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Shoot-supplied ammonium targets the root auxin influx carrier AUX1 and inhibits lateral root emergence in *Arabidopsis*

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ABSTRACT

Deposition of ammonium (NH_4^+) from the atmosphere is a substantial environmental problem. While toxicity resulting from root exposure to NH_4^+ is well studied, little is known about how shoot-supplied ammonium (SSA) affects root growth. In this study, we show that SSA significantly affects lateral root (LR) development. We show that SSA inhibits lateral root primordium (LRP) emergence, but not LRP initiation, resulting in significantly impaired LR number. We show that the inhibition is independent of abscisic acid (ABA) signalling and sucrose uptake in shoots but relates to the auxin response in roots. Expression analyses of an auxin-responsive reporter, *DR5:GUS*, and direct assays of auxin transport demonstrated that SSA inhibits root acropetal (rootward) auxin transport while not affecting basipetal (shootward) transport or auxin sensitivity of root cells. Mutant analyses indicated that the auxin influx carrier AUX1, but not the auxin efflux carriers PIN-FORMED (PIN)1 or PIN2, is required for this inhibition of LRP emergence and the observed auxin response. We found that AUX1 expression was modulated by SSA in vascular tissues rather than LR cap cells in roots. Taken together, our results suggest that SSA inhibits LRP emergence in *Arabidopsis* by interfering with AUX1-dependent auxin transport from shoot to root.

Key-words: ABA; ammonium toxicity; auxin transport; root; shoot-derived signal.

INTRODUCTION

NH_3 is a major air pollutant, accounting for 80% of total N deposition in Europe (Krupa 2003; Castro, Stulen & DeKok 2005). NH_3 pollution is the result of intensive agricultural and farming practices and, together with excess N fertilizer applications, has been shown to lead to phytotoxic effects in crops (Britto & Kronzucker 2002) and many wild species (Kronzucker *et al*. 2003; Stevens *et al*. 2004; Clark & Tilman 2008). While the toxic effects of NH_3, and resultant NH_4^+, from root exposure are well studied (Britto & Kronzucker 2002; Balkos, Britto & Kronzucker 2010; Barth *et al*. 2010; Li *et al*. 2010), significantly less is known about the effects of shoot exposure to NH_3/NH_4^+. It is well demonstrated that shoot cells can take up both NH_3 and NH_4^+ (Raven & Farquhar 1981; Lockyer & Whitehead 1986; Wollenweber & Raven 1993; Peuke *et al*. 1998; Hanstein & Felle 1999; Hanstein *et al*. 1999), and that, indeed, rates of influx in leaf cells can rival or exceed those in root cells (Nielsen & Schjoerring 1998; Britto *et al*. 2002). In traditional agriculture, foliar application of nitrogen is a common method to improve nitrogen utilization efficiency. However, this method can often result in visual damage, commonly described as leaf 'scorching', 'burning' or 'tipping' (Poulton *et al*. 1990; Gooding & Davies 1992; Phillips & Mullins 2004).

Several important physiological roles have been linked to excessive NH_4^+ exposure, such as cellular pH and ionic imbalance, relationships with carbon biochemistry and energy consumption and modifications of hormonal balance (Britto & Kronzucker 2002). Recently, in the model system *Arabidopsis*, it has been shown that NH_4^+, when supplied directly to roots, targets chiefly the elongation growth of the primary root, and that direct contact of the root tip with NH_4^+ is essential to the development of NH_4^+ toxicity in the root system (Li *et al*. 2010). The inhibition of root growth has been shown to involve impaired N-glycosylation of proteins via the enzyme guanosine 5′-diphosphate (GDP)-mannose pyrophosphorylase (Qin *et al*. 2008; Barth *et al*. 2010) and futile NH_4^+ cycling at the root plasma membrane (Britto *et al*. 2001; Kronzucker *et al*. 2003; Szczerba *et al*. 2008; Balkos *et al*. 2010; Li *et al*. 2010). By contrast, the characteristics and underlying mechanisms of NH_3/NH_4^+ toxicity triggered by shoot uptake of NH_3/NH_4^+ are still largely unknown.

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Lateral roots (LRs), which typically comprise the majority of the root systems in many higher plants, contribute greatly to nutrient acquisition from soil as well as to responses to environmental stresses. In *Arabidopsis*, the development of LRs proceeds through the following stages: lateral root primordia (LRP) initiation, establishment, emergence, activation into mature LRs and final maintenance of LR elongation (Malamy & Benfey 1997; Casimiro *et al.* 2003). The hormones abscisic acid (ABA) and auxin are important internal negative and positive regulators during LR development, respectively (Fukaki & Tasaka 2009). ABA has been implicated in LRP emergence and meristem activation independent of auxin (De Smet *et al.* 2003). On the other hand, auxin is involved in every stage of LR formation (Benkova *et al.* 2003; Dubrovsky *et al.* 2008), and auxin transport is critical (Blilou *et al.* 2005; Grieneisen *et al.* 2007; Lucas *et al.* 2008). Mutants in auxin efflux carriers such as PIN-FORMED (PIN)s and P-Glycoprotein (PGP)s show significant defects in LR formation (Fukaki & Tasaka 2009; Peret *et al.* 2009). Similarly, Auxin Resistant 1 (AUX1), an auxin influx carrier also regulates LRP positioning and initiation (Swarup *et al.* 2001; Marchant *et al.* 2002; De Smet *et al.* 2007; Laskowski *et al.* 2008). There is strong evidence that basipetally transported auxin is needed for LRP initiation (Casimiro *et al.* 2001; De Smet *et al.* 2007), whereas acropetally transported auxin is required for LRP emergence and/or elongation (Reed, Brady & Muday 1998; Casimiro *et al.* 2001; Bhalerao *et al.* 2002; Swarup *et al.* 2008). The role of AUX1 has been investigated mainly in the context of LRP initiation (Swarup *et al.* 2005; De Smet *et al.* 2007). However, AUX1 has also been suggested to be involved in acropetal auxin transport and LRP emergence (Swarup *et al.* 2001; Marchant *et al.* 2002; Laskowski *et al.* 2008). Particularly, it was interesting that AUX1 expression occurred in the vasculature of mature roots (Laskowski *et al.* 2008), and that effect of AUX1 on acropetal auxin transport is dependent on ethylene (Negi, Ivanchenko & Muday 2008).

The ABA-mediated pathway of LR development is important for root responses to several stress conditions, such as high nitrate and osmotic stress (Signora *et al.* 2001; Deak & Malamy 2005; Xiong *et al.* 2006; MacGregor *et al.* 2008). NH_4^+ toxicity is an important stress for plants (Britto & Kronzucker 2002). In addition to the external NH_4^+, abiotic stresses also could trigger accumulations of high intracellular NH_4^+ and subsequent toxicity if not efficiently removed (Lutts, Majerus & Kinet 1999; Skopelitis *et al.* 2006). Therefore, the ABA signal may be involved in the responses of NH_4^+ toxicity. There is contradictory evidence on the role of auxin signalling in response to nitrogen. Reductions in auxin translocation from shoots to roots have also been proposed to act as a long-range signal, mediating the inhibition of LR development on high nitrate (Forde 2002), whereas increased auxin transport to roots has been proposed to occur under ammonium nutrition (Gerendas *et al.* 1997). However, a suppression of root auxin content has been associated with ammonium nutrition (Kudoyarova, Farkhutdinov & Veselov 1997). In this study, we examine whether shoot-supplied ammonium (SSA) in agar plate culture, designed to simulate the effect of atmospheric NH_4^+ deposition, inhibits LR formation in *Arabidopsis*. We test the following hypotheses: (1) SSA inhibits LRP initiation and/or LRP emergence; (2) SSA-mediated inhibition is involved with altered ABA signalling as high nitrate and osmotic stresses; (3) SSA-mediated inhibition reduces cellular auxin response in roots; (4) SSA-mediated inhibition is linked to altered auxin signal, auxin basipetal or acropetal transport in roots; (5) the role of auxin transporters AUX1, PIN1 and PIN2 in SSA-mediated inhibition of LR formation; and (6) SSA decreases the expression of AUX1. The goal of the study was to identify chief targets and mediators of SSA inhibition of root growth.

