New Phytologist

Research

GSA-1/ARG1 protects root gravitropism in *Arabidopsis* under ammonium stress

Na Zou[1,2]*, Baohai Li[2]*, Hao Chen[3], Yanhua Su[2], Herbert J. Kronzucker[4], Liming Xiong[3], František Baluška[5] and Weiming Shi[2]

[1]College of Landscape and Art, Jiangxi Agricultural University, Nanchang 330045, China; [2]State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, No. 71 East Beijing Road, Nanjing 210008, China; [3]Donald Danforth Plant Science Center, St Louis, MO 63132, USA; [4]Department of Biological Sciences, University of Toronto, 1265 Military Trail, Toronto, ON M1C 1A4, Canada; [5]Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

Author for correspondence:
Weiming Shi
Tel: +86 25 86881566
Email: wmshi@issas.ac.cn

Received: *31 January 2013*
Accepted: *11 May 2013*

New Phytologist (2013)
doi: 10.1111/nph.12365

Key words: ammonium, AUX1, auxin transport, *GSA-1/ARG1*, PIN2, root gravitropism.

Summary

- Gravitropism plays a critical role in plant growth and development, plant stability and acclimation to changes in water and nutrient availability. Ammonium (NH_4^+) is well known to have profound effects on root growth, but its impacts on gravitropism are poorly understood.
- To determine which genes are essential for the maintenance of root gravitropism under NH_4^+ stress, we isolated and identified an NH_4^+-sensitive mutant, *gsa-1* (gravitropism sensitive to ammonium-*1*), in *Arabidopsis thaliana*, using an agar plate root reorientation assay.
- We found that, under NH_4^+ stress, *gsa-1* displayed increased loss of root gravitropism. Gene cloning and sequencing revealed that *gsa-1* contains a G to C transversion mutation at the highly conserved 5′-GT splice position of intron 10 of *ARG1* (ALTERED *R*ESPONSE TO *G*RAVITY*1*), known to participate in the transduction of the root gravity signal. Genetic complement tests established the locus of *GSA-1/ARG1* and its role in resistance to NH_4^+ inhibition on root gravitropism. GSA-1/ARG1 is required for normal AUX1 expression and basipetal auxin transport in root apices. In addition, PIN-FORMED2 (PIN2) is proposed as a target in the reduction of root gravitropism under NH_4^+ stress, a response which can be antagonized by the GSA-1/ARG1-dependent pathway.
- These results suggest that GSA-1/ARG1 protects root gravitropism in *Arabidopsis thaliana* under ammonium stress.

Introduction

Gravitropism ensures the growth of plant organs along a specific vector relative to gravity, and dictates the upward growth of shoots and that of roots down into the soil. Root gravitropism plays an important role in determining the distribution of the root system in the soil, and is therefore critical to the physical anchorage of the plant, as well as to water and nutrient acquisition (Forde & Lorenzo, 2001; Perrin *et al.*, 2005). Changes in orientation relative to gravity (gravistimulation) induce root bending towards the original growth direction, a process that can be conceptually divided into four successive steps: gravity perception, signal transduction, signal transmission and curvature response (Perrin *et al.*, 2005). Columella cells containing sedimentable amyloplasts in the root cap are the principal gravity-perceptive sites in roots (Caspar & Pickard, 1989; Kiss *et al.*, 1989; Blancaflor *et al.*, 1998). The sedimentation of amyloplasts in the root cap has long been thought to trigger a signal transduction pathway that promotes the development of a lateral gradient of auxin, which is then transported to the elongation zone (EZ), where it leads to differential cellular elongation on opposite flanks of the corresponding EZ (Chen *et al.*, 2002).

The formation and maintenance of auxin gradients are dependent on polar auxin transport, mediated by special transporters, including auxin influx carriers (in particular AUX1) and efflux facilitators (PIN-FORMED3 (PIN3) and homologous protein PIN2/AGR1 (AGRAVITROPIC1)/WAV6 (WAVY ROOTS 6)/EIR1 (ETHYLENE INSENSITIVE ROOT 1); Ge *et al.*, 2010). For example, AUX1 encodes an auxin influx-mediating transmembrane protein, localized in the stele, the apical side of protophloem cells, columella, epidermis and lateral root cap tissues (Swarup *et al.*, 2001). By contrast, PINs encode auxin efflux-mediating proteins, and polar PIN localization directs auxin flow (Wisniewska *et al.*, 2006). A relocation of PIN3 within statocytes from a symmetrical distribution at the plasma membrane is believed to represent the initial step in the establishment of the lateral auxin gradient on gravistimulation (Friml *et al.*, 2002; Ottenschläger *et al.*, 2003; Harrison & Masson, 2008). Following this, the resulting lateral auxin

