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RESEARCH PAPER

Ammonium-induced shoot ethylene production is associated with the inhibition of lateral root formation in *Arabidopsis*

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Abstract

Foliar NH_4^+ exposure is linked to inhibition of lateral root (LR) formation. Here, the role of shoot ethylene in NH_4^+-induced inhibition of LR formation in *Arabidopsis* was investigated using wild-type and mutant lines that show either blocked ethylene signalling (*etr1*) or enhanced ethylene synthesis (*eto1*, *xbat32*). NH_4^+ exposure of wild-type *Arabidopsis* led to pronounced inhibition of LR production chiefly in the distal root, and triggered ethylene evolution and enhanced activity of the ethylene reporter *EBS:GUS* in the shoot. It is shown that shoot contact with NH_4^+ is necessary to stimulate shoot ethylene evolution. The ethylene antagonists Ag^+ and aminoethoxyvinylglycine (AVG) mitigated LR inhibition under NH_4^+ treatment. The decrease in LR production was significantly greater for *eto1-1* and *xbat32* and significantly less for *etr1-3*. Enhanced shoot ethylene synthesis/signalling blocked recovery of LR production when auxin was applied in the presence of NH_4^+ and negatively impacted shoot AUX1 expression. The findings highlight the important role of shoot ethylene evolution in NH_4^+-mediated inhibition of LR formation.

Key words: Ammonium toxicity, *Arabidopsis*, auxin, ethylene, lateral root.

Introduction

Ammonium (NH_4^+), an important source of nitrogen for many species (Kronzucker *et al*., 1997, 2003; Balkos *et al*., 2010), is frequently present in soils and in the atmosphere in significant quantities (Britto and Kronzucker, 2002; Dupre *et al*., 2009). The primary mechanisms underlying NH_4^+ toxicity in plants are still under debate (Britto *et al*., 2001; Cruz *et al*., 2006; Szczerba *et al*., 2006; ten Hoopen *et al*., 2010; Kempinski *et al*., 2011; Li *et al*., 2012), but alterations in hormonal balance, including that of ethylene, have been clearly implicated (Barker and Corey, 1991; Barker, 1999*a*, *b*; You and Barker, 2005; Barth *et al*., 2010; Li *et al*., 2011*b*). Ethylene production was shown to increase linearly with tissue NH_4^+ accumulation (Barker, 1999*a*). You and Barker (2002, 2005) reported that ethylene evolution was preceded by NH_4^+ accumulation and was detected before, or concurrent with, the development of toxicity symptoms. In addition, it has been shown that the application of aminooxyacetic acid and silver thiosulphate, inhibitors of ethylene synthesis and action, can ameliorate symptoms of NH_4^+ toxicity (Barker and Corey, 1991; Feng and Barker, 1992*b*, *d*; You and Barker, 2005). However, the detailed characteristics and mechanisms of ethylene signalling in NH_4^+ stress responses remain largely unclear.

A second major phytohormone that has been linked to NH_4^+ toxicity is auxin [indole acetic acid (IAA)], which serves as an important positive regulator during lateral root (LR) development (Peret *et al*., 2009). Rootward (acropetal) auxin transport through the central cylinder of the root provides the auxin essential for LR emergence and the subsequent elongation or growth, and inhibition of acropetal auxin movement reduces LR number and is reversible by auxin (Bhalerao *et al*., 2002). AUX1, an auxin influx carrier, has been suggested to

be involved in acropetal auxin transport and LR primodia (LRP) emergence (Swarup *et al.*, 2001; Laskowski *et al.*, 2008). AUX1 mutants have significantly reduced acropetal auxin transport (Rashotte *et al.*, 2003) and LR formation (Marchant *et al.*, 2002). The function of AUX1 is furthermore dependent on ethylene (Negi *et al.*, 2008), and ethylene is clearly implicated in LR formation (Ivanchenko *et al.*, 2008; Negi *et al.*, 2008, 2010; Lewis *et al.*, 2011). Root treatment with ethylene or its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) reduces LR formation in *Arabidopsis* and tomato (Negi *et al.*, 2008, 2010). Additionally, LR number is reduced in the ethylene-overproducing mutants *eto1* and *xbat32*, while the ethylene-insensitive mutants *etr1-3* (*Arabidopsis*) and *Nr* (tomato) have increased LR number (Negi *et al.*, 2008, 2010; Prasad *et al.*, 2010; Strader *et al.*, 2010). High levels of ethylene in the root environment promote both acropetal and basipetal auxin transport in the root, preventing the localized accumulation of auxin needed to drive LR production (Negi *et al.*, 2008; Lewis *et al.*, 2011). However, high levels of ethylene in shoots negatively regulate auxin transport in the shoot (Negi *et al.*, 2010).

A stunted root system is a significant symptom of NH_4^+ toxicity (Britto and Kronzucker, 2002; Balkos *et al.*, 2010), but little is known about the effects of NH_4^+ on LR development. Li *et al.* (2011*b*) found that the effect of NH_4^+ on LR formation was dependent on the locus of NH_4^+ exposure. Shoot-supplied ammonium (SSA) reduced LR formation, whereas root-supplied ammonium (RSA) increased it (Li *et al.*, 2010). The study also suggested that SSA inhibits LR number by interfering with AUX1-dependent auxin transport from shoot to root. In contrast, there has been no study to evaluate the role of ethylene and its interaction with auxin in high-NH_4^+-induced suppression of LR development.

To elucidate the mechanism underlying the inhibition of LR development by SSA, the following research questions were posed in this study. (i) Is LR development in response to SSA equally affected in mature and newly formed root portions? (ii) Is the inhibition of LR number by SSA independent of shoot reactive oxygen species (ROS) accumulation? (iii) Is NH_4^+ contact with the shoot necessary to stimulate shoot ethylene evolution? (iv) Does shoot ethylene modulate LR development under SSA conditions? (v) Is shoot ethylene linked to the SSA-related inhibition of auxin transport from shoot to root? (vi) Is the AUX1 gene in shoots regulated by ethylene under exposure to SSA?

Materials and methods

Plant materials and growth conditions

All experiments were conducted using *Arabidopsis thaliana* (Columbia ecotype). Seeds of *etr1-3* (Guzman and Ecker, 1990), *eto1-1* (Alonso and Stepanova, 2004), and *xbat32* (Nodzon *et al.*, 2004; Prasad *et al.*, 2010) mutants, all in the Col-0 background, were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). The *EBS:GUS* line was kindly provided by J. Alonso, and was originally generated by Dr Anna Stepanova (Stepanova *et al.*, 2005). The *DR5:GUS* line was provided by Dr Tom J. Guilfoyle (University of Missouri). 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 *et al.* (1993), and was composed of 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-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^{-1} 2-(*N*-morpholino) ethanesulphonic acid (MES), and 0.8% agar (adjusted to pH 5.7 with 1 M NaOH). The day of sowing was considered as day 0. Seedlings were grown, oriented vertically, on the surface of the culture plates in a growth chamber set at a 16 h light/8 h dark photoperiod, an irradiance of 100 µmol m^{-2} s^{-1}, and a constant temperature of 23 ± 1 °C.