MATERIALS AND METHODS

Plant material and growth conditions

All experiments were conducted on *Arabidopsis thaliana* (Columbia ecotype). Seeds of *aux1-7* (Pickett, Wilson & Estelle 1990), *eir1-1* (Roman *et al.* 1995), *pin1-1* (Okada *et al.* 1991), *abi4-1* (Finkelstein *et al.* 1998), *aba3-1* (Léon-Kloosterziel *et al.* 1996) and *aba2-3* (Laby *et al.* 2000) mutants, all in the Col-0 background, were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). Transgenic lines, *DR5::GUS* in *aux1-7* and *DR5::GUS* in *eir1-1* (Sabatini *et al.* 1999), and *DR5::GFP* line were kindly provided by Dr Ben Scheres (Utrecht University). The *DR5::GUS* line was provided by Dr Tom J. Guilfoyle (University of Missouri); *aux1-22* and *ProAUX1::AUX1-YFP* in *aux1-22* (Swarup *et al.* 2004) and the *ProAUX1::GUS* line (Marchant *et al.* 1999) were obtained from Dr Malcolm Bennett (University of Nottingham). Seeds were surface-sterilized and cold-treated at 4 °C for 48 h before being sown on standard growth medium. The standard growth medium has been described previously (Li *et al.* 2010), modified from Cao, Class & Crawford (1993), was composed of 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-ethylenediaminetetraacetic acid (EDTA), 50 μM H_3BO_3, 12 μm $MnSO_4$, 1 μM $ZnC1_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 1% sucrose, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (MES) and 0.8% agar (adjusted to pH 5.7 with 1 M NaOH). Plates were kept vertically in a growth chamber at 23 ± 1 °C under a light intensity of 100 μmol m^{-2} s^{-1}, with a photoperiod of 16 h light and 8 h dark.

Shoot-supply experiments

Custom-made segmented plates (13 × 13 cm) were separated into the upper and bottom parts with a 3 mm air gap (Zhang & Forde 1998), using two fixed plastic strips of 2 mm in height and a movable glass strip of 3 mm in width. The plates could prevent efficiently the upper substance from moving to the bottom part. Standard growth medium (i.e. control medium) was poured into the bottom part, whereas standard growth medium supplemented with

various chemicals was poured into the upper part. The pH is controlled the same between the upper and bottom media. For example, SSA was achieved by pouring control medium supplemented with $(NH_4)_2SO_4$ into the upper part, whereas control medium alone was used in the bottom part. Unless otherwise stated, SSA was performed as described above; '$-NH_4^+$' refers to the control (0 mM NH_4^+) and '$+NH_4^+$' refers to the SSA treatment (NH_4^+ was supplied at 60 mM, except in concentration dependence experiments, where concentrations were as indicated). Seedlings were transferred to the segmented plates for treatment when their primary roots reached 2 cm in length [about 5 d after germination (DAG)] and positioned such that only the shoots were in contact with the upper medium.

Root measurements

Only those roots confined in the bottom agar surface were chosen for analysis. The number of mature LRs (longer than 0.5 mm in length) was counted (Zhang *et al*. 1999). Roots were scanned, and total root length was analysed using image analysis software (WinRHIZO Pro, version 2004b, Regent Instruments Inc., Quebec, Canada). The primary root of individual seedlings was carefully straightened along the side of a ruler, and root length was recorded (Zhang *et al*. 1999). The length of LRs was determined by subtracting the primary root length from the total root length. The densities of LR number and length were indicated by dividing LR number and length by the primary root length. LRP were counted and classified using the methods and nomenclature described in Malamy & Benfey (1997).

Image analysis

Histochemical analysis of the β-glucuronidase (GUS) reporter enzyme activity was performed as described elsewhere (Weigel & Glazebrook 2002). Assessment of shoot tissue permeability to toluidine blue O (TB) was according to the method by MacGregor *et al*. (2008). GUS staining patterns in roots were analysed using an Olympus BX51 microscope with differential interference contrast (DIC) optics, and GUS or TB staining in the shoot using an Olympus SZX10 stereo microscope (Olympus Corporation, Tokyo, Japan). The micrographs were obtained with an Olympus DP71 camera, and whole seedlings were photographed with a Canon G7 camera (Canon Inc., Tokyo, Japan). The *ProAUX1::AUX1-YFP* reporter was analysed using a Zeiss LSM710 confocal microscope, and image analysis was performed using Zeiss 2009 software (Carl Zeiss AG, Jena, Germany). Images were representatives of at least 10 individual plants from each treatment. Experiments were repeated at least twice. All the images and graphs were arranged using Adobe Photoshop.

DR5::GUS-based auxin transport assay

The method, as described by Lewis & Muday (2009), was used to measure auxin transport. In brief, to measure acropetal auxin transport, plates containing the control seedlings (5 DAG *DR5::GUS* plants), or indole acetic acid (IAA)-treated seedlings, were inverted and incubated in the dark for 5 h. IAA treatment was conducted by placing agar solidified with 3-μM IAA (IAA-solidified) on shoots. To measure basipetal auxin transport, plates with the control seedlings (5 DAG *DR5::GUS* plants) or IAA-treated seedlings were incubated in the dark for 2 h. IAA treatment was conducted by placing a solidified agar block containing 1 μM IAA such that it overlapped with the root tip by ~0.5 mm. The entire seedling was then subjected to GUS staining for 16 h at 37 °C. Auxin transport was determined by comparing the distance of GUS staining from the site of IAA application of the treated seedlings with that of the controls. At least 10 seedlings for each treatment were measured and the experiments were repeated twice independently.

Radioactive auxin transport assay

The measurements were performed according to the procedure by Lewis & Muday (2009) using ^{3}H-labelled IAA (American Radiolabeled Chemical, St. Louis, MO, USA). In brief, for acropetal assays, 5-day-old seedlings grown on standard growth media were treated with either 0 or 60 mM shoot-supplied NH_4^+ for 1 day, then the shoots were incubated with 1% agar blocks containing 100 nM ^{3}H-IAA. Plates were then inverted and incubated for 18 h in the dark. Following the incubation, the apical 5 mm of the root tip was placed in a vial containing 3 mL of scintillation fluid. After overnight incubation, radioactivity in the vial was counted with a scintillation counter. For basipetal assays, seedlings were grown and treated with shoot-supplied NH_4^+ as in acropetal assays. One percent agar blocks containing 100 nM ^{3}H-IAA were then placed in contact with the root tips (0.5 mm) for 5 h in the dark. The apical 2 mm of the roots were discarded, and the apical 5 mm sections of the remaining roots were excised for radioactivity counting, as described above. Four replicates, each with 10 seedlings, were carried out in the experiments.

Real-time quantitative PCR analysis

The seedlings (5 DAG) were treated for 6 h with or without 60 mM NH_4^+. The whole seedlings were collected and protected in RNAlater solutions (Ambion, Austin, TX, USA). Total RNA was isolated with RNAiso Reagent (TaKaRa, Kyoto, Japan). cDNA was synthesized from aliquots of 1 μg total RNA with Superscript transcriptase M-MLV (TaKaRa) and used as the template for PCR amplification with specific primers for the selected genes. PCR was amplified with the primers of AUX1, LAX1, LAX2, LAX3 and CBP20 (Supporting Information Table S1), performed on Opticon Monitor 2 (Bio-Rad, Hercules, CA, USA) with a real-time quantification PCR kit (SYBR Premix Ex Taq™; TaKaRa) in 25 μL reactions, according to the manufacturer's instructions. PCR cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 20 s and

72 °C for 15 s. CBP20 encoding nuclear cap-binding protein was used as the housekeeping gene, and relative RNA abundance was normalized to the CBP20 internal control ($[mRNA]_{gene}/[mRNA]_{CBP20}$). Primers were designed across exon–exon junctions of cDNA to avoid potential contamination with genomic DNA. There were 100 pooled seedlings for each treatment, and two independent experiments were performed. Three repeats for each pool were carried for the PCR.

Statistical and graphical analyses

For all experiments, the data were statistically analysed using SPSS version 13.0 (SPSS, Chicago, IL, USA). The detail was presented in the figure legends. Graphs were produced using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA, USA) except Fig. 2a in Origin 8.0 software. All graphs and images were arranged using Adobe Photoshop 7.0.