*These authors contributed equally to this work.

gradient is shifted basipetally through the combined actions of AUX1 and PIN2 via lateral root cap and epidermal cells towards EZ (Friml, 2003; Swarup *et al.*, 2005). AUX1 is present in the same cells as PIN3 and PIN2, and probably facilitates the uptake of auxin into the lateral root cap and the epidermal region, and PIN2 is believed to mediate its directional translocation towards EZ (Friml, 2003). However, differential cellular elongation on opposite flanks of the central EZ induced by the lateral auxin gradient may be responsible for only part of the gravitropic curvature (Chen *et al.*, 2002). Recent findings have suggested the involvement of the transition zone (TZ), also known as the distal elongation zone (DEZ), as a secondary site/mechanism of gravity sensing for root gravitropism, which may be independent of the auxin gradient (Wolverton *et al.*, 2002; Chavarría-Krauser *et al.*, 2008; Baluška *et al.*, 2010). Roots of *pin3* mutants lack the bending response in the elongation region, but bending is not affected in TZ (Chavarría-Krauser *et al.*, 2008; Baluška *et al.*, 2010).

Given that the appropriate distribution of roots within the soil greatly affects plant survival, roots have evolved to sense adverse environmental cues and to modulate their growth direction through a variety of pathways. For example, moisture gradients and water stress can cause the desensitization of gravitropism in *Arabidopsis* by the degradation of amyloplasts in root columella cells, allowing roots to exhibit positive hydrotropism (Takahashi *et al.*, 2003). Similarly, roots exposed to salt stress show inhibited gravitropism, which permits the active avoidance of stressed regions (Li & Zhang, 2008; Sun *et al.*, 2008). In addition, the availability of phosphorus has been shown to regulate the root configuration of legumes by altering the growth angle of basal roots, so as to facilitate improved acquisition of phosphorus from the soil (Bonser *et al.*, 1996; Liao *et al.*, 2001). Similarly, reductions in external potassium have been observed to trigger agravitropic growth in *Arabidopsis*, so that roots grow away from potassium-impoverished regions, which may well represent a mechanism by which plants respond to mineral deficiencies in general (Vicente-Agullo *et al.*, 2004). Furthermore, ammonium (NH_4^+), both a major nitrogen source and common toxicant (Kronzucker *et al.*, 1997), frequently produces the inhibition of root growth and lateral root formation (Gerendás *et al.*, 1997; Britto & Kronzucker, 2002; Qin *et al.*, 2008; Li *et al.*, 2010, 2011a,b, 2012, 2013; Kempinski *et al.*, 2011), but has also been shown to affect the root gravitropism response (Zou *et al.*, 2012).

Here, we report a novel *Arabidopsis thaliana* mutant, *gsa-1* (*g*ravitropism *s*ensitive to *a*mmonium-*1*), which displays reduced root gravitropism in response to NH_4^+ stress. Gene cloning shows *gsa-1* to be allelic to *ARG1* (*A*LTERED *R*ESPONSE TO *G*RAVITY *1*), which is required for the establishment of the lateral auxin gradient across the root cap following gravistimulation (Boonsirichai *et al.*, 2003; Harrison & Masson, 2008).Our results further demonstrate that the disruption of *GSA-1*/*ARG1* can reduce basipetal auxin transport and the expression of AUX1 protein in root apices. Moreover, we demonstrate the involvement of PIN2 in the NH_4^+ regulation of the gravitropic response.

Materials and Methods

Plants and growth conditions

The plant material used in this work included the Columbia (Col-0) and Landsberg erecta (Ler) ecotypes of *Arabidopsis thaliana* (L.) Heynh, T-DNA-transformed mutants derived from Col-0, and the mutants *arg1-3* (SALK_024542C), *eir1-1* (Roman *et al.*, 1995) and *aux1-22* (Roman *et al.*, 1995); the transgenic *DR5::GUS* (Ulmasov *et al.*, 1997) was in the Col-0 background, *proPIN2::PIN2-GFP* in *eir1-1* (Blilou *et al.*, 2005) and *proAUX1::AUX1-YFP* in *aux1-22* (Swarup *et al.*, 2004). *gsa-1* plants carrying the *proAUX1::AUX1-YFP* and *proPIN2::PIN2-GFP* constructs were derived from crosses between *gsa-1* and the corresponding constructs of the transformed plants, respectively, and homozygous plants for both *gsa-1* and the *proAUX1::AUX1-YFP* or *proPIN2::PIN2-GFP* insertion were used.