Shoot supply experiments

The shoot supply experiments were analysed as described in a previous report (Li *et al.*, 2011*b*), and briefly as follows: custom-made segmented plates (13 × 13 cm) were separated into the upper and bottom parts with a 3 mm air gap (Zhang and Forde, 1998), using two fixed plastic strips of 2 mm in height and a movable glass strip of 3 mm in width. The plates effectively prevented mixing of the upper and bottom parts. Normal growth medium (control medium) was poured into the bottom part, and control medium with various concentrations of $(NH_4)_2SO_4$ supplemented with or without various chemicals, as indicated in the figure legends, was poured into the upper part (see Supplementary Fig. S1 available at *JXB* online). The pH was held at 5.7 in both the upper and bottom media. In the whole study, ammonium was applied to shoots as described above; '–NH_4^+' signifies the control (0 mM NH_4^+) and '+NH_4^+' the shoot ammonium treatment. To study the effect of ethylene inhibitors, ROS inhibitors, and IAA, the upper medium was supplemented with or without NH_4^+ plus the indicated concentrations of silver nitrate ($AgNO_3$), aminoethoxyvinylglycine (AVG), diphenyleneiodonium (DPI), dimethylthiourea (DMTU), hydrogen peroxide (H_2O_2), ACC, or IAA. All chemicals used were obtained from Sigma-Aldrich. Seedlings were transferred to the segmented plates for treatment at 5 days after germination (5 DAG) (primary root length was ~2 cm at this stage of development), and positioned such that only the shoots were in contact with the upper medium. As discussed previously (Li and Shi, 2007; Li *et al.*, 2010), to achieve growth suppression and tissue NH_4^+ contents in agar media similar to those seen in hydroponic NH_4^+ toxicity studies (Britto *et al.*, 2001; Szczerba *et al.*, 2008; Balkos *et al.*, 2010), 20–40 mM NH_4^+ had to be applied.

Lateral root quantification

Five-day-old seedlings were transferred to new media, containing either no additions or varying concentrations of $(NH_4)_2SO_4$ with or without the indicated amounts of $AgNO_3$, AVG, DPI, DMTU, H_2O_2, ACC, or IAA. In this study, the emerged but not activated LRP are still referred to as LRP (Malamy and Benfey, 1997), and only mature LRs (>0.5 mm in length) are denoted as LRs (Zhang *et al.*, 1999). The number of mature LRs was counted under a dissecting microscope after an additional 3–5 d of growth. For LRP analysis, root portions that were formed before or during the treatments were harvested separately, and then LRP were counted and classified using the methods and nomenclature described in Malamy and Benfey (1997).

Histochemical staining and image analysis

Histochemical staining of H_2O_2 was performed as previously described (Dong *et al.*, 2009) with minor modifications. Leaves were vacuum-infiltrated with 0.1 mg ml^{-1} 3, 3'-diaminobenzidine (DAB) in 50 mM TRIS-acetate buffer, at pH 5.0. Samples were incubated for 24 h at room temperature in the dark prior to transfer to 80% ethanol. Histochemical analysis of β-glucuronidase (GUS) reporter enzyme activity was performed as described elsewhere (Weigel and

Glazebrook, 2002). GUS staining patterns in roots were analysed using an Olympus BX51 microscope with differential interference contrast (DIC) optics, and GUS or ROS staining in the shoot using an Olympus SZX10 stereo microscope. For photographs of whole seedlings, seedlings were transferred to agar plates to keep them moist, scanned using a desktop scanner, and then photographs were taken. All staining and image analysis procedures were repeated at least twice. All the images and graphs were arranged in Adobe Photoshop.

Ethylene measurements

After seedling exposure to 30 mM NH_4^+ for 1 d, roots and shoots were weighed separately and put into 5 ml gas-tight vials containing 1 ml of agar medium (0.7% agar). Headspace samples (1 ml) were withdrawn and analysed using a GC-6850 gas chromatograph (Agilent Technologies Japan, Ltd), which was equipped with an FID detector.

DR5:GUS-based auxin transport assay

For measurement of auxin transport, the method as described by Lewis and Muday (2009) was used. In brief, to estimate acropetal auxin transport, plates containing control seedlings (5 DAG *DR5:GUS* plants), IAA-treated or IAA- and $AgNO_3$-co-treated seedlings were incubated for 48 h. IAA or IAA and $AgNO_3$ co-treatments were conducted by placing agar solidified with 3 μM IAA or 50 μM $AgNO_3$ on shoots. Entire seedlings were then subjected to GUS staining for 16 h at 37 °C. At least 10 seedlings for each treatment were measured, and experiments were repeated twice.

Real-time quantitative PCR analysis

Seedlings (6 DAG) were treated for 6 h with or without 40 mM NH_4^+. The shoots 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 of total RNA with Superscript transcriptase M-MLV (TaKaRa) and used as the template for PCR amplification with specific primers for the selected genes. The sequences of the primers used are given in Supplementary Table S1 at *JXB* online.

PCR was amplified with the primers of two genes and 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 (Xu and Shi, 2006). CBP20 encoding nuclear cap-binding protein was used as the housekeeping gene, and relative RNA abundance was normalized to the CBP20 internal control ($[mRNA]_{gene}/[mRNA]_{CBP20}$).

Statistical and graphical analyses

For all experiments, data were statistically analysed using the SPSS 13.0 program (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) with a Duncan post-hoc test was used. Graphs were produced using Origin 8.0. All graphs and images were arranged using Adobe Photoshop 7.0.

Results

SSA inhibits lateral root number in both proximal and distal portions

To analyse the effect of SSA on LR development, only *Arabidopsis* shoots were allowed to come into contact with NH_4^+ (see the Materials and methods; Supplementary Fig. S1 at *JXB* online). As shown in Fig. 1A, LR number was reduced by SSA, and this was positively related to NH_4^+

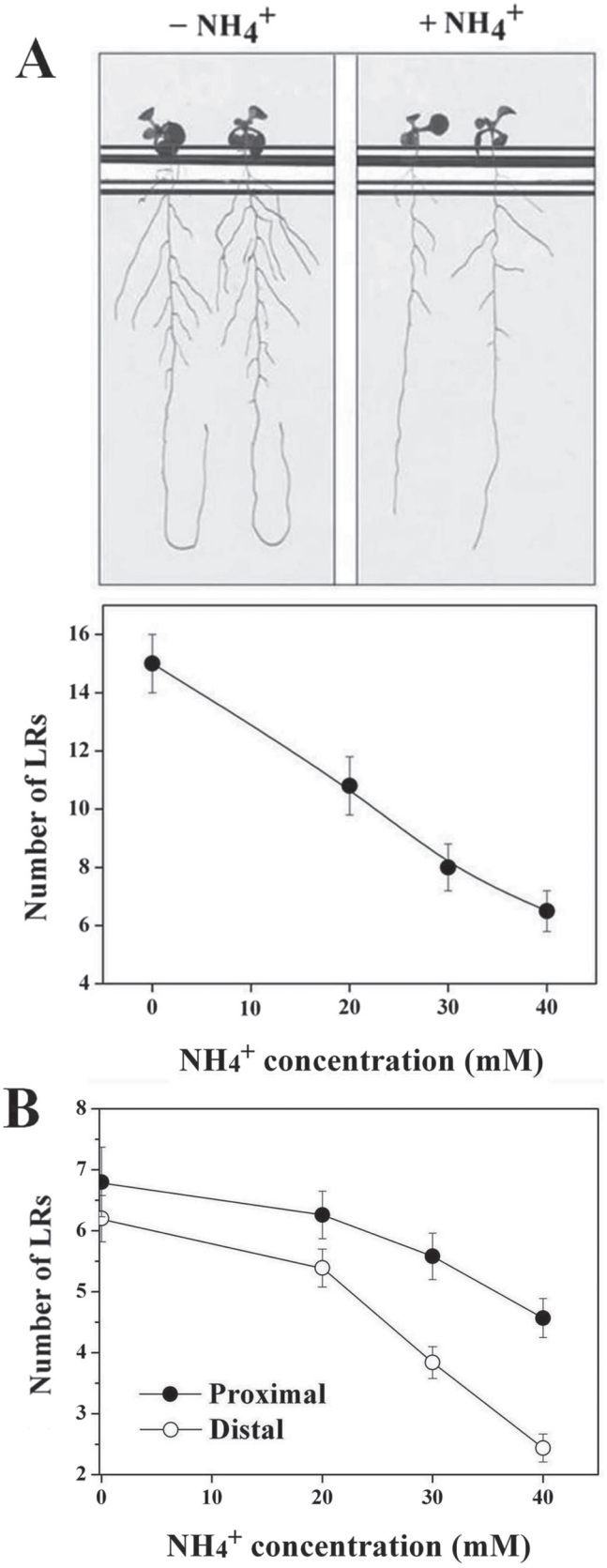

Fig. 1. Inhibitory effect of SSA on LR number in *Arabidopsis* (Col-0). Seedlings at 5 day after germination (5 DAG) were transferred to the SSA treatment medium and grown for an additional 5 d. (A) Photograph of representative seedlings after 5 d of vertical growth in medium lacking or supplemented with 30 mM NH_4^+