RESULTS

SSA causes reduction of LR length and number

We designed a segmented agar plate system that allows only *Arabidopsis* shoots to come into contact with NH_4^+ (see Experimental procedures). It is known that NH_4^+ can directly enter shoot cells, and that shoot uptake rates can be substantial (see Introduction). SSA led to marked reduction of both shoot and root growth in Col-0 (Fig. 1a). Fifty percent inhibition of growth (EC_{50} – see dashed lines in Fig. 1b) was lowest for LR parameters. EC_{50} for LR length was approximately 32 mM, and 49 mM for LR number, whereas EC_{50} for shoot weight was about 63 mM, and >80 mM for the primary root (Fig. 1b). SSA inhibition of LR length and number was very similar to that affected by NH_4^+ exposure of whole plants (where both shoots and roots come into contact with NH_4^+; Li & Shi 2007; Supporting Information Fig. S1a).

Clearly, much higher concentrations of NH_4^+ are necessary in diffusion-limited agar systems as used here to produce growth inhibitions (e.g. 50% suppression of shoot growth) similar to those seen in hydroponic or soil cultures at much lower concentrations (Britto *et al*. 2001; Szczerba *et al*. 2008; Balkos *et al*. 2010). Indeed, typically, in agar plate studies, nutrient levels are significantly greater than those in natural environments (e.g. potassium in Murashige–Skoog medium is 20 mM, whereas typical soil concentrations are one to two orders of magnitude lower; Britto & Kronzucker 2008). Similarly, NH_4^+ toxicity thresholds on agar media can be substantially higher, as described elsewhere (Li *et al*. 2010). When all nutrients are lowered, however, NH_4^+ effects can become evident at lower concentrations even in agar medium. In 1/50-strength nutrient medium, 1 mM NH_4^+ inhibited LR formation (Supporting Information Fig. S1b). However, in this medium, the roots of *Arabidopsis* seedlings grew slowly and more variably, not suitable to study LR development. Therefore, standard growth medium was used throughout in subsequent experiments.

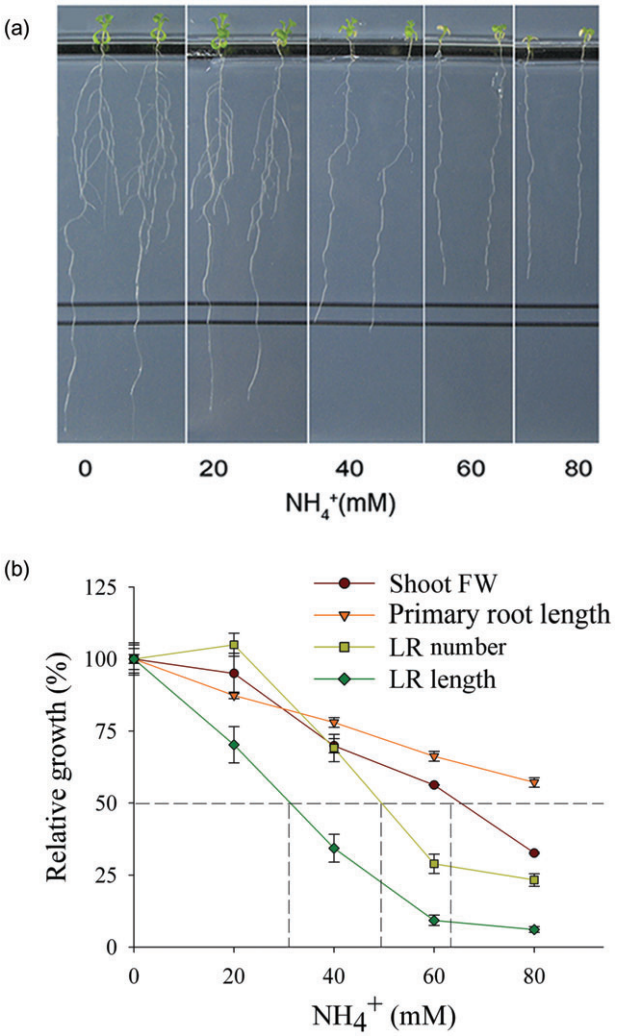

Figure 1. Inhibitory effect of shoot-supplied ammonium (SSA) on root and shoot growth in *Arabidopsis* (Col-0). Seedlings at 5 d after germination (5 DAG) were transferred to the NH_4^+ treatment medium and grown for an additional 6 d. (a) Two representative plants are shown from each SSA treatment. (b) Percentage inhibition of shoot fresh weight (FW), primary root length, lateral root (LR) length and number as affected by SSA. Because of the low weight of a single plant, the shoot fresh weights of all seedlings were pooled for accuracy. Data from two experiments are combined. Data represent means of 20 or more plants ± SE. Dashed lines indicate the NH_4^+ concentrations at which 50% growth suppression (EC_{50}) of the various parameters was seen. One hundred percent corresponds to 5.50 ± 0.27 mg $plant^{-1}$ for shoot FW, 8.60 ± 0.13 cm $plant^{-1}$ for the primary root length, 1.71 ± 0.06 no. cm^{-1} primary root for LRs, and 1.50 ± 0.08 cm cm^{-1} primary root for LR length.

SSA inhibitory of LR formation was reversible. When NH_4^+ was withdrawn, the LR numbers were recovered to the control level (Supporting Information Fig. S1c). This suggests that SSA indeed is responsible for the inhibition of

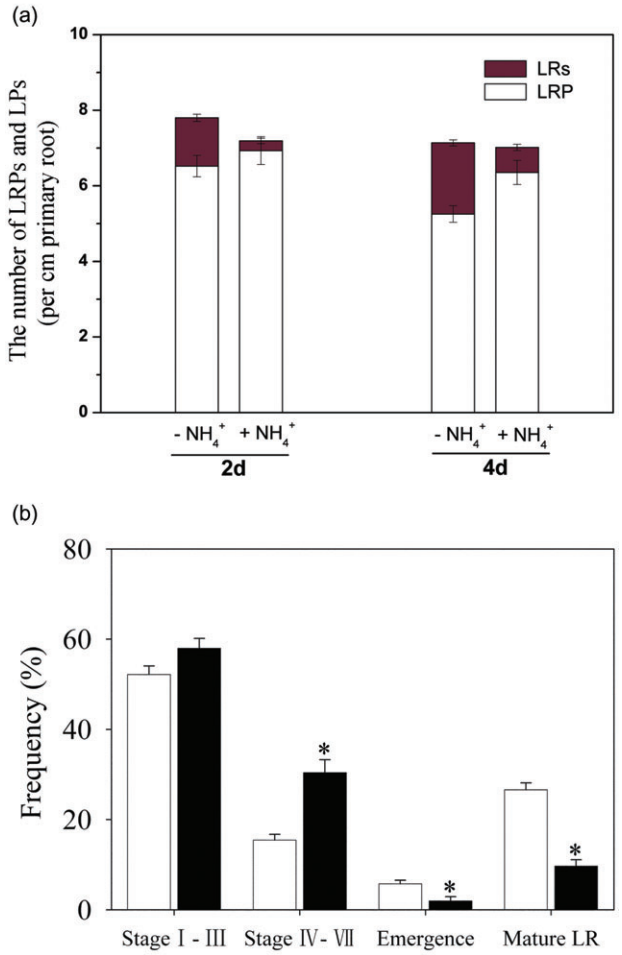

Figure 2. Effect of SSA on lateral root primordium (LRP) and LRs development. Transgenic *DR5::GUS* in Col-0 seedlings (4 DAG) were transferred to the treatment medium without or with SSA and incubated for 2 or 4 d prior to staining for glucuronidase (GUS). (a) The number of the LRP and LRs (Statistics indicated in text). (b) Distribution proportions of different stages of LRP and LRs (at eight days). Data from two experiments are combined. Bars represent averages of 10 plants ± SE. The asterisk represents means that significantly differ between control ($-NH_4^+$) and treatment ($+NH_4^+$) conditions (Student's *t*-test, $P < 0.05$).

LR formation. NH_4^+ was supplied as $(NH_4)_2SO_4$, which, at the concentrations applied, may cause sulphur-related or osmotic stress. Furthermore, NH_4^+ is assimilated into metabolites, such as glutamate (Glu) and glutamine (Gln). It was, thus, necessary to determine whether root growth inhibition was directly caused by NH_4^+. Therefore, K_2SO_4, KNO_3, Glu, Gln and mannitol were examined. Reductions in LR length and number in media containing equivalent K_2SO_4 or KNO_3 concentrations did not reach those with $(NH_4)_2SO_4$ (Supporting Information Fig. S2a,b). With high concentrations of Glu or Gln, LR length was only marginally inhibited, and LR number remained unaffected (Supporting Information Fig. S2c,d). Similarly, mannitol, near-iso-osmotic to $(NH_4)_2SO_4$, did not suppress LR length and number (Supporting Information Fig. S2e,f). Hence, the inhibition of LR formation could be principally attributed to NH_4^+.

