0.2× MS medium and, 12–14 h later, histochemically stained for GUS activity.

Serotonin determination by GC-MS

The extraction of serotonin from leaves and roots of *A. thaliana* Col-0 (0.1 g) was done with 3 ml of methanol with continuous agitation for 3 h. The extract was evaporated to complete dryness under a stream of nitrogen. Serotonin was acetylated with acetic anhydride (1.5 ml) and 1 ml of dichloromethane, and then sonicated for 15 min and heated at 75°C for 1.5 h. The *N*-acetylserotonin was evaporated under a stream of nitrogen and redissolved in 50 μl of dichloromethane. *N*-Acetylserotonin was analyzed in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973, and a 30 m × 0.2 mm × 0.25 mm, 5% phenyl methyl silicone capillary column (HP-5 MS). Operating conditions used helium as carrier gas, 1 ml min^{-1}; detector temperature of 300°C and injector temperature of 250°C. The volume of injected sample was 1 μl. The column was held for 3 min at 150°C and programmed at 6°C min^{-1} to a final temperature of 278°C for 5 min. *N*-Acetylserotonin was identified by comparison with a mass spectra library (NIST/EPA/NIH, 'Chem Station' Hewlett Packard). The identity of the *N*-acetylserotonin was further confirmed by the comparison of the retention time in the tissue extract with a sample of the pure serotonin standard (Sigma) derivatized following the same procedure mentioned above. A SIM analysis was used to verify the presence of this compound in the sample. The molecular ions were monitored after electron impact ionization (70 eV). They were *m/z* 218, *m/z* 159 and *m/z* 146. To estimate the amount of compound produced by the plant, we constructed an individual calibration curve for the derivatized standard using concentrations from 0.1 to 30 μg.

Microscopy

The *A. thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6, Leica Microsystems). Total LRs were counted at 30× magnification. Images were captured with a Sony Cyber-shot DSC-S75 digital camera (Sony Electronics Inc.) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss). For confocal microscopy, solvent- or serotonin-treated transgenic Arabidopsis seedlings expressing the histone *H2B:YFP* construct (Boisnard-Lorig et al. 2001) were mounted on microscope slides into a solution of propidium iodide (10 mg ml^{-1}). Primary root meristems were analyzed by imaging mounted samples with an inverted confocal microscope (Zeiss Axiovert 200 LSM). For propidium iodide detection, wavelengths employed were an excitation line of 568 nm with an emission window of 585–610 nm. YFP was excited with 488 nm line and emission detected at 505–550 nm.

Data analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All LRs emerging from the primary root and observed under the 30× objective were taken into account for LR number data. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with Tukey's post-hoc test were used for testing differences in growth and root developmental responses in WT and mutant plants. In the figures, different letters are used to indicate means that differ significantly ($P = 0.05$).

Supplementary data

Supplementary data are available at PCP online.

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