concentrations. This SSA inhibition was very similar to that affected by whole-plant-supplied ammonium (WSA) (where both shoots and roots come into contact with NH_4^+; Li *et al.*, 2011*b*; Supplementary Fig. S2A). However, RSA increased LR number even when the NH_4^+ concentration was raised to 50 mM (Supplementary Fig. S2A; Li *et al.*, 2010). Since LR number was inhibited in response to $(NH_4)_2SO_4$ applications, it was necessary to determine the role of the SO_4^{2-} ion, a high nitrogen level *per se*, and nitrogen metabolites (such as glutamate and glutamine), to establish whether LR number inhibition was directly caused by NH_4^+. K_2SO_4, KNO_3, glutamate, and glutamine were examined. Reductions in LR number in media containing equivalent K_2SO_4 or KNO_3 concentrations did not reach those with $(NH_4)_2SO_4$ (Supplementary Fig. S3). With high concentrations of glutamate or glutamine, LR number remained unaffected (Supplementary Fig. S3).

To explore further the effect of SSA on LR development, two root portions were analysed separately: the portion that existed at the time of transfer, named as 'proximal', and the portion that formed after transfer, named as 'distal'. NH_4^+-treated proximal and distal portions displayed a dose-dependent inhibitory effect, but LRs in the distal portion were inhibited more significantly than those in the proximal portion at all concentrations (~60% reduction in distal versus ~35% in proximal, respectively, at 40 mM NH_4^+) (Fig. 1B). Next, the effects of SSA on LRP development and LR emergence in the proximal and distal regions were investigated. LRP development was examined in *DR5:GUS* seedlings by monitoring GUS activities, based on previous observations that *DR5* is active at all stages of LRP (Benkova *et al.*, 2003; Dubrovsky *et al.*, 2008). The developmental stage of each LRP was classified according to Zhang *et al.* (1999): stage A, up to three cell layers; stage B, unemerged, more than three cell layers; stage C, emerged LRs, <0.5 mm in length; stage D, LRs, >0.5 mm. Total LRP and LRs under exposure to high NH_4^+ were similar to those in the absence of NH_4^+, and the percentage of proximal to distal portions was undifferentiated (Fig. 2A). In proximal portions grown in the absence of NH_4^+, most LRP were classified as stage D and almost no stage A LRP were observed (Fig. 2B). Interestingly, in SSA conditions, a decrease in LRP transition from early to late stages of development was observed; the percentage of stage A LRP in SSA-treated seedlings was significantly increased compared with that in control media, whereas the percentage of stage D LRP developed in the reverse manner (Fig. 2B). In contrast, it was observed that, in the distal portions, unemerged stage B LRP increased significantly, whereas the number of stage C and stage D LRP was reduced markedly in SSA-treated seedlings (Fig. 2B).

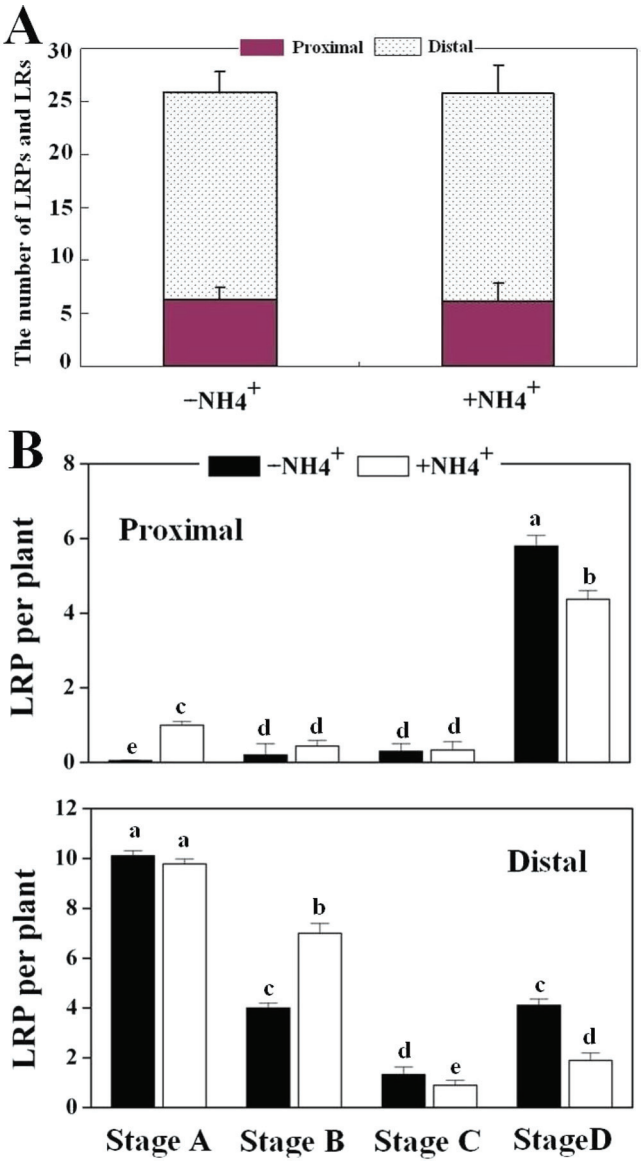

Fig. 2. Effect of SSA on LRP and LR development in proximal and distal portions. Transgenic *DR5::GUS* in Col-0 seedlings (5 DAG) were transferred to the treatment medium with or without 40 mM NH_4^+ in the shoots and incubated for 4 d prior to staining for GUS. (A) Number of LRP and LRs in proximal and distal portions. (B) LR stage distribution (at 9 d) in proximal and distal portions. Data from two experiments are combined. Bars represent averages of 10 plants ±SE. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).

(upper panel), and quantification of LR number inhibition by 20, 30, and 40 mM NH_4^+ (bottom panel). (B) The inhibition of LR number in the proximal and distal root portions as affected by varying concentrations of NH_4^+. Data from three experiments are combined. Data represent means of 15 or more plants ±SE.

Inhibitory action of SSA on lateral root number does not involve ROS accumulation in the shoot

To evaluate the role of shoot ROS in NH_4^+-induced inhibition of LR number, attempts were first made to detect *in situ* accumulation of H_2O_2, using DAB staining. Without SSA treatment, the shoot showed minimal DAB staining. However, following a 2 d NH_4^+ treatment, a significant increase in H_2O_2 was detected in the shoot (Fig. 3A). To examine whether the