SSA does not inhibit LRP initiation but causes arrest of subsequent LRP emergence

We further examined LRP initiation and LRP emergence in *DR5::GUS* seedlings by monitoring GUS activities, based on the observation that *DR5* is active at all stages of LRP (Benkova *et al*. 2003; Dubrovsky *et al*. 2008). In this study, the emerged but not activated LRP is still called LRP (Malamy & Benfey 1997), and only mature LRs (longer than 0.5 mm) are denoted as LRs (Zhang *et al*. 1999). The total LRP and LRs density of seedlings in $+NH_4^+$ medium were similar to those in $-NH_4^+$ medium at both 2 and 4 d after transfer; however, percentages of LRs in $+NH_4^+$-treated seedlings (3.4 and 9.7%, respectively) were significantly (Student's *t*-test, $P < 0.05$) less than those in control media, particularly 4 d after transfer (16.4 and 26.6%, respectively), whereas percentages of LRP developed reversely (Fig. 2a). In detail, accumulations of unemerged LRP, especially at the advanced stage, increased significantly, whereas the number of emerged but not activated LRP (shorter than 0.5 mm) and LRs was reduced markedly in $+NH_4^+$-treated seedlings (Fig. 2b). Taken together, these data indicate that SSA does not affect LRP initiation but rather a later stage of LR development. Targets may be LRP organization, LRP emergence, LR meristem activation or elongation (or a combination of these).

Inhibitory action of SSA on LR development does not involve either the ABA-mediated pathway or sucrose uptake in shoots

ABA is a negative regulator of LR development (De Smet *et al*. 2003), and has been suggested to mediate inhibitory effects of nitrate and osmotica (Signora *et al*. 2001; Deak & Malamy 2005; Xiong *et al*. 2006; MacGregor *et al*. 2008). Therefore, ABA mutants, including *abi4-1* (ABA-insensitive), *aba3-1* and *aba2-3* (ABA-deficient), resistant to the inhibitory effects of nitrate and osmotica (Signora *et al*. 2001; Deak & Malamy 2005; MacGregor *et al*. 2008), were used to test whether SSA inhibition of LR growth involves ABA mediation. We hypothesized that these ABA mutants should display resistance to the SSA inhibition, as was the case with applications of high nitrate and osmotic treatment. However, LR length and number in the *abi4-1*, *aba2-3* and *aba3-1* mutants showed slight sensitivity compared to wild type in response to SSA (Supporting Information Fig. S3).

Recently, it was found that one ABA pathway can repress LR formation by targeting sucrose uptake in shoots and decreasing shoot permeability in agar culture (MacGregor *et al*. 2008). However, SSA slightly increased shoot permeability, as indicated by TB (Supporting Information Fig. S4a), suggesting that shoots in $+NH_4^+$ medium are more

sucrose-permeable to uptake (MacGregor *et al*. 2008). Furthermore, elevated sucrose in the shoot medium markedly promoted LR formation in $-NH_4^+$ medium but did not alleviate the SSA-mediated inhibition of LR formation (Supporting Information Fig. S4b). SSA also repressed LR formation in the sucrose-free medium (Supporting Information Fig. S1b). These observations indicate that SSA does not inhibit LR formation by reducing shoot sucrose uptake in medium, although we cannot exclude possible indirect effects on sucrose metabolism or transport.

The influence of SSA on LR formation involves a reduced auxin response

Given the established role of auxin in LR formation (Casimiro *et al*. 2003; Fukaki & Tasaka 2009; Peret *et al*. 2009), we examined whether auxin is involved in SSA inhibition of LR development. Although the suppression of LR length in $+NH_4^+$ medium was not rescued by naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D; Supporting Information Fig. S5a,c), LR numbers in $+NH_4^+$ medium recovered as those in $-NH_4^+$ medium following the application of 0.05 μM NAA or 0.1 μM 2,4-D (Supporting Information Fig. S5b,d). These results suggest that SSA inhibits LR number by interfering with auxin signalling.

To determine how SSA affects the auxin response, we examined the spatial expression of the *DR5::GUS* reporter gene, which indicates the sensitivity of the auxin response inside the plant (Ulmasov *et al*. 1997), and is used widely in studying the role of auxin in LR development (Benkova *et al*. 2003; Dubrovsky *et al*. 2008). In $+NH_4^+$ medium, *DR5::GUS* expression was reduced markedly in young leaves and vascular tissues of cotyledons and the primary root apex, compared with $-NH_4^+$ controls (Fig. 3a,b). *DR5::GUS* expression in LRP (mainly the advanced stages), and partially in adjacent cells, was also reduced markedly (Fig. 3c). The *DR5* expression in adjacent cells

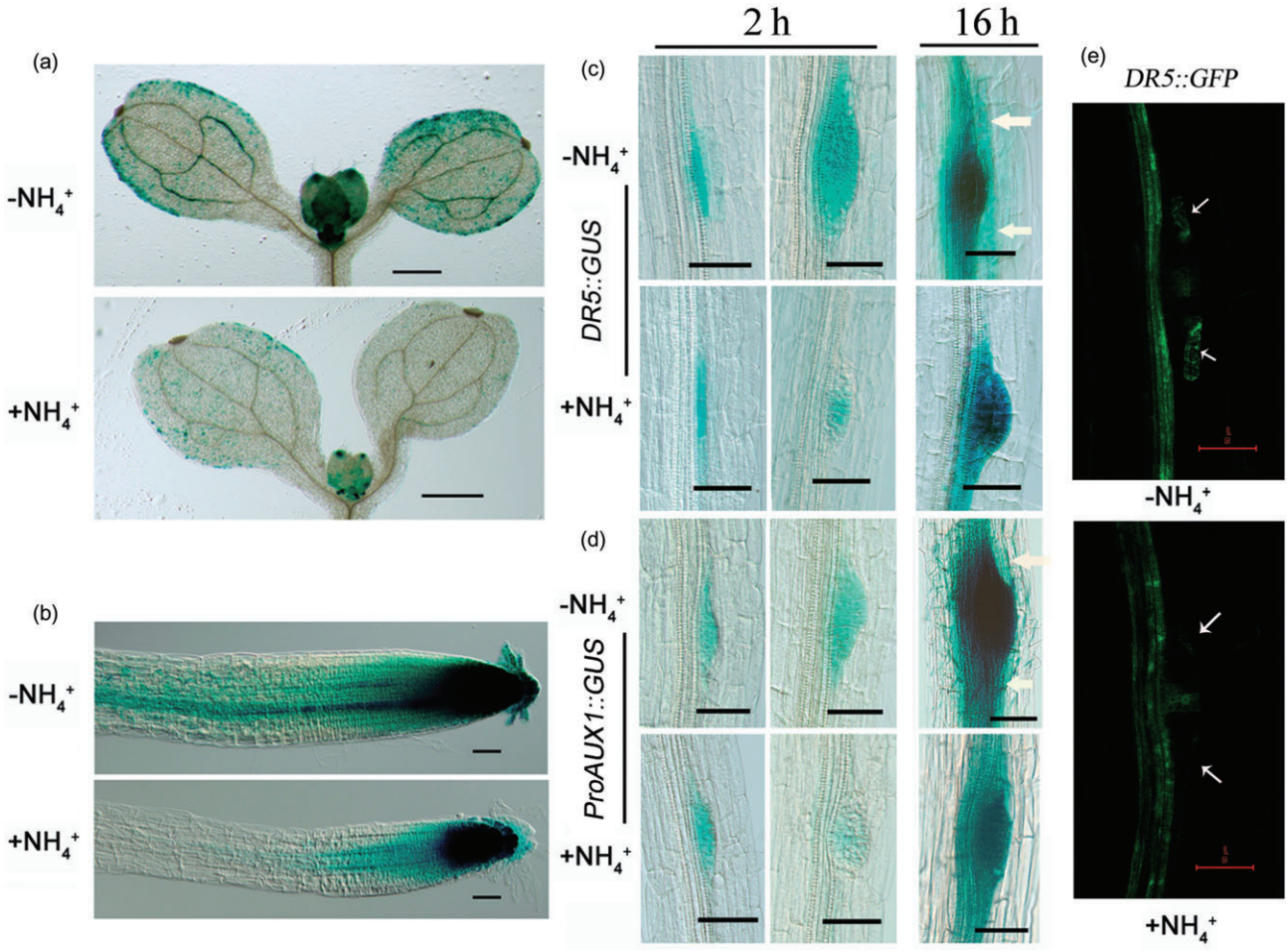

Figure 3. Effect of SSA on auxin response (*DR5::GUS*) and AUX1 (*ProAUX1::GUS*) expression in Col-0. (a, b) *DR5::GUS* expression in (a) shoots and (b) primary root apices in seedlings (4 DAG) with SSA treatment for an additional 2 days. One representative sample from each treatment (12 plants) is shown. Scale of bars = 1 mm (a), 50 μm (b). (c–e) Expression patterns of (c) *DR5::GUS*, (d) *ProAUX1::GUS* and (e) *DR5::GFP* in LRP and their adjacent cells (indicated by the arrows). The GUS was stained for 2 and 16 h, respectively, as indicated. The treatment was the same as in (a, b). LRP was mainly at the advanced stage except the first column (early stage LRP) in (c, d). One representative sample from each treatment (12 plants) is shown. Scale of bars = 50 μm.