The double mutants *gsa-1aux1-22* and *gsa-1eir1-1*, carrying both the *gsa-1* and either the *aux1-22* or *eir1-1* mutation in the homozygous state, were obtained by crossing single mutants and selfing the corresponding F1 progeny. Plants homozygous for *aux1-22* and *eir1-1* were identified by germination on normal agar growth medium, with the addition of 0.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM 1-aminocyclopropane-1-carboxylate (ACC), respectively (Luschnig *et al.*, 1998; Marchant *et al.*, 1999). Several rosette leaves were excised from a single 2,4-D- or ACC-resistant F2 plant and prepared for total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) amplification using the primers F2R2, as described below for the identification of *gsa-1*. The homozygous *gsa-1* mutation displayed larger RT-PCR amplification products than did Col-0.

After being surface sterilized and cold treated at 4°C for 2 d, the seeds were sown on normal *Arabidopsis thaliana* growth medium, as described by Li *et al.* (2010). The nutrient medium contained 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 $ZnCl_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 1% (w/v) sucrose, 0.5 g l^{-1} MES and 0.8% (w/v) agar (adjusted to pH 5.7 with 1 M NaOH). The culture plates were placed vertically in a growth chamber at 23 ± 1°C under a light intensity of 100 μmol photons m^{-2} s^{-1}, with a 16 h : 8 h light : dark cycle. Five-day-old seedlings germinated on normal growth medium, with relatively straight root tips and *c.* 1.5 cm in length, were selected for gravity stimulation experiments. The NH_4^+ treatment medium was prepared by the addition of varying concentrations of $(NH_4)_2SO_4$ to the normal growth medium. Ion effects were analyzed using NH_4Cl (with the same NH_4^+ concentration as in $(NH_4)_2SO_4$), K_2SO_4 (with the same SO_4^{2-} concentration) and KNO_3 (with the same N concentration). For tests of general osmotic substitution of $(NH_4)_2SO_4$, different concentrations of mannitol were used.

Gravity stimulation

Five-day-old seedlings of similar size were transferred to new agar plates containing the appropriate treatments. Roots were

placed vertically in rows, after recording the initial positions of the root tips, the plates were rotated by 90° and placed vertically for gravistimulation under different concentrations of NH_4^+ (0, 10, 20 and 30 mM $(NH_4)_2SO_4$) in a cultivation chamber at time zero. Digital images of seedling growth were captured at regular, specified time points (as defined in the text) following gravistimulation with a Canon G7 (Canon Inc., Tokyo, Japan). The root elongation and tip angles from the vertical were determined as described by Sun *et al.* (2008). Root elongation refers to the length of the primary root after transfer to treatment solution, whereas the gravitropic angle refers to the angle of the root tip relative to the gravity vector.

Isolation of the *gsa-1* mutant

A total of 5160 T-DNA insertional mutant lines in the Col-0 background, as described previously (Zuo *et al.*, 2000; Zhang *et al.*, 2005; Li *et al.*, 2012), were used in *gsa-1* isolation. Five-day-old seedlings with roots *c.* 1.5 cm in length were transferred onto new medium supplemented with 30 mM $(NH_4)_2SO_4$, arranged in rows and then gravistimulated to screen for NH_4^+-sensitive phenotypes of root gravitropism. The gravitropic bending during the following 72 h was captured, and putative mutants with a reduced gravitropic response were selected and transferred to soil to grow to maturity and to self-fertilize. One of these putative mutants, called *gsa-1*, was selected for analysis. The homozygous M4 *gsa-1* mutant was backcrossed against the parental wild-type (Col-0) another two times to remove the unlinked mutations caused by the mutagenesis.

Genetic analysis

Although rosettes and inflorescences of *gsa-1* were similar to those of Col-0 in morphology, its root displayed a defect in gravitropism when grown on agar plates for 3 d after germination (DAG) (Supporting Information Fig. S1). F1 and F2 progenies derived from the crosses of Col-0 × *gsa-1* and Ler × *gsa-1* were grown on normal growth medium for 3 d; agravitropic individuals were scored and compared statistically (χ^2 test). Segregation of the root gravitropic phenotype of crossed F2 progeny seedlings at 5 DAG on agar medium supplied with 30 mM $(NH_4)_2SO_4$ was also analyzed and compared statistically.