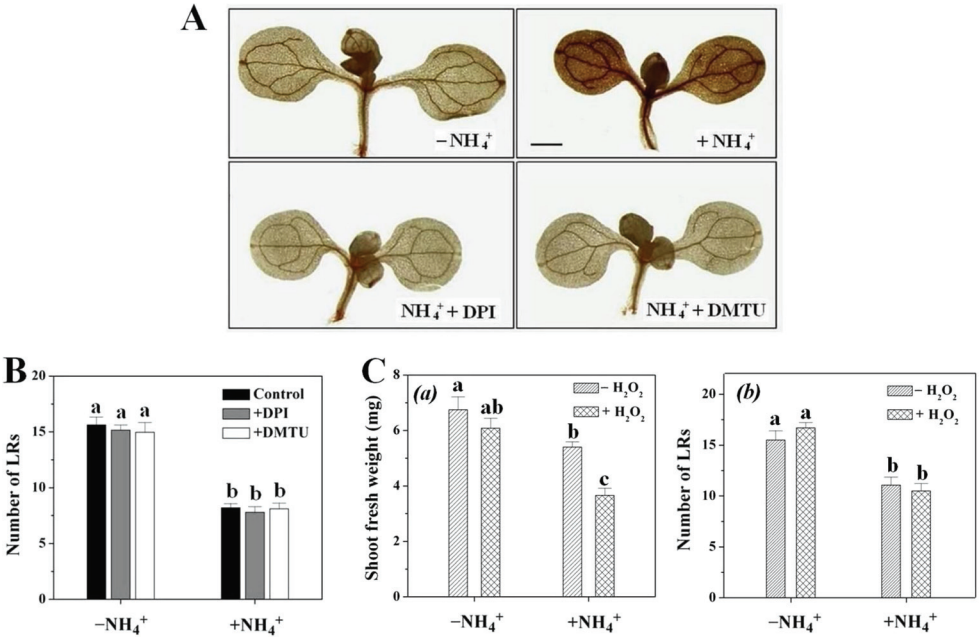

Fig. 3. Effect of DPI or DMTU on SSA-induced shoot ROS accumulation and LR number inhibition in wild-type seedlings. (A) *In situ* detection of leaf hydrogen peroxide. Seedlings at 5 DAG were exposed to 30 mM NH_4^+ alone or in combination with 100 μM DPI or 5 mM DMTU in the shoots for 2 d, and then DAB staining of shoots was performed. Scale bars=0.5 mm. (B) LR number in different treatments. (C) Shoot fresh weight (a) and LR number (b) in different treatments. Five-day-old seedlings were transferred to medium and shoots were supplemented with or without 30 mM NH_4^+ alone or in combination with 100 μM DPI, 5 mM DMTU, or 2 mM H_2O_2 for 5 d. Values are the mean ±SE, *n*=8–11. Different letters represent means statistically different at the 0.01 level (one-way ANOVA with Duncan post-hoc test).

inhibitory effect of SSA on *Arabidopsis* LR formation was related to the accumulation of ROS in the shoot, the effects of the NADPH oxidase inhibitor DPI and the ROS scavenger DMTU on shoot ROS accumulation and on LR formation were examined. As shown in Fig. 3A, both DPI and DMTU abolished NH_4^+-induced H_2O_2 shoot accumulation. However, the abolition of NH_4^+-induced H_2O_2 accumulation did not alleviate the inhibition of LR number (Fig. 3B). In the absence of NH_4^+, LR number was also not affected by the two scavengers (Fig. 3B). The effects of SSA on wild-type growth were also examined in combination with external H_2O_2. The combination treatment of NH_4^+ and H_2O_2 in the shoot medium markedly inhibited shoot growth but did not suppress LR number to any greater extent than NH_4^+ treatment alone (Fig. 3C).

Shoot ethylene evolution is enhanced under SSA conditions

In order to examine whether ethylene plays a role in SSA inhibition of LR formation, the effect of SSA on ethylene accumulation was first analysed in different tissues of *Arabidopsis* by the use of the ethylene reporter *EBS:GUS* (an ethylene reporter construct in which the GUS reporter gene is driven by a synthetic EIN3-responsive promoter). It was hypothesized that SSA might enhance ethylene accumulation in the whole seedling or at least in the root. As shown in Fig. 4A, on NH_4^+-treated medium, shoot *EBS:GUS* expression was enhanced markedly, compared with controls grown in the absence of NH_4^+. In contrast, no changes in root *EBS:GUS* activities were observed in response to NH_4^+ (Fig. 4B). Ethylene evolution was additionally investigated by use of gas chromatography. As shown in Fig. 4C, shoot ethylene production under NH_4^+ exposure was significantly greater than in the absence of NH_4^+, whereas root ethylene production was unaffected, consistent with the results from GUS staining. To examine further how SSA might induce endogenous ethylene production, a split-shoot experiment was devised, transferring 5-day-old *Arabidopsis EBS:GUS* seedlings with one cotyledon positioned on $-NH_4^+$ medium and the other on high NH_4^+ medium (Fig. 5A). After 48 h, no visible expression of *EBS:GUS* was observed in the untreated cotyledon (Fig. 5B). Surprisingly, the cotyledon supplied with NH_4^+ displayed significantly increased *EBS:GUS* expression (Fig. 5B).

As ACC synthase (ACS) and ACC oxidase (ACO) are key enzymes of the ethylene biosynthetic pathway in plants, the question was asked whether the observed induction of ethylene production by high NH_4^+ is due to regulation of expression of the genes encoding ACS and ACO in shoots. To answer this question, four genes for ACS, *AtACS2*, *AtACS7*, *AtACS8*, and *AtACS11*, and two for ACO, *AtACO1* and *AtACO2*, were examined using quantitative real-time PCR. As shown in Fig. 5C, all ACS genes responded with increased transcript abundance following 6 h SSA treatment. Expression of *AtACO1* and *AtACO2* was also rapidly up-regulated in response to SSA treatment after 6 h (Fig. 5C).

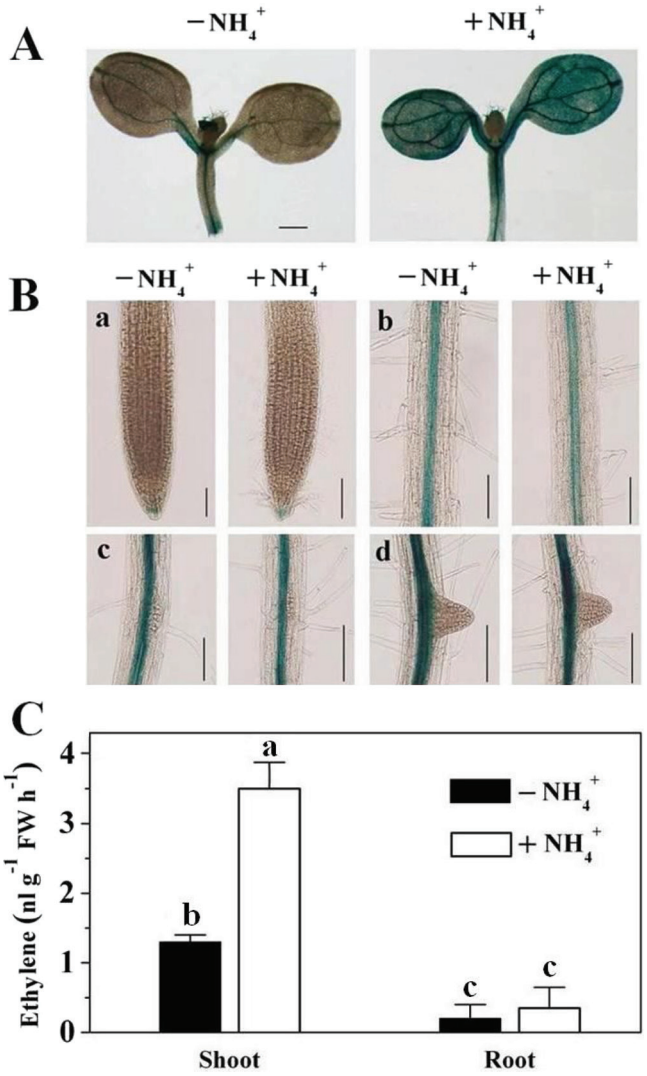

Fig. 4. Effect of SSA on ethylene response in wild-type *Arabidopsis* seedlings. Seedlings at 5 DAG were exposed to 30 mM NH_4^+ in the shoots for 24 h, and then ethylene reporter *EBS::GUS* activity (A, B) and ethylene evolution (C) were determined. (A) Activity of *EBS::GUS* in *Arabidopsis* shoot tissue. One representative sample from each treatment (10 plants) is shown. Scale bars=0.5 mm. (B) Activity of *EBS::GUS* in *Arabidopsis* root tissue. One representative sample from each treatment (10 plants) is shown. Scale bars=50 µm. (a) Primary root apex; (b) stele of primary root; (c, d) immature lateral roots. (C) Ethylene evolution in *Arabidopsis* shoot and root. Values are means ±SE of three replicates. Different letters represent means statistically different at the 0.01 level (one-way ANOVA with Duncan post-hoc test).