was more pronounced in *DR5::GFP* seedlings (Fig. 3e); this reduction was observed as 37.5% (15/40) LRP at the advanced stage of seedlings in $+NH_4^+$ medium compared with 9% (3/33) LRP at the advanced stage of seedlings in $-NH_4^+$ controls. The altered *DR5::GUS* expression pattern indicates that SSA impairs auxin phloem transport from shoot to root (Haritatos, Ayre & Turgeon 2000; Marchant *et al*. 2002) or reduces cell sensitivity to auxin (Perez-Torres *et al*. 2008).

SSA impairs acropetal but not basipetal auxin transport

We assayed acropetal auxin transport and basipetal auxin transport by examining auxin induction of *DR5::GUS* expression (Lewis *et al*. 2007; Lewis & Muday 2009). SSA strongly reduced shoot-applied IAA induction of *DR5::GUS* in vascular tissues of root tips (Fig. 4a,b), suggesting a reduction in acropetal auxin transport. However, the induction of *DR5::GUS* expression was identical in $-NH_4^+$ and $+NH_4^+$ media when IAA was applied to root tips (Fig. 4a,c), indicating that basipetal auxin transport was unaffected. The results that SSA interferes mainly with acropetal, but not basipetal, auxin transport in the root were further confirmed by using the ^{3}H-IAA-labelling method (Fig. 4d). Finally, the application of external auxin to the root, but not the shoot, led to recovery of LR numbers in $+NH_4^+$ medium(Fig. 4e), whereas shoot application of IAA did significantly increase LR formation in the absence of SSA (Fig. 4e, Student's *t*-test, $P < 0.05$). This result is consistent with limited auxin movement from shoots to roots under SSA and enhancement of this movement by shoot IAA application. This is also consistent with the *DR5::GUS* images of shoot IAA application, showing

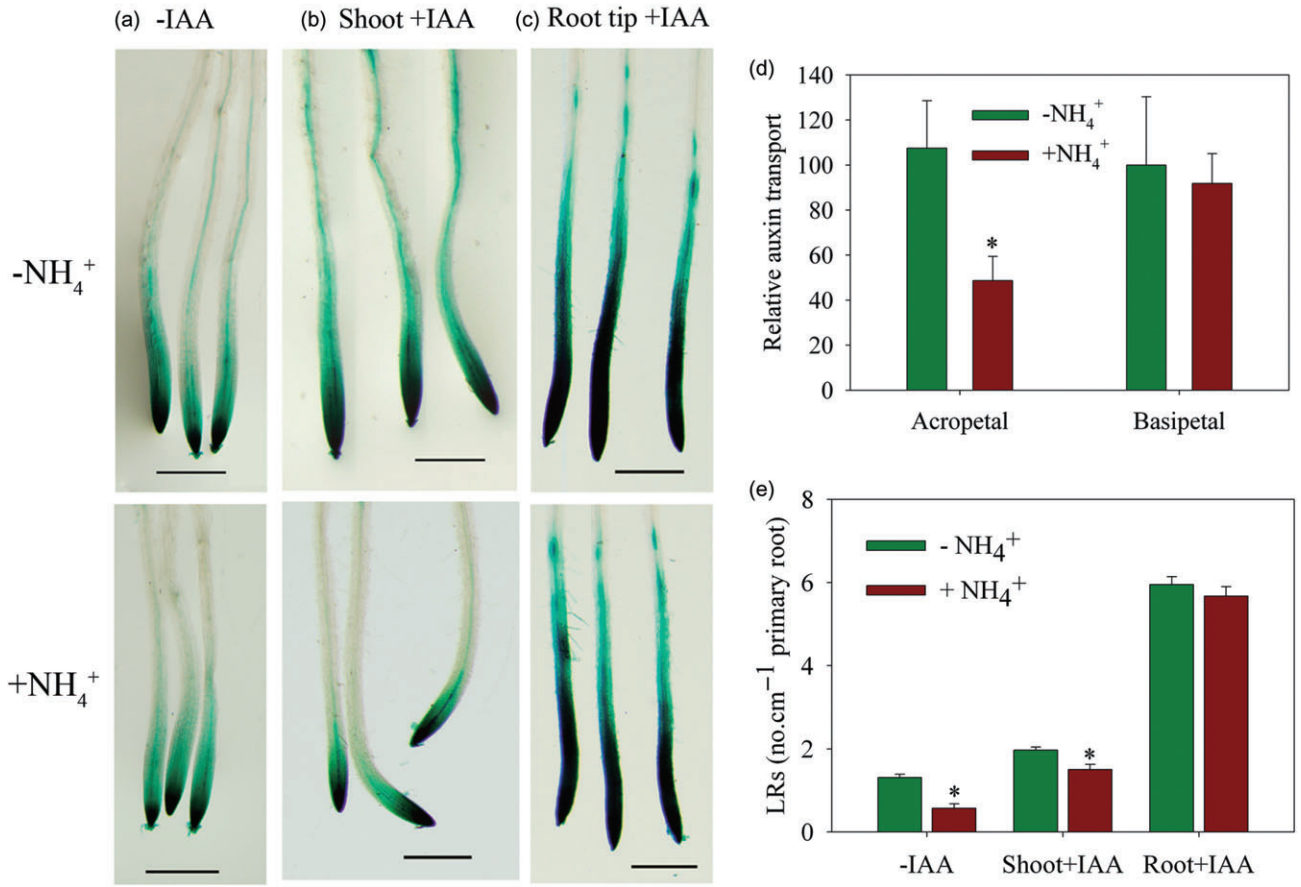

Figure 4. Auxin induction of *DR5::GUS* expression, lateral root (LR) number, and auxin transport in Col-0, with or without SSA treatment. (a) Expression of *DR5::GUS* in the primary apex of 5 DAG seedlings treated with or without SSA for 5 h (the response was similar at 2 h; data not shown) in the dark prior to staining for GUS expression. (b) Effects of the application of 3 μM indole acetic acid (IAA) to shoots on *DR5::GUS* expression in (a). (c) Expression of *DR5::GUS* after application of 1 μM IAA to the root tip. Three representative plants from each treatment (12 plants) are shown (a–c). Scale of bars = 0.5 mm. (d) Effect of SSA on relative acropetal and basipetal auxin transport in 5 DAG seedlings of Col-0 using ^{3}H-IAA-labelling assays. Bars represent averages ± SE (four replicates, each with 10 seedlings). The asterisk represents statistical significance (Student's *t*-test, $P < 0.05$). (e) Effect of SSA on auxin stimulation of LR production with auxin application to shoot and root. IAA was applied at 3 or 0.1 μM to shoots and roots, respectively, with or without SSA, and incubation occurred for 3 d prior to determination of LR number. Data are from one of two experiments. Bars represent averages of 12 plants ± SE. The asterisk represents statistical significance between NH_4^+ and $-NH_4^+$ treatment (Student's *t*-test, $P < 0.05$).