Mapping of *gsa-1*

Mapping populations were created by crossing the *gsa-1* mutant (Col-0) with wild-type plants of Ler, and their F1 progeny were propagated by self-fertilization. F3 progenies from individual F2 plants were subjected to a gravitropism assay to determine the corresponding gravitropic genotype of the F2 parents. Homozygous F2 progeny mutant plants were selected and used to map *gsa-1* by bulked segregation analysis. The protocol of mapping has been described previously (Li *et al.*, 2012). DNA was extracted from each pool and subjected to PCR amplification with InDel (Insertion/Deletion) primers (Salathia *et al.*, 2007). The candidate gene *GSA-1* was linked with CER464751 (F: AAACCCCTTCCAGGATGAAC; R: ACGTTTTGAACCAC CGCTAC), where a well-known agravitropic mutant, *arg1*, has been mapped previously (Sedbrook *et al.*, 1999). Thus, the *gsa-1* and *arg1-3* mutants were crossed with each other for allelic testing.

Cloning of *ARG1* genomic and cDNA sequences

DNA for PCR amplification was extracted according to the procedure of Weigel & Glazebrook (2002). Total RNA was extracted from roots of 7-d-old seedlings using the Trizol reagent (Invitrogen). One microgram of DNase-treated total RNA was used as a template for first-strand cDNA synthesis with reverse transcriptase M-MLV (TaKaRa Biochemicals, Dalian, China) and an oligo(dT) primer.

PCR and RT-PCR amplification were performed with Col-0 and *gsa-1* genomic DNA and cDNA, respectively, using the primers F1/R1 (5′-CGAGAAGATGAGCGCGAAAAAGCTTGA A-3′/5′-TATATCATCAATCCACCATCACAAT-3′) (Harrison & Masson, 2008) and F2/R2 (5′-TCAACCAAGCTTCTT-ATCAGA-3′/5′-GCTTTGGCGCGGTTTCAAGA-3′), respectively, for the candidate gene, *ARG1*. PCR and RT-PCR amplification were performed in a 20-μl volume containing 10–100 ng μl^{-1} genomic DNA or cDNA (2 μl), 10 μM of each primer (0.4 μl), 2.5 mM deoxynucleoside triphosphates (dNTPs) (1.6 μl), 25 mM Mg^{2+} (1.2 μl), 10× PCR buffer (2.0 μl) and 0.2 units of Taq DNA polymerase (TaKaRa). The conditions for the PCR program used in the cloning of the *ARG1* sequence were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 3 min, and final extension at 72°C for 7 min. The following program was used for the RT-PCR amplification of *ARG1*: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and final extension at 72°C for 7 min. The PCR and RT-PCR products were cloned into a pMD®18-T vector and sequenced.

Complementation of *gsa-1*

To verify that the gravitropic defect of *gsa-1* was associated with the mutations in *ARG1*, the *ARG1* coding region, including the corresponding untranslated region (UTR) sequences, was amplified from Col-0 and *gsa-1* cDNA, respectively, using the primer pairs 5′-CGGC*TCTAGA*TTTTTTTCCTCGATCTTCTTCT TC-3′ and 5′-GGC*GAGCTC*GAAGCAAGTCTGGTATTATA ATT-3′ with the *Xba*I and *Sac*I restriction sites in italic, and cloned into the *Xba*I–*Sac*I-digested pBI121 binary vector, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator sequences to create the transformation construct. These constructs were transformed into *Agrobacterium,* strain C58, and then introduced into the *gsa-1* mutants by the floral dip method for the complementation assay (Clough & Bent, 1998), and the defect-carrying *GSA-1* cDNA and pBI21 vector were transformed as a negative control. Transformants were identified by kanamycin resistance; eight, two and five independent homozygous and genetically stable transgenic lines were developed for wild-type *GSA-1 cDNA*/

gsa-1, defective *GSA-1 cDNA*/*gsa-1* and pBI21 vector/*gsa-1*, respectively. All of the wild-type *GSA-1 cDNA*/*gsa-1* transgenic lines, but not the defective *GSA-1 cDNA*/*gsa-1* and pBI21 vector/*gsa-1* transgenic lines, complemented the *gsa-1* phenotype, although only one representative from each is presented in this study.