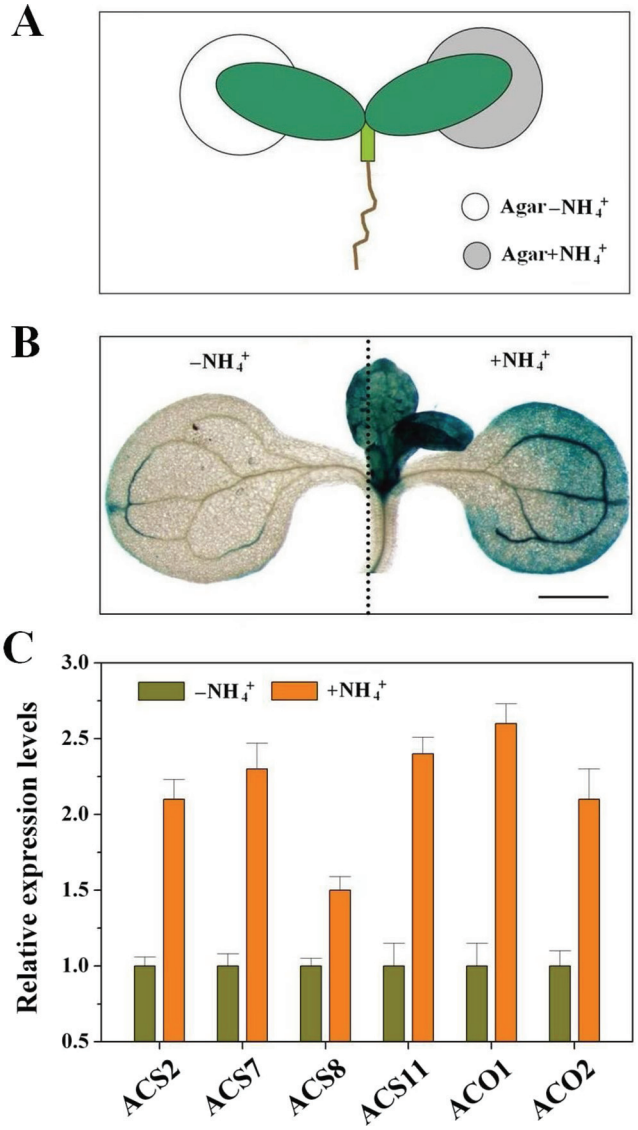

Fig. 5. *EBS:GUS* expression in the split-shoot experiment (A and B) and effect of SSA on expression of shoot aminocyclopropane carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) (C) of wild-type *Arabidopsis* seedlings. (A) Schematic diagram of the experimental set-up for the split-shoot experiment. *Arabidopsis* seedlings were germinated on the growth medium. At day 5, half of the cotyledon was positioned on 40 mM NH_4^+ medium, while the other half of the cotyledon remained in normal growth medium. After 48 h, *EBS:GUS* activity was measured. (B) The expression of *EBS:GUS* in the split-shoot experiment. One representative sample from each treatment (10 plants) is shown. Scale bars=1 mm. (C) Effect of high NH_4^+ concentration on expression of shoot ACS and ACO. Expression of shoot ACS and ACO was determined by quantitative real-time PCR after exposure of 6-day-old wild-type seedlings to 40 mM NH_4^+ for 6 h. Values are means ±SE of three replicates.

Enhanced shoot ethylene signalling and/or synthesis is involved in SSA-mediated inhibition of lateral root number

To examine whether increased shoot ethylene signalling and/or biosynthesis is involved in the inhibitory effect of SSA on LR number, the effects of antagonists of ethylene perception ($AgNO_3$) and ethylene biosynthesis (AVG) on LR number in the presence of SSA were investigated. As shown in Fig. 6A, LR number increased by ~54% and ~47% in SSA medium plus 50 µM $AgNO_3$ or 1 µM AVG in the shoot, respectively. Given that SSA markedly inhibited LR number in the distal primary root portion formed during the treatment (cf. Fig. 1),

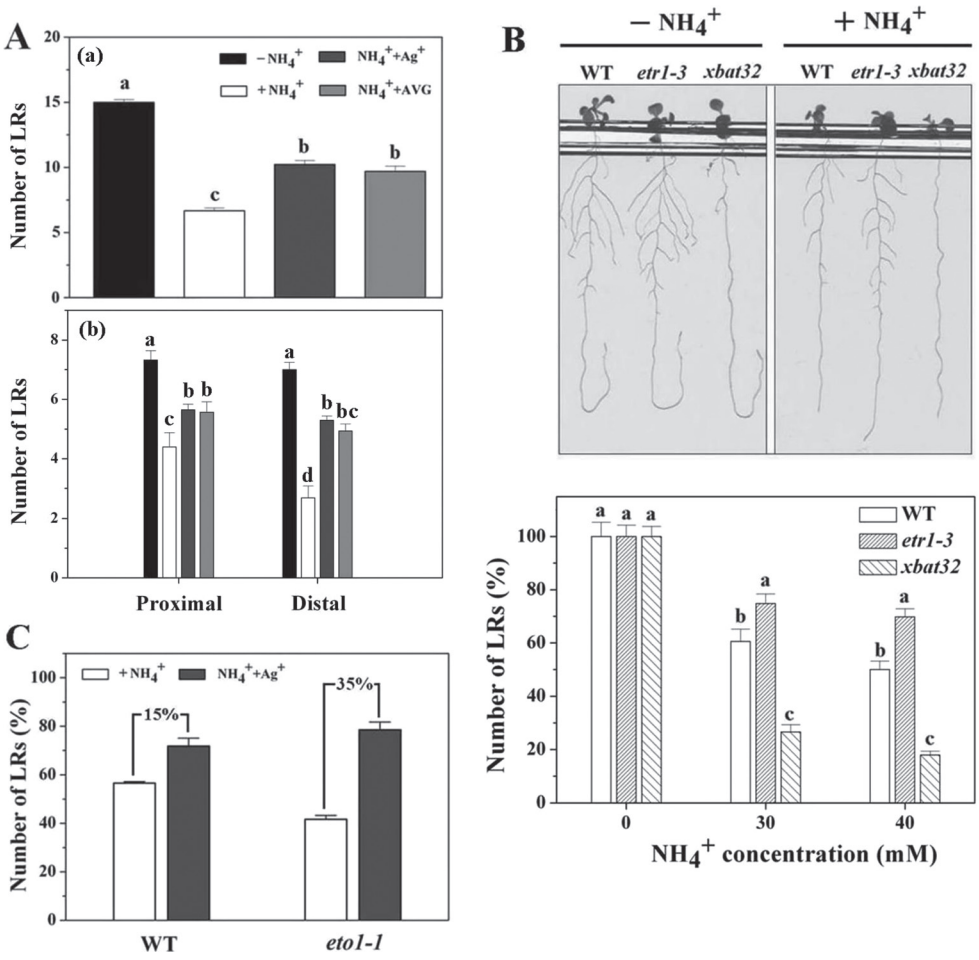

Fig. 6. Enhanced shoot ethylene evolution involves SSA-mediated inhibition of LR number. Five-day-old wild-type, *etr1-3*, *xbat32*, and *eto1-1* seedlings were transferred to medium and shoots were supplemented with 30 mM or 40 mM NH_4^+ alone or in combination with 50 μM $AgNO_3$ or 1 μM AVG for 5 d, after which LRs produced were quantified. (A) Effect of shoot-supplied ethylene inhibitors $AgNO_3$ and AVG on LR number of wild-type *Arabidopsis* seedlings grown in 40 mM NH_4^+ treatment medium. (a) LR number per seedling; (b) LR number in the proximal and distal root portions per seedling. Values are the means ±SE, *n*=10–15. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test). (B) Effect of SSA on LR number in the wild type, the ethylene-overproducing mutant, *xbat32*, and the ethylene-insensitive mutant, *etr1-3*. Photograph of a representative seedlings after 5 d of vertical growth in medium lacking or supplemented with 30 mM NH_4^+ (upper panel). Data are from one of two experiments. Values are the means ±SE, *n*=8–12. Different letters represent means statistically different at the 0.01 level (one-way ANOVA with Duncan post-hoc test). The LR number in the wild type, *etr1-3*, and *xbat32* in control was 14.85 ± 0.80, 16.83 ± 0.72, and 5.23 ± 0.19, respectively. (C) Effect of the shoot-supplied ethylene inhibitor $AgNO_3$ on LR number in the wild type and the ethylene-overproducing mutant *eto1-1* grown in 40 mM NH_4^+ treatment medium. Mean LR number in $-NH_4^+$ control in the same experiment was 14.14 ± 0.85 for wild-type seedlings and 8.25 ± 1.1 for *eto1-1*. Values are the means ±SE, *n*=6–10.