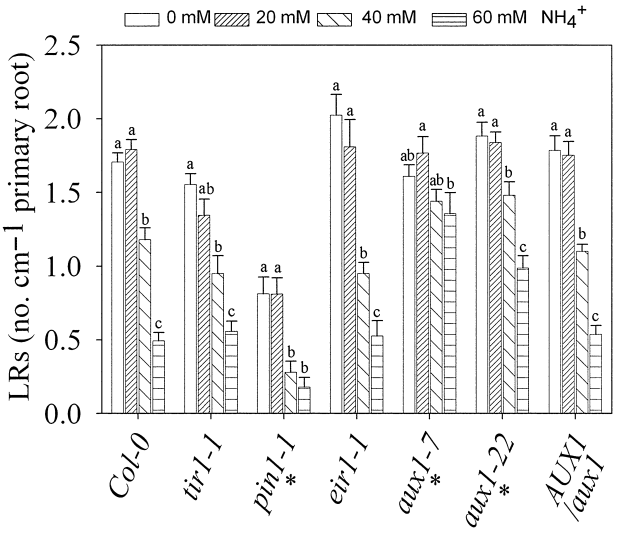

Figure 5. Effect of SSA on lateral root (LR) numbers in auxin-response and -transport mutants. Seedlings (5 DAG) of the indicated genotypes were treated with SSA for an additional 6 d. Data are from one of two experiments. Bars represent averages of 12 or more plants ± SE. Letters indicate significantly different means between treatments within a given genotype (one-way analysis of variance with Tukey multiple comparison test, $P < 0.05$). The asterisk indicates significant difference between mutants and Col-0; there was no significant difference between *aux1-7* and *aux1-22* (univariate analysis with a Tukey post hoc test, $P < 0.05$).

more IAA movement to the root. These results indicate that an interruption of auxin signalling or auxin transport from shoot to root is indeed responsible for SSA inhibition of LR formation.

The above observation suggests that the sensitivity of root cells to auxin may be not altered by SSA (Fig. 4a,c). It has been demonstrated that transport inhibitor response 1 (TIR1) acts as an auxin receptor (Dharmasiri, Dharmasiri & Estelle 2005) and the *tir1-1* mutant shows reduced sensitivity to auxin and reduced LR formation (Ruegger *et al.* 1998). Here, we found that the response of LR number to SSA in the *tir1-1* mutant was similar to wild-type controls (Fig. 5), which further supports that SSA may not affect the sensitivity of root cells to auxin. Taken together, our experimental data supported that SSA may inhibit LRP emergence by impairing auxin transport.

The auxin influx carrier AUX1 is required for the inhibition by SSA of the emergence of LRP and of acropetal auxin transport

If the inhibitory effect of SSA on LR number involves the auxin transport pathway, the number of LRs in mutants defective in auxin transport should differ from wild type following SSA. Mutants, *pin1-1* and *eir1-1*, are disrupted in the auxin exporters PIN1 or Ethylene Insensitive Root 1 (EIR1)/PIN2, and are defective in acropetal or basipetal auxin transport in roots, respectively (Blilou *et al.* 2005), while the auxin importer AUX1 functions in acropetal and basipetal auxin transport in roots (Swarup *et al.* 2001; Marchant *et al.* 2002). LR numbers in *pin1-1*and *eir1-1* mutants were inhibited more by SSA than controls, while LR numbers in *aux1-7* and *aux1-22* were less inhibited than controls (Fig. 5). Furthermore, wild-type and AUX1-complemented *aux1* mutants showed similar LR number sensitivity to SSA as controls (Fig. 5). This suggests that the inhibition by SSA of LR number requires the normal operation of AUX1, but is independent of PIN1 and PIN2. Notably, LR numbers of *aux1-7* and *aux1-22* (11-DAG) were similar to those of controls, as described elsewhere (Dubrovsky *et al.* 2006; Swarup *et al.* 2008; *cf.* Marchant *et al.* 2002; the discrepancy between studies may be attributable to different seedling ages, methods or growth conditions; Dubrovsky *et al.* 2006; Laskowski *et al.* 2008).

We further investigated LRP development and auxin response and transport in the *aux1-7* mutant. Both the total LRP and LRs and the percentage of LRs in *aux1-7* (6 DAG seedlings) were significantly ($P \leq 0.05$) less than those of controls (Fig. 6a,b). However, neither these LRP measures nor LR number were significantly ($P > 0.05$) different in *aux1-7* from controls with or without NH_4^+ (Fig. 6a,b), whereas significant reduction in the percentage of mature LRs was observed in wild-type seedlings treated with NH_4^+ ($P \leq 0.05$) compared with $-NH_4^+$ controls (Figs 2b & 6b). These observations not only confirm that AUX1 is required for LRP initiation and emergence, as reported previously (Marchant *et al.* 2002), but demonstrates that AUX1 is required for SSA inhibition of LRP emergence.

We also noted that *DR5::GUS* expression levels in vascular tissues of shoots, LRP (especially during later stages) and the primary root apex were reduced markedly in *aux1-7* (Fig. 6c) compared with controls (Fig. 3a–c, $-NH_4^+$), indicating reduced auxin-responsive signals. However, unlike in wild type (Fig. 3a–c), the *DR5::GUS* expression was markedly induced in cotyledons and slightly increased in cells adjacent to the LRP of *aux1-7* in $+NH_4^+$ medium, but was unaffected in the primary root apex (Fig. 6c). Consistently, acropetal auxin transport in *aux1-7* roots was unaffected by SSA (Fig. 6d). These results collectively demonstrate that SSA arrests LRP emergence and reduces the auxin response by reducing AUX1-dependent acropetal auxin transport in roots.

We also examined LRP emergence and auxin response in the *eir1-1* mutant. Under control conditions (without SSA), expression of *DR5::GUS* was enhanced significantly in LRP (Supporting Information Fig. S6), as described previously and attributed to a defect in basipetal auxin transport in LRP (Swarup *et al.* 2008), and also in the primary root apex in the mutant (Supporting Information Fig. S6) relative to wild type (Fig. 3b,c). As expected, SSA treatment markedly decreased both the expression of *DR5::GUS* in LRP and the primary root apex and LRP emergence in *eir1* (Supporting Information Fig. S6). These effects were more pronounced in *eir1* than in the wild type, indicating that SSA reduces LRP emergence and