Construction of *proAtARG1:GUS* fusion genes

To investigate the expression of *GSA-1/ARG1*, the promoter of *AtGSA-1/AtARG1* was fused to a β-glucuronidase (GUS) reporter gene, and the recombinant was introduced into Col-0 to produce *proAtARG1:GUS* transgenic plants: DNA fragments covering the 5′ flanking regions, from −1405 to −1 of the *ARG1* gene (At1 g68370), were amplified from Col-0 genomic DNA using the primers 5′-ACAT*AAGCTT*TCACTCTCTTCCCCCTTCG TCCAA-3′ and 5′-GCGGC*TCTAGA*CTTCTCGAAGAATTT GAAATCAC-3′, with the *Hind*III and *Xba*I sites in italic, and then inserted into a binary pBI121 vector, creating a recombinant transcription. The recombinant *proAtARG1:GUS* fusion gene was introduced into Col-0 plants.

Image analysis

Histochemical analyses of *GUS* gene enzyme activity of the transgenic plants containing the *DR5::GUS* reporter gene and the *proAtARG1:GUS* fusion construct were carried out according to Weigel & Glazebrook (2002). Histochemical staining, observation and measurement of amyloplasts in the columella cells of the root cap were performed as described by Takahashi *et al.* (2003). Images were obtained using an Olympus BX51 optical microscope equipped with differential interference contrast (DIC) for observation and an Olympus DP71 system for photography (Olympus Optical, Tokyo, Japan). The images shown are representative of at least 10 plants for each treatment, and the experiments were repeated at least twice. *ProPIN2::PIN2-GFP* and *ProAUX1::AUX1*-YFP were visualized using a Zeiss LSM710 confocal laser scanning microscope. The excitation wavelengths were 514 and 488 nm for yellow (YFP) and green (GFP) fluorescent proteins, respectively, and emission was detected at 520–550 nm. Images were taken under the same conditions and were representative of at least 10 individual plants from each treatment. Each experiment was repeated at least twice.

Basipetal ^{3}[H]IAA transport

To measure basipetal auxin transport, 5-d-old seedlings were transferred to new agar plates with 0 or 30 mM $(NH_4)_2SO_4$ for 1 d, and then the root tips were incubated with 1% agar blocks containing 100 nM ^{3}H-labelled IAA (American Radiolabeled Chemical, St Louis, MO, USA) 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 by Li *et al.* (2011a), and following the procedure by Lewis & Muday (2009). The results are pooled from three experiments of 10 seedlings per treatment.

Data analysis

Data were analyzed statistically using SPSS version 13.0 (SPSS, Chicago, IL, USA). One-way ANOVA with an LSD (least-significance difference) test was used for the analysis of differences in root growth and gravitropism following NH_4^+ treatments. Sigma Plot 13.0 was used for the generation of graphs and Photoshop for photocomposition.

Results

Isolation of *gsa-1* and genetic analysis

Our time-dependence results show that 30 mM $(NH_4)_2SO_4$ inhibits significantly the gravitropic bending of Col-0 at shorter exposure times of < 24 h. However, at prolonged exposure times (longer than 24 h), no significant difference was found in root gravitropic angles between treatments and controls (Zou *et al.*, 2012). On the basis of these findings, and using the experimental system shown in Fig. 1(a), we transferred 5-d-old seedlings, screened 5160 T-DNA insertion mutants and isolated a mutant with distinct NH_4^+ sensitivity in the root gravitropic response, which we refer to as *gsa-1*. In contrast with Col-0, the differences in root gravitropic angle between NH_4^+-treated plants and *gsa-1* controls did not diminish with time, and 30 mM $(NH_4)_2SO_4$ inhibited significantly the root gravitropic response of *gsa-1* at each time point tested (Fig. 1b).

gsa-1 displayed reduced root gravitropic curvature dynamics relative to those of Col-0 under NH_4^+-free control conditions (i.e. on normal growth medium, Fig. 1b), indicating that *gsa-1* represents a root-sensing or gravitropism-associated mutant. In observations of the process of seed germination, *gsa-1* was seen to grow in random directions and without the regular wavy root growth habit relative to Col-0. These differences were more apparent in 3-d-old seedlings (Fig. S1a,b). The F1 seedlings generated from *gsa-1* crossed with wild-type Col-0 displayed a similar gravitropism response to the wild-type, and self-fertilized F2 seedlings revealed a 3 : 1 segregation ratio of wild-type/*gsa-1* (Table 1; Fig. S1c). Therefore, the *gsa-1* mutant was caused by a single recessive nuclear mutation.