it was thus asked whether $AgNO_3$ and AVG may reduce inhibition. Indeed, both ethylene inhibitors increased LR number, and did so more significantly in the distal than in the proximal portion (Fig. 6A).

To examine further the role of ethylene in the SSA-induced suppression of LR number, a genetic approach was adopted, using an ethylene-overproduction mutant, *xbat32*, and an ethylene-insensitive mutant, *etr1-3*. The *etr1-3* mutant has a reduced ethylene response resulting from the dominant-negative versions of membrane ethylene receptors described in Bleecker *et al.* (1998) and O'Malley *et al.* (2005). The *xbat32* mutant has a defect in a RING-type E3 ligase gene that negatively regulates ethylene biosynthesis by modulating the abundance of ACS proteins, thereby enhancing ethylene synthesis (Prasad *et al.*, 2010; Lyzenga *et al.*, 2012). When shoots were exposed to media with varying concentrations of NH_4^+, a clear trend of decreasing LR number was observed in the three genotypes, but there were significant differences in the extent of inhibition among them: the decrease in LR number was more significant in the *xbat32* mutant compared with wild-type seedlings (Col-0); in contrast to *xbat32* plants, the *etr1-3* mutant had less inhibition of LR number compared with the wild type (Fig. 6B). For example, the LR number of wild-type plants was reduced by ~50% when exposed to 40 mM NH_4^+, while the same treatment reduced the LR number by ~31% and ~85% in *etr1-3* and *xbat32* plants,

respectively (Fig. 6B). The effect of external $AgNO_3$ addition on LR number under SSA stress in wild-type and the ethylene-overproduction mutant *eto1-1* was also examined (Alonso and Stepanova, 2004). The suppression of the LR number induced by SSA was alleviated by $AgNO_3$ addition in both wild-type and *eto1-1* plants, but LR number was increased more significantly in *eto1-1*. Compared with plants grown in SSA medium, *eto1-1* mutant LR number increased ~35%, as compared with ~15% in the wild type, in SSA medium plus 50 μM $AgNO_3$ in the shoot (Fig. 6C).

Inhibition of ethylene perception or signalling enhances auxin's ability to rescue SSA-mediated inhibition of lateral root number

Under SSA condition, the application of external IAA to the shoot of wild-type seedlings only partially rescued the reduced LR number and partially enhanced auxin transport from shoot to root, as illustrated by auxin induction of *DR5:GUS* expression in the central cylinder of root tips (Fig. 7A, B; Li *et al*., 2011*b*). Given the known interaction between ethylene and auxin in LR development, it was reasoned that enhanced ethylene signalling and/or synthesis induced by SSA might, by interfering with auxin transport from shoot to root, prevent a better rescue of shoot-applied auxin. To test this hypothesis, the combined effect of blocking ethylene perception and/or signalling and increasing shoot auxin levels was analysed. On $-NH_4^+$ medium, treatment with IAA in addition to $AgNO_3$ did not significantly enhance *DR5:GUS* expression in the central cylinder of root tips, and the number of LRs was also not increased compared with IAA alone (Fig. 7A, B), consistent with ethylene being at limiting concentrations in wild-type seedlings grown in untreated medium. In contrast, the combined effects of blocking ethylene perception and

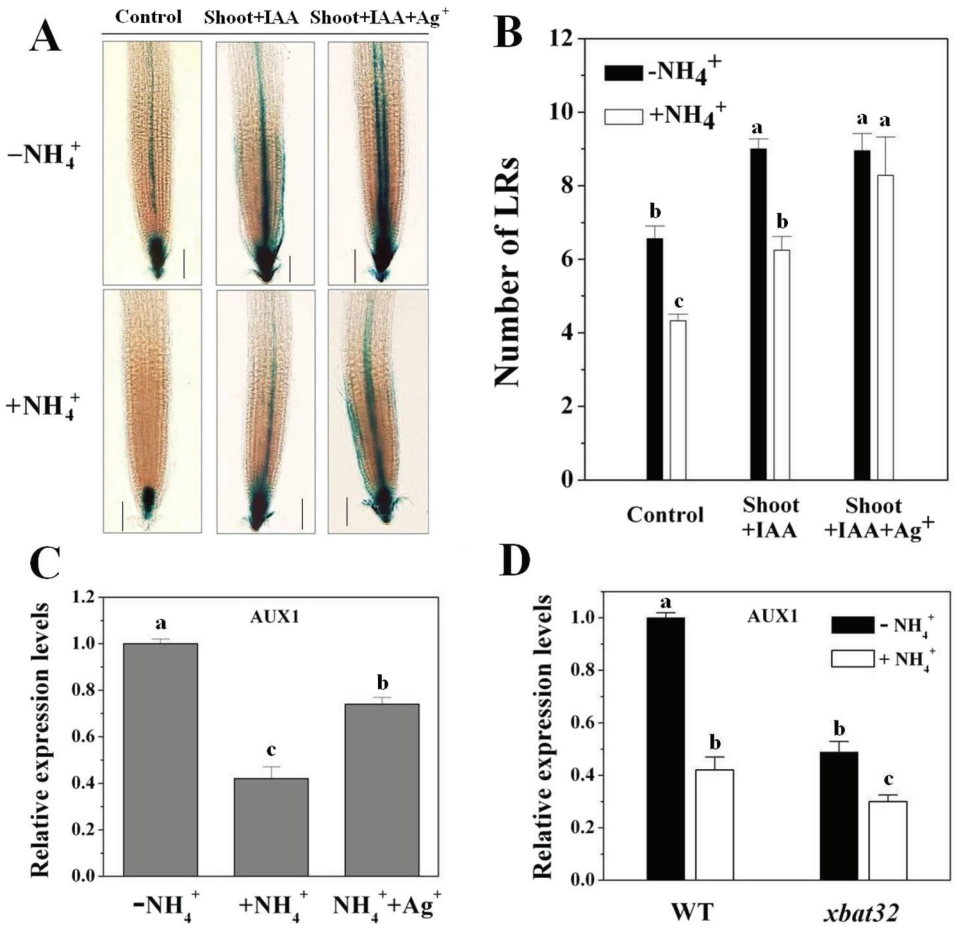

Fig. 7. Effect of inhibition of shoot ethylene signalling and/or increase in auxin levels on NH_4^+-induced *DR5:GUS* expression, lateral root numbers, and expression of the shoot auxin influx transporter AUX1. (A and B) Five-day-old Col-0 seedlings were transferred to medium and shoots supplemented with 3 μM IAA alone or in combination, 3 μM IAA plus 50 μM $AgNO_3$, with or without 30 mM NH_4^+ treatment of shoots. *DR5:GUS* roots imaged 2 d after transfer. Scale bars=50 μm. LR numbers were assessed 3 d after transfer. Data are from one of two experiments, and bars represent averages of 7–10 plants ±SE. (C) Effect of shoot-supplied $AgNO_3$ (50 μM) on NH_4^+-induced down-regulation of AUX1 from aerial parts. Values are means ±SE of three replicates. (D) Effect of shoot NH_4^+ on AUX1 expression in aerial parts of Col-0 and the ethylene-overproducing mutant *xbat32*. Values are means ±SE of three replicates. Expression of AUX1 was determined by quantitative real-time PCR after exposure of shoot of 6-day-old Col-0 and *xbat32* seedlings to 40 mM NH_4^+ alone or in combination with 50 μM $AgNO_3$ for 6 h. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).

increasing auxin levels significantly increased shoot auxin-induced *DR5:GUS* gene expression in the central cylinder of root tips in SSA medium (Fig. 7A). As acropetal IAA transport occurs in the central cylinder, the results indicate that more IAA moved from shoot to root. Meanwhile, $AgNO_3$ and IAA co-treatment brought about a greater rescue of the LR number in the wild type compared with auxin alone and led to recovery of LR number in SSA medium (Fig. 7B).