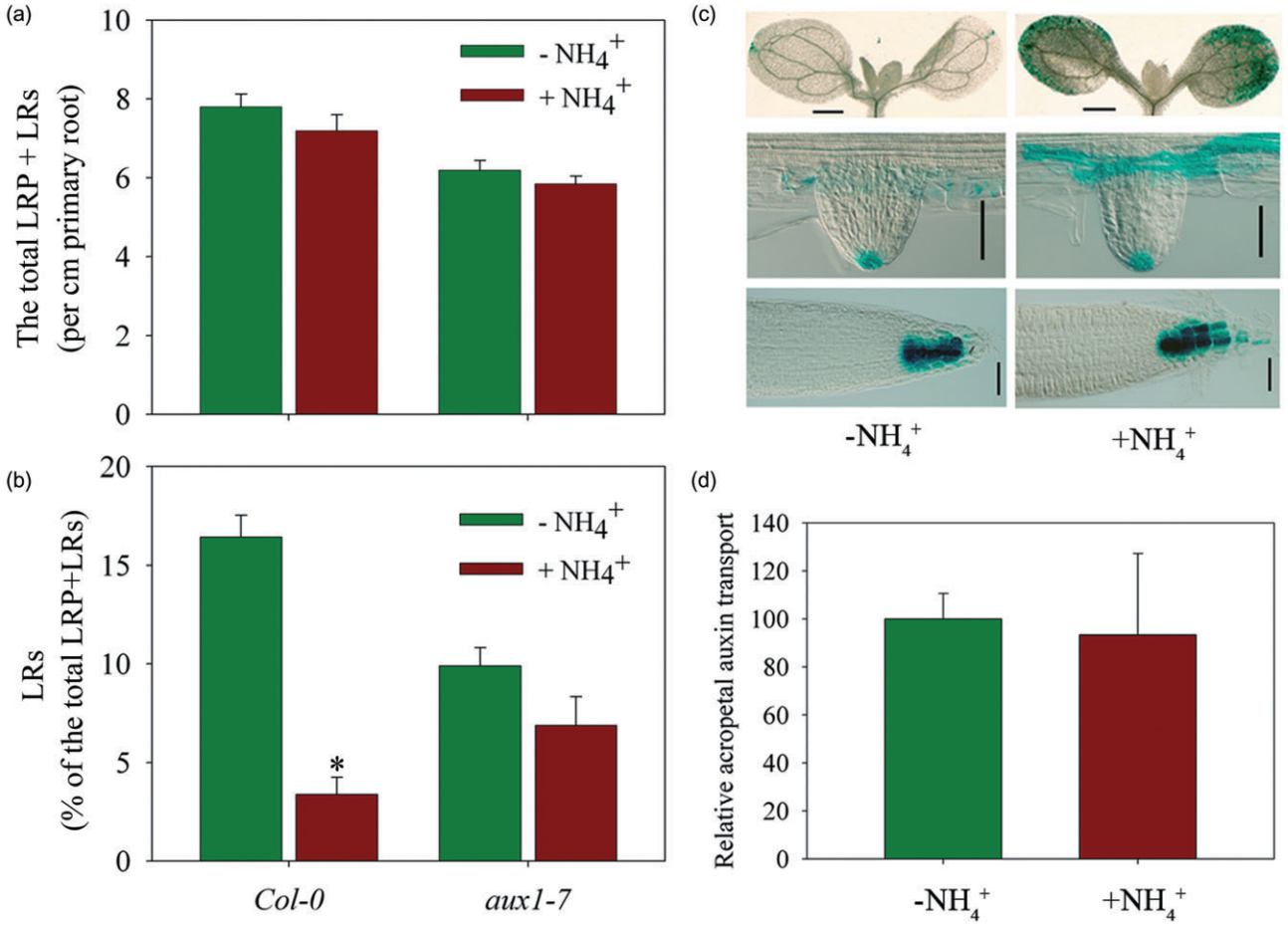

Figure 6. Effect of SSA on lateral root primordium (LRP) development, auxin response and acropetal auxin transport in *aux1-7*. (a) Comparison of the total LRP + LRs in *aux1-7* and Col-0 seedlings. (b) Comparison of the percentage of LRs in the total LRP + LRs in *aux1-7* and Col-0 seedlings. Data are from one of two experiments. Bars represent averages of 10 plants ± SE. The asterisk represents statistical significance (Student's *t*-test, $P < 0.05$). (c) Expression of *DR5::GUS* in shoots (upper panels), the adjacent cells of the just-emerged LRP (middle panels), and the primary root apex (lower panels) in *aux1-7*. One representative sample from each treatment (12 plants) is shown. Scale of bars = 1 mm (upper panels), 50 μm (middle and bottom panels). In (a–c), 4-day-old seedlings were transferred to treatment medium with or without SSA and incubated for 2 more days. (d) Effect of SSA on acropetal auxin transport in *aux1-7*, measured using ^{3}H-IAA-labelling assays. The acropetal auxin transport of *aux1-7* in $-NH_4^+$ denoted as 100%. Bars represent averages ± SE (four replicates, each with 10 seedlings).

auxin accumulation independently of the PIN2/EIR1 auxin exporter, and supporting the idea that the reduction in acropetal auxin transport is responsible for SSA inhibition of LRP emergence.

SSA modulates the expression of AUX1 at both mRNA and protein levels

To gain further insight into SSA regulation of AUX1 during LR development, expression of AUX1 in whole seedlings was analysed using the real-time PCR. *AUX1* expression was reduced by 54.6% in seedlings treated with 60 mM NH_4^+ for 6 h (Fig. 7a). To study the spatial regulation of *AUX1* by SSA, expression patterns of *ProAUX1::GUS* were examined. In $+NH_4^+$ medium, *ProAUX1::GUS* expression markedly decreased in the vascular tissues of the primary root (Fig. 7b), the LRP (mainly in the advanced stage) and in neighbouring cells (Fig. 3d). This supports the notion that AUX1 is critical to both LRP development and emergence (Marchant *et al.* 2002). On $+NH_4^+$ medium, expression of *ProAUX1::GUS* was very similar to *DR5::GUS* in wild type (Fig. 3b,c), underscoring that SSA affects the auxin response by regulating AUX1. However, *ProAUX1::GUS* expression in the root apex was not different between $-NH_4^+$ and $+NH_4^+$ medium (Fig. 7b). Moreover, the expression of the *ProAUX1::AUX1-YFP* reporter in stelar tissues of just-mature root zones, but not in LR cap cells, was reduced in $+NH_4^+$ medium (Fig. 7c,d), identical to the result obtained with *ProAUX1::GUS* (Fig. 7b). Taken together, we infer that SSA suppresses the expression of the auxin influx carrier AUX1 in vascular tissues rather than LR cap cells in roots.

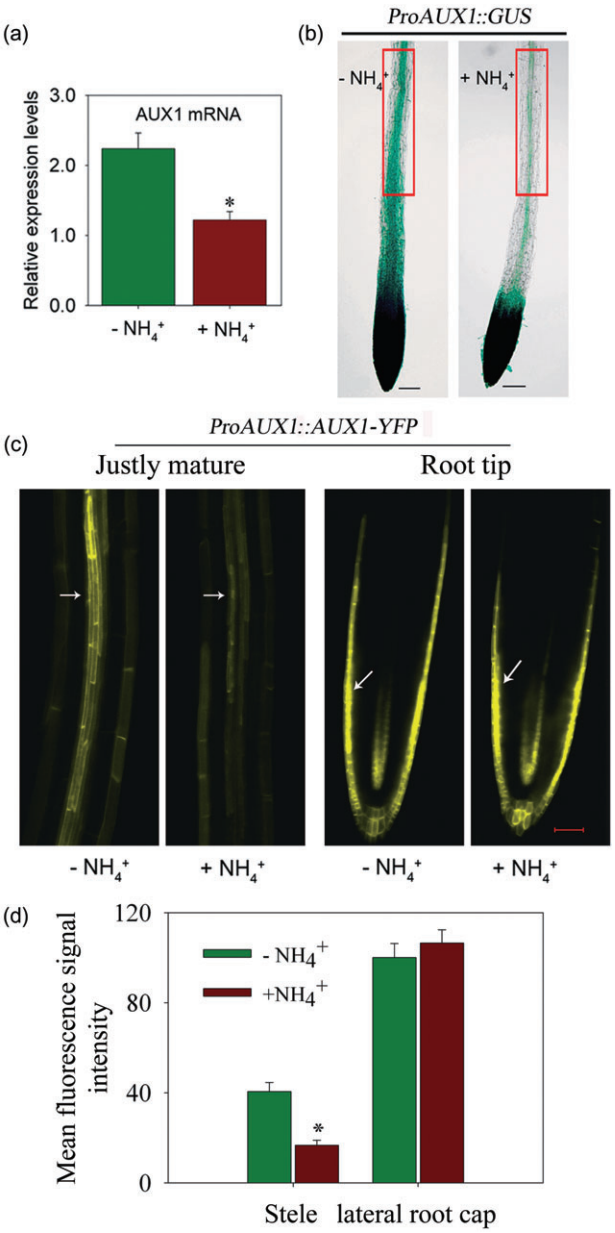

Figure 7. Effect of SSA on mRNA and protein levels of the auxin influx transporter AUX1. (a) Effect of SSA on *AUX1* mRNA levels in whole seedlings for 6 h, as measured by real-time PCR. Bars represent SE ($n = 6$). Asterisk denotes statistical significance ($P < 0.01$ in a Student's *t*-test). (b) GUS staining of the *ProAUX1::GUS* in the primary root apex. The highlighted region of the root and root tip was further examined for *ProAUX1::AUX1-YFP* expression in (c). (c) Expression of *ProAUX1::AUX1-YFP* in stele tissues and lateral root cap cells as indicated of roots. One representative sample from each treatment (10 plants) is shown in (b, c). Scale of bars = 50 μm. (d) Quantification of YFP fluorescence in stele tissues in just-matured primary root and lateral root cap cells in root tip as indicated by red arrows in (c) by image analysis of confocal sections. Bars represent SE ($n = 10$). Asterisk denotes statistical significance ($P < 0.01$ in a Student's *t*-test). The seedlings (b–d) were 4-day-old seedlings treated with or without SSA for 2 days.

DISCUSSION

SSA negatively modulates LR development by interfering with auxin transport

Our results show that SSA in *Arabidopsis* not only directly impairs the growth of shoots but also strongly arrests root development, in particular the number of LRs and their elongation. This effect contrasts with that seen with NH_4^+ applied directly to roots where the elongation of the root rather than LR number represented the principal target of inhibition (Li *et al.* 2010).

We found that SSA exerts its inhibition of LR number independently of known ABA signalling pathways, recognized as essential for LR responses to high nitrate and osmotic stresses (Signora *et al.* 2001; Deak & Malamy 2005; Xiong *et al.* 2006; MacGregor *et al.* 2008). Firstly, the inhibitory effect of SSA on LR number is more severe than that of equimolar concentrations of nitrate and K_2SO_4, osmotic stress. Secondly, SSA arrests LRP emergence without affecting LRP initiation, different from the inhibitory effect of high nitrate or ABA on meristem activation of LRP (Signora *et al.* 2001; De Smet *et al.* 2003). Moreover, ABA response and biosynthesis mutants such as *abi4-1*, *aba2-3* and *aba3-1* still displayed LR growth sensitivity to SSA similar to wild type. The LR length of *aba2-3* and *aba3-1* mutants is even more sensitive compared to wild type in response to SSA, which is in contrast to high nitrate and osmotic stresses. In addition, ABA signal is recently supposed to repress LR formation via targeting sucrose uptake in shoots by reducing the permeability in agar culture (MacGregor *et al.* 2008), whereas the increased permeability of shoots and the lack of alleviation of LR growth inhibition by sucrose application indicate that the ABA signal is not principally involved.

The inhibitory effects of SSA on LRP emergence, but not LRP initiation, resemble the phenotypes of seedlings defective in shoot-derived auxin signals (Reed *et al.* 1998; Casimiro *et al.* 2001; Bhalerao *et al.* 2002). Analysis of the effect of SSA on auxin response and auxin transport in roots indeed suggests that an important part of the NH_4^+-driven inhibition on LR formation is mediated by the auxin pathway. We confirmed that SSA decreased auxin transport from the shoot to the root apex and that this process requires the auxin importer AUX1. Interestingly, although application of auxin to roots almost completely restored the LR number in SSA-treated wild-type seedlings, SSA treatment still exerted some inhibition on LR number in *aux1* mutants, suggesting that there are additional AUX1-independent, or, more generally, auxin-independent mechanisms involved in the NH_4^+ inhibition of LR formation. It should be noted that LAX3, of the AUX1/LAX families, is also involved in LR formation, but acts at different steps (Swarup *et al.* 2008). Whether SSA also affects the expression of other members in AUX1/LAX families? Indeed, SSA reduced the expression levels of other AUX/LAX family members both in the wild type and in *aux1* seedlings (Supporting Information Fig. S7). SSA may, thus, regulate both AUX1 and other AUX/LAX members to modulate LR development.

SSA reduces the auxin response in roots via a long-distance regulatory mechanism mediated by the auxin importer AUX1

The NH_4^+-induced changes in growth and development are undoubtedly linked to hormonal balance including auxin, despite many contradictory literature and arguments regarding this (Britto & Kronzucker 2002). Here, we showed that the SSA inhibition of LR number can be rescued by exogenous auxin application, indicating a direct link between NH_4^+ toxicity and an auxin signal. Consistently, SSA caused a dramatic reduction in *DR5::GUS* expression levels in the vascular tissues of the shoot, LRP, their adjacent cell and the primary root apex. Moreover, SSA modulated auxin transport from shoot to root (acropetal auxin transport) rather than impairing basipetal auxin transport in roots or auxin biosynthesis in shoots or reducing root-cell sensitivity to auxin by using both the *DR5::GUS*-based method and ^{3}H-IAA-labelling method.

Many *Arabidopsis* mutants disrupted in auxin transport exhibit disturbed LRP emergence (Peret *et al*. 2009). Mutant analysis indicated that the auxin importer AUX1, but not the auxin exporters PIN1 or PIN2, is required for SSA inhibition of LR number. AUX1 has been shown to be required for NH_4^+ inhibition of primary root growth in a low potassium medium (Cao *et al*. 1993). Furthermore, the inhibitory effects of SSA on the auxin response, acropetal auxin transport and LRP emergence were alleviated in *aux1* mutants. Additionally, SSA treatments abolished the increase in *DR5::GUS* activity in the root apex of the *eir1-1* mutant caused by its defective basipetal auxin transport, further supporting the notion that SSA modulates acropetal auxin transport. Indeed, SSA decreased the expression of both AUX1 mRNA and protein in vascular tissues rather than LR cap cells in roots, which is consistent with the results of reductions of acropetal auxin transport but not basipetal auxin transport in SSA treatment. Collectively, this suggests that SSA generates a signal to modulate LRP emergence by reducing auxin transport from shoot to root apex via AUX1; they also suggest that there are independent regulatory mechanisms for auxin import and export (Kleine-Vehn *et al*. 2006; Vieten *et al*. 2007). Moreover, the strikingly similar expression patterns of *DR5::GUS* and *ProAUX1::GUS* in LRP and adjacent cells and vascular tissues of the primary root suggest a feedback loop between auxin levels and *AUX1* expression (Laskowski *et al*. 2008). Therefore, SSA might first modulate local expression of AUX1 to reduce auxin influx, and consequently, lower auxin levels might then further decrease AUX1, affecting long-distance auxin transport. Thus, the effect of SSA on AUX1 expression in the root could be a consequence of reduced auxin transport from the shoot.

The role of AUX1 in acropetal, unlike in well-confirmed basipetal (Swarup *et al*. 2005; De Smet *et al*. 2007), auxin transport has just been clarified in recent reports (Laskowski *et al*. 2008; Negi *et al*. 2008). The strong expression of AUX1 in mature zones of roots (our results, and see also Laskowski *et al*. 2008) indicates that AUX1 plays an important role in acropetal auxin transport and LR formation. The role of AUX1 in acropetal IAA transport is further supported by direct measurements under elevated ethylene treatment (Negi *et al*. 2008). Our results show that AUX1 is important for acropetal auxin transport and LR formation under SSA conditions.

Notably, SSA increased the auxin response in cotyledons and LRP-adjacent cells in the *aux1-7* mutant. A simple explanation is that, when AUX1 is defective, auxin synthesis or other transport pathways may be up-regulated and therefore replenish auxin transport in response to SSA. However, this could be masked by inhibition of AUX1-mediated auxin transport in the wild-type or other auxin transport mutants that retain functional AUX1 in response to SSA. Thus, pathway replenishment by SSA in the *aux1-7* background warrants further investigation. In conclusion, we propose a regulatory pathway that accounts for a large portion of the SSA effects on LR development. SSA strongly inhibits LR formation independent of either the ABA-mediated pathway or the shoot sucrose uptake pathway. Rather, SSA diminishes auxin transport from shoots to the root tip (acropetal auxin transport) via modulating the expression of the auxin importer AUX1. This results in reduced root auxin responses and causes the arrest of LRP emergence. Our study may provide an attractive experimental framework to study the regulation of root development by aboveground environmental signals in *Arabidopsis*. The mechanism of NH_4^+ toxicity described here is expected to operate in addition to other physiological changes under NH_4^+ exposure such as disturbance of cellular pH or ion balance (Wollenweber & Raven 1993; Hanstein & Felle 1999; Britto & Kronzucker 2002), the energy cost associated with plasma-membrane NH_4^+ fluxes (Britto *et al*. 2001; Kronzucker *et al*. 2001) or reduced efficiencies in protein glycosylation (Yang & Butler 2000; Marcaggi & Coles 2001; Hess *et al*. 2006; Qin *et al*. 2008; Barth *et al*. 2010; Li *et al*. 2010). Excessive atmospheric NH_4^+ deposition, in addition to excessive foliar nitrogen fertilizer applications, is an emerging issue (Krupa 2003; Stevens *et al*. 2004; Castro *et al*. 2005; Clark & Tilman 2008), compounding excessive soil NH_4^+ (Wolt 1994; Kronzucker *et al*. 2003) and subjecting crop leaf systems to very high nitrogen levels, in particular in the stage of germination or in species with low-lying canopies, such as members of the Brassicaceae. Our study suggests that plants may have evolved limited regulatory strategies to cope with the environmental challenge of canopy exposure to high NH_4^+.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Characterization of the NH_4^+-induced inhibition of LR number and length.

Figure S2. Effects of shoot-supplied K^+ (as K_2SO_4), NO_3^- (as KNO_3), L-glutamate (Glu), L-glutamine (Gln), and mannitol on LR number and length in Col-0.

Figure S3. Effect of SSA on abscisic acid mutants.

Figure S4. Toluidine blue O (TB) staining of shoots, and the effect of external sucrose on lateral root (LR) formation under SSA treatment.

Figure S5. Effects of external auxin application on LR length and number in Col-0 with or without SSA.

Figure S6. Effect of SSA on LRP Emergence and *DR5::GUS* expression in *eir1-1*.

Figure S7. Real-time quantitative PCR analysis of the expression of the *AUX1/LAX* gene family members *LAX1*, *LAX2*, and *LAX3* as affected by SSA.

Table S1. Primers used in this study.

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