Characteristics of root gravitropism in Col-0 and *gsa-1* in response to ammonium

To better understand the gravitropic response of *gsa-1* roots to NH_4^+, we monitored root elongation and gravitropic curvature over time in Col-0 and *gsa-1* in response to different concentrations of NH_4^+ (10, 20 and 30 mM $(NH_4)_2SO_4$). In the short term (< 12 h), all concentrations of $(NH_4)_2SO_4$ applied reduced significantly root gravitropism, but the effect decreased in Col-0 with extended (24 h and beyond) treatment (Fig. 2a). After 48 h of gravistimulation, 10 mM $(NH_4)_2SO_4$ even increased the maximum curvature of treated roots (displaying smaller gravitropic angle) in contrast with controls. Roots treated with 20 and 30 mM $(NH_4)_2SO_4$ displayed no significant difference in root gravitropic angles relative to those of controls at 48/72 h. After

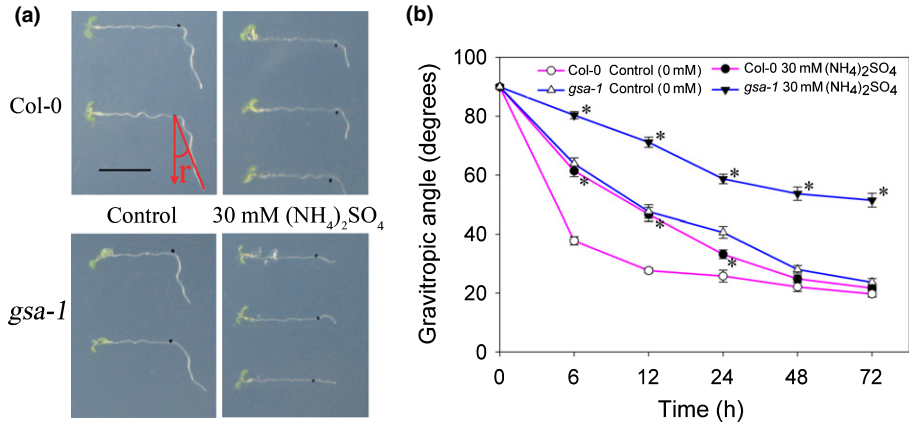

Fig. 1 Isolation and characterization of ammonium-sensitive mutants in root gravitropism. Five-day-old *Arabidopsis thaliana* seedlings with roots of *c.* 1.5 cm in length, grown on vertically oriented germination agar plates, were transferred onto new plates supplemented with 30 mM $(NH_4)_2SO_4$, with the root tips marked; the plates were rotated by 90° to initiate gravistimulation at 'time zero' and to screen for seedlings exhibiting reduced root gravitropism in response to NH_4^+ stress. (a) Diagram of the experimental set-up for the isolation of the NH_4^+-sensitive mutant *gsa-1,* and showing the effects of 30 mM $(NH_4)_2SO_4$ on root gravitropism in Col-0 and *gsa-1* following 48 h of gravity stimulation. Bar, 1 cm. (b) Time course of the root gravitropic response under NH_4^+ exposure in Col-0 and *gsa-1*. The gravitropic angle is defined as the angle of the root tip relative to the gravity vector (r). Data are from a minimum of three independent experiments with 10–12 seedlings per experiment. Error bars indicate ± SE. *Significant differences ($P < 0.05$) compared with the corresponding controls, determined by one-way ANOVA.

Table 1 Genetic analysis of *Arabidopsis thaliana* mutant *gsa-1*

Crosses (♀×♂)	Generation	Total seedlings tested	Wild-type phenotype[a]	Mutant phenotype	$\chi^{2\,b}$
Col-0 × *gsa-1*	F1	47	47	0	0.14
	F2	291	221	70	
gsa-1 × Col-0	F1	50	50	0	0.08
	F2	335	249	86	
gsa-1 × Ler	F1	40	40	0	0.11
	F2	196	149	47	

[a]Wild-type phenotype or mutant phenotype was determined for seedlings at 3 d after germination (DAG).
[b]The calculated value was based on the expected ratio of three wild-type seedlings to one mutant seedling ($P > 0.05$).