To examine whether the AUX1 gene in shoots is regulated by SSA-induced ethylene, the effect of $AgNO_3$ on expression of this gene was examined in the SSA medium using quantitative real-time PCR. Exposure of wild-type seedlings to SSA led to a marked decrease in transcript for the shoot AUX1 gene, and AUX1 expression was reduced by 57% in shoots treated with 40 mM NH_4^+ for 6 h (Fig. 7C). More importantly, it was found that the SSA-induced down-regulation of shoot AUX1 expression was significantly stimulated by $AgNO_3$ (Fig. 7C). The response of AUX1 expression to SSA was also investigated using the ethylene-overproducing mutant *xbat32*. Compared with wild-type seedlings, the transcript level of AUX1 was markedly deceased in the *xbat32* shoot in the absence of NH_4^+ (Fig. 7D). In SSA medium, the AUX1 expression of the *xbat32* mutant was also significantly lower than that of the wild type (Fig. 7D).

Discussion

NH_4^+ toxicity affects many, if not most, plant species and leads to serious root growth inhibition (Britto and Kronzucker, 2002). A role for ethylene evolution has long been suggested (Barker and Corey, 1991; Barker, 1999*a*, *b*), but its involvement remains incompletely understood. Recent studies have demonstrated that ethylene plays a major role in regulating LR development, especially in the distal primary root portion (Ivanchenko *et al*., 2008; Negi *et al*., 2008). Additionally, SSA, in simulated NH_4^+ toxicity culture environments, inhibits LR development in *Arabidopsis* (Li *et al*., 2011*b*). Therefore, it was imperative to examine whether ethylene is involved in the SSA-dependent impairment of LR development. It was found that exposure of *Arabidopsis* shoots to high NH_4^+ concentrations inhibited LR number, especially so in the distal portion of the root (Fig. 1B). It was demonstrated furthermore that this inhibitory effect is related to enhanced shoot ethylene accumulation and the up-regulation of genes encoding the enzymes AtACS and AtACO, the two key enzymes in ethylene synthesis. More importantly, it was found that inhibition of ethylene perception or signalling enhances the ability of the plant hormone auxin (IAA) to rescue SSA-mediated inhibition of LR number, stimulates auxin transport from shoot to root, and up-regulates the shoot expression of the gene coding for the auxin transporter AUX1 previously suppressed by ethylene. Therefore, it was shown that SSA-induced shoot ethylene production underpins NH_4^+ toxicity-induced inhibition of LR number, and that AUX1-mediated auxin transport is implicated in the signal transduction pathway in shoots when ethylene levels are elevated.

To date, little is known about LR development under NH_4^+ toxicity. Li *et al*. (2011*b*) and the present data (Supplementary Fig. S3 at *JXB* online) show that shoot-supplied K^+ (as K_2SO_4), NO_3^- (as KNO_3), and nitrogen metabolites (glutamate and glutamine) do not mimic the SSA-mediated inhibition of LR number in *Arabidopsis* seedlings. Additionally, the effect of NH_4^+ on LR formation has been shown to depend on the locus of exposure (Li *et al*., 2010; Li *et al*., 2011*a*, *b*; Supplementary Fig. S2A). *Arabidopsis* plants grown under WSA and SSA conditions show high shoot NH_4^+ accumulation compared with RSA (Li *et al*., 2011*a*; Supplementary Fig. S2B). Shoot NH_4^+ accumulation is widely considered to be critical to NH_4^+ toxicity (Baker, 1999*b*; Britto and Kronzucker, 2002; Li *et al*., 2012). Hence, the greatly increased shoot NH_4^+ content may serve as the intrinsic trigger that leads to the reduced LR formation under SSA conditions (Li *et al*., 2011*a*). Li *et al*. (2011*b*) examined the inhibitory effect of SSA on LR number but without separately analysing the root portions formed prior to and during the SSA treatment. Here it is shown that LRs in newly formed distal root portions were inhibited to a greater extent than those in the proximal roots formed prior to the transfer to high NH_4^+, although SSA could suppress LR number in both portions (Fig. 1B). This finding is indicative that LR development between mature and newly formed root portions is differentially sensitive to SSA. This contention is strengthened by the detailed analysis of LRP, showing that the inhibitory effect of SSA on LR development in these two portions occurs at different stages (Fig. 2B). Li *et al*. (2011*b*) and the present data (Fig. 2B) show that SSA inhibits LRP emergence from the primary root, but the current study further reveals that this negative effect on emergence is mainly seen in newly elongating root regions during NH_4^+ exposure. It is also shown that SSA had no effect on total LR initiation events in both proximal and distal portions (Fig. 2A).

The present data indicate an enhanced ethylene evolution upon exposure of *Arabidopsis* seedlings to SSA. This finding is consistent with earlier reports (Feng and Barker, 1992*a*, *b*, *c*, *d*, Barker, 1999*a*, *b*; You and Barker, 2005). It was found that SSA enhanced the expression of *AtACS2*, *AtACS7*, *AtACS8*, *AtACS11*, *AtACO1*, and *AtACO2* genes encoding ACS and ACO, the two key enzymes responsible for ethylene synthesis (Fig. 5C). The enhanced expression of these genes was correlated with the observed rapid SSA-induced ethylene production (Fig. 4C) and the stimulation of shoot *EBS:GUS* activity (Figs 4A, 5B). Additionally, SSA only enhanced shoot ethylene production, while root ethylene was unaffected (Fig. 4). Furthermore, shoot ethylene evolution required direct shoot contact with the +NH_4^+ medium (Fig. 5B). It is possible that greatly increased shoot NH_4^+ content by direct shoot uptake triggers ethylene evolution. A previous study showed that foliar ethylene evolution increased sharply in tomato when foliar NH_4^+ accumulation passed a critical value (Barker, 1999*a*). Alternatively, acidification, a general response to NH_4^+ nutrition (Gerendas *et al*., 1997; Britto and Kronzucker, 2002), may act as a trigger for ethylene evolution. However, in the present study, medium pH was maintained within narrow limits with the buffering agent MES during the growth period

of 5 d (Supplementary Fig. S4 at *JXB* online); that is, the buffer capacity of MES was sufficient to avoid NH_4^+-induced medium acidification. Furthermore, a previous observation that high ethylene evolution correlated with high tissue NH_4^+ but was independent of nitrogen form and pH regime (Feng and Barker, 1992*c*) also seems to discount this possibility. The observation that shoot contact with NH_4^+ is necessary to stimulate shoot ethylene evolution might provide useful information and insights for future studies of the role of ethylene in the development of the NH_4^+ toxicity syndrome.

Despite the well-known role of ethylene in root development under various nutritional stimuli and stresses (Schmidt, 2001; Shin and Schachtman, 2004; Visser *et al.*, 2007; Ivanchenko *et al.*, 2008; Negi *et al.*, 2008; Tian *et al.*, 2009; Lewis *et al.*, 2011), it was not known whether elevated shoot ethylene may modulate LR development under NH_4^+ stress. The present data show that shoot-supplied ACC, a precursor of ethylene, can inhibit LR number in wild-type seedlings on $-NH_4^+$ medium (Supplementary Fig. S6A at *JXB* online). This result provides a first indication that elevated shoot ethylene may be involved in the regulation of LR number. Next, pharmacological and genetic approaches were employed to examine the links between SSA-induced shoot ethylene accumulation and the inhibition of LR number by SSA. Remarkably, the SSA-induced inhibition of LR number was alleviated in the presence of an antagonist of ethylene perception (Ag^+) and an inhibitor of ethylene biosynthesis (AVG) (Fig. 6A). The dual evidence that LR number was inhibited by SSA to a greater extent in the ethylene-overproduction mutant *xbat32* than in wild-type plants and that the mutant exhibiting reduced ethylene sensitivity (*etr1-3*) showed increased LR number compared with the wild type under SSA conditions (Fig. 6B) supports this notion. The observation that an external, shoot-supplied ethylene inhibitor alleviated SSA-suppressed LR number in the ethylene-overproduction mutant *eto1-1* more significantly than in the wild type (Fig. 6C) further demonstrates the important role of shoot ethylene. Furthermore, it was found that ethylene inhibitors increased LR number in both proximal and distal portions under SSA, consistent with the above-described LR-suppressive phenotype induced by SSA (Fig. 6A), and that shoot-supplied ACC reduces LR number in both portions on $-NH_4^+$ medium (Supplementary Fig. S6B). These results suggest that shoot ethylene regulates LR number in both proximal and distal portions, different from the inhibitory effect of root ethylene on LR number only in the distal portion (Ivanchenko *et al.*, 2008; Lewis *et al.*, 2011). Together, these findings indicate that inhibition of LR number by SSA at least partially results from elevated shoot ethylene production.

Auxin applied exogenously to shoots partially rescued the LR phenotype under SSA conditions (Li *et al.*, 2011*b*; this study). Based on the present findings, one explanation for the partial rescue of SSA-suppressed LR number may be the presence of high shoot ethylene induced by NH_4^+. Several reports have found that ethylene inhibits polar auxin transport in shoot tissues (Morgan and Gausman, 1966; Suttle, 1988). In contrast, auxin transport was significantly increased in tomato stem tissue treated with $AgNO_3$, an ethylene signalling antagonist (Negi *et al.*, 2010). Consistent with this, by examining auxin induction of *DR5:GUS* expression under SSA conditions, it is also shown that blocking ethylene perception increased auxin transport from shoot to root (Fig. 7A). Li *et al.* (2011*b*) suggested that altered auxin transport is required for NH_4^+-driven inhibition of LR number. It is therefore hypothesized that blocking shoot ethylene signalling will restore proper auxin transport, allowing exogenously supplied auxin to be more available for LR production under SSA. This hypothesis is borne out by the finding that $AgNO_3$ and IAA co-treatment bring about a greater rescue of LR number in Col-0 seedlings compared with IAA alone and lead to recovery of LR number in SSA medium (Fig. 7B). Further, the down-regulation of root AUX1 expression is required for SSA inhibition of acropetal auxin transport (Li *et al.*, 2011*b*). The present pharmacological and genetic data reveal that SSA-induced shoot ethylene evolution suppresses shoot AUX1 expression (Fig. 7C, D). Ethylene also inhibits AUX1 expression in the mature region of the root but stimulates PIN3 and PIN7, resulting in blocking the formation of the auxin gradient required for LR development (Lewis *et al.*, 2011). Shoot AUX1 facilitates IAA loading into the leaf vasculature, and mutations in AUX1 cause a delay in this process, resulting in a reduced-LR phenotype (Marchant *et al.*, 2002). Thus, we suggest that negative regulation of shoot AUX1 expression by SSA-induced ethylene signalling and/or synthesis may trigger the inhibition of auxin transport from shoot to root.

Under SSA conditions, significant shoot H_2O_2 accumulation was detected (Fig. 3A). The present results are in agreement with studies demonstrating that NH_4^+ stress induces excessive ROS generation in leaves (Nimptsch and Pflugmacher, 2007; Wang *et al.*, 2008, 2010). In addition, NH_4^+-induced H_2O_2 accumulation was sensitive to DPI, a potent inhibitor of NADPH oxidase (Fig. 3A). This result suggests that NH_4^+-induced H_2O_2 accumulation may be caused by increased activity of NADPH oxidase. This is supported by recent results showing that NADH oxidase activity increased significantly in *Hydrilla verticillata* treated with high NH_4^+ (Wang *et al.*, 2011). Previous results indicate that ROS may be formed as signalling intermediaries between environmental stress and root development (Potters *et al.*, 2007). ROS accumulation has been shown to be involved in the LR development response to K^+ deprivation (Shin and Schachtman, 2004), and LR formation was more severely influenced by abscisic acid (ABA) in ROS-overaccumulating transgenic plants, but was less influenced in ROS-reduced mutants (Lee *et al.*, 2012). However, the present study did not reveal any significant influence of shoot ROS accumulation on LR number (Fig. 3B, C). These results suggest that SSA inhibition of LR number is unlikely to be caused by shoot ROS accumulation. Nevertheless, the possibility that root ROS accumulation may be involved in SSA-induced LR inhibition cannot be excluded. However, it is currently unclear whether SSA might induce ROS accumulation in the root, and more research is warranted to examine this.

Additionally, disruption of foliar cation nutrition is a typically observed component of the NH_4^+ toxicity syndrome

(Szczerba *et al*., 2006; Li *et al*., 2012; Supplementary Fig. S5 at *JXB* online) and may interfere with shoot-derived signals, thus influencing LR development. However, the present data indicate that shoot-supplied K^+, which also disrupts foliar cation nutrition, does not produce significant LR effects (Supplementary Fig. S5). Furthermore, high NO_3^- supply is also known to affect shoot cation content significantly (Roosta and Schjoerring, 2007; Hermans *et al*., 2010), but it, too, does not mimic the NH_4^+-mediated inhibition of LR number in *Arabidopsis* (Li *et al*., 2011*b*; Supplementary Fig. S3).

In conclusion, the present results show that shoot contact with NH_4^+ is necessary to stimulate shoot ethylene evolution. Furthermore, it is reported that elevated shoot ethylene is closely associated with a reduction in LR number under SSA challenge, as demonstrated by mitigation with ethylene antagonists, lower SSA sensitivity in ethylene-insensitive mutants, and greater sensitivity in ethylene-overproduction mutants. The exact mechanism by which shoot ethylene inhibits LR development under SSA conditions remains to be resolved; however, AUX1-mediated auxin transport may be one component of the signal transduction pathway in shoots enriched in ethylene. The results provide novel insight into how LRs are regulated in response to NH_4^+ stress. Further research into the mechanisms of interplay between NH_4^+ and ethylene evolution of plants will enable fuller understanding of how plants respond to various degrees of NH_4^+ stress, and will be instrumental in the development of strategies to improve the NH_4^+ tolerance of crops.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Experimental set-up for supplying NH_4^+ to shoots.

Figure S2. The effect on LR number and tissue NH_4^+ content of exposure of different seedling parts to NH_4^+.

Figure S3. Effects of NH_4^+, K^+, and NO_3^- (a), and glutamate and glutamine (b) on the number of LRs.

Figure S4. pH medium with the buffering agent MES during the experimental period.

Figure S5. The effect of shoot-supplied NH_4^+ [$(NH_4^+)_2SO_4$] and K^+ (as K_2SO_4) on mineral contents in shoots of *Arabidopsis* seedlings.

Figure S6. Shoot-supplied ACC inhibits LR number in –NH_4^+ medium.

Table S1. Gene-specific primers used for PCR.

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