gravistimulation for 48 h, 10 mM $(NH_4)_2SO_4$ had no significant impact on the root gravitropic angle in *gsa-1*, whereas 20 and 30 mM $(NH_4)_2SO_4$ inhibited significantly *gsa-1* root gravitropic bending, an effect not seen in Col-0 (Fig. 2b). These results show that the root gravitropic response of *gsa-1* is more sensitive to NH_4^+ relative to that of Col-0. Nevertheless, the tracking of root growth over time at all concentrations of $(NH_4)_2SO_4$ showed the effect of NH_4^+ on root elongation to be similar between Col-0 and *gsa-1* (Fig. 2c,d). After a 3-d treatment with 10, 20 and 30 mM $(NH_4)_2SO_4$, the mean root elongation values of Col-0 and *gsa-1* were reduced to 71.5%, 47.4% and 30.1%, and 67.9%, 45.6% and 24.7%, respectively (Fig. 2c,d). The lack of correlation between the effects of NH_4^+ on root gravitropic angle and root elongation illustrates that the influence of NH_4^+ on the gravitropic response of *gsa-1* is not a secondary effect of its inhibition of root elongation.

Given that NH_4^+ applications were in the form of $(NH_4)_2SO_4$, it was necessary to ascertain whether the observed effects were indeed attributable to the NH_4^+ ion, and not the counterion (SO_4^{2-}), highly concentrated N or the osmotic strength of the applied salts. To address this issue, NH_4Cl applications with identical concentrations of NH_4^+, K_2SO_4 applications with identical concentrations of SO_4^{2-}, KNO_3 applications with identical concentrations of N and isosmotic applications of mannitol were substituted for $(NH_4)_2SO_4$ in the treatment medium. *gsa-1* roots displayed largely indistinguishable gravitropic kinetics under NH_4Cl as under $(NH_4)_2SO_4$ (Figs S2a, 2a), whereas both K_2SO_4 and KNO_3 at the indicated concentrations had little impact on *gsa-1* root gravitropism (Fig. S2b,c). Further, mannitol produced no inhibitory effect on root gravitropism at the concentrations applied (Fig. S2d). Thus, the action of $(NH_4)_2SO_4$ on *gsa-1* root gravitropism could be attributed to NH_4^+.

Identification of *GSA-1*

As T-DNA insertion loci isolated by thermal asymmetric interlaced-PCR (TAIL-PCR) were not associated with the NH_4^+ response phenotype, a map-based cloning strategy was pursued to isolate the mutated gene. With preliminary positional cloning analysis, *gsa-1* showed linkage to CER464751 (at base pair

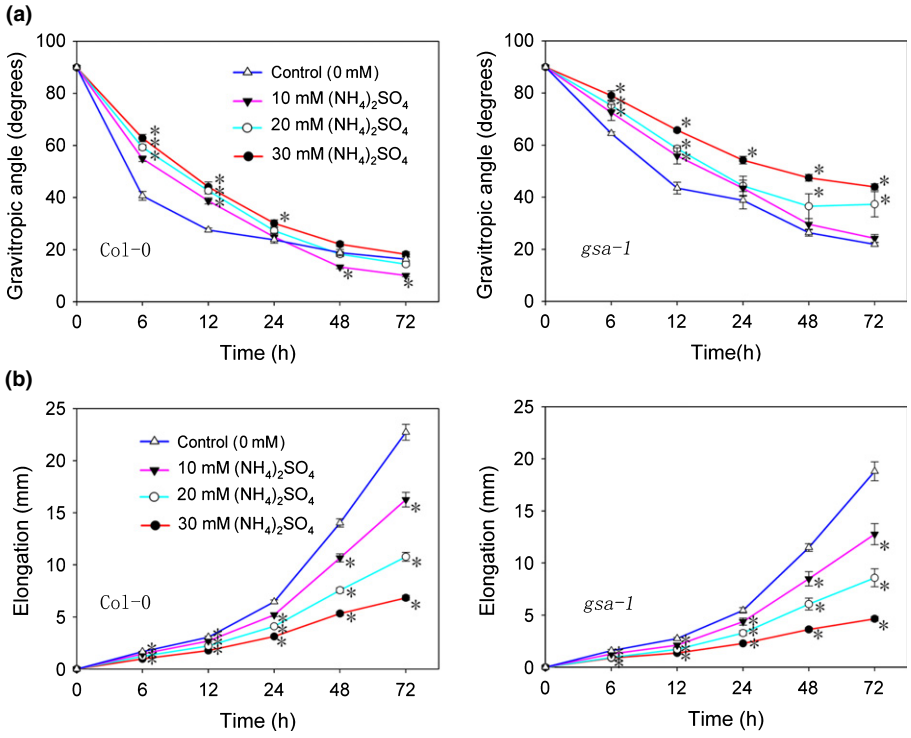

Fig. 2 Time–concentration kinetics of root gravitropism and elongation of *Arabidopsis thaliana* Col-0 and *gsa-1* on ammonium exposure. Five-day-old Col-0 and *gsa-1* seedlings were transferred to medium with varying concentrations of $(NH_4)_2SO_4$ (10, 20 and 30 mM) for gravistimulation, by rotating the plates by 90°. Root elongation and gravitropic angles were measured at the indicated time points following the treatments. (a) Effects of different concentrations of $(NH_4)_2SO_4$ on the root gravitropic response in Col-0 and *gsa-1*. (b) Effects of different concentrations of $(NH_4)_2SO_4$ on root elongation in Col-0 and *gsa-1*. Data are from four independent experiments with 10–12 seedlings per experiment. Error bars indicate ± SE. *Significant differences ($P < 0.05$) compared with the controls, as determined by one-way ANOVA.

26 628 510 of chromosome 1), where a well-known mutant, *arg1* (*altered response to gravity1*), displaying a similar gravitropism phenotype to that of *gsa-1*, has been identified. Genetic analysis by crossing *gsa-1* and *arg1-3* containing a T-DNA disruption of the *ARG1* gene demonstrated that *gsa-1* failed to complement *arg1-3* (Fig. S3), indicating that the two mutants are allelic to each other. The genomic sequence displayed a point mutation of G to C at base pair 2274 of *AtARG1* (the universally conserved 5′ GT splice site of intron 10 of *AtARG1*) (Fig. 3a), thus abolishing the GU splice site consensus, leading to the retention of base pair 172 in intron 10. Semi-quantitative RT-PCR expression analysis of total mRNA extracted from 7-d-old seedlings of the indicated genotype, using the *ARG1* primers F1/R1, as described by Harrison & Masson (2008), detected a larger *AtARG1 cDNA* product in *gsa-1* relative to Col-0, which was further different from the null allele *arg1-3* (Fig. 3b). Primers of F2/R2 across intron 10 of *ARG1*, as shown in Fig. 3(a), were further used to amplify the genomic DNA and mRNA isolated from Col-0 and *gsa-1*, respectively. PCR and RT-PCR products of *gsa-1* had the same sizes as the Col-0 genomic DNA amplification products, which were larger than the RT-PCR product of Col-0 (Fig. 3c). Corresponding sequencing confirmed the mutation of G to C at the 5′ conserved GT splice site of intron 10. It was inferred that the disruption of GU caused a frameshift and premature stop codons in the C-terminus of the *gsa-1* mutant, and thus destroyed the predicted coiled-coil region of *ARG1* (Fig. 3a). Transformed *gsa-1* plants expressing wild-type *AtARG1* cDNA under the control of the CaMV 35S promoter oriented in a similar manner to Col-0, whereas those carrying the defective *AtARG1* cDNA or pBI121 construct could not rescue the agravitropic phenotype of *gsa-1* (Figs S4, 3d). To further determine the role of *GSA-1/ARG1* in NH_4^+-induced root agravitropism, we examined the gravitropic dynamics in Col-0, *gsa-1*, T3 generation homozygous lines of transgenic *Arabidopsis thaliana* wild-type *GSA-1 cDNA*/*gsa-1*, defective *GSA-1 cDNA*/*gsa-1* and pBI21 vector/*gsa-1*. Transgenic lines of wild-type *GSA-1 cDNA*/*gsa-1* displayed gravitropic curvature kinetics similar to those of Col-0 under NH_4^+ stress, whereas transgenic lines of defective *GSA-1 cDNA*/*gsa-1* and pBI21 vector/*gsa-1* were similar to *gsa-1* (Fig. 3e). These results indicate that the agravitropism phenotype of *gsa-1* is indeed caused by the mutation of *GSA-1/ARG1*, and that *GSA-1/ARG1* plays an important role in the resistance to NH_4^+-induced reduction of root gravitropism.

gsa-1 displays similar amyloplast degradation kinetics to Col-0 under ammonium stress

As reported previously by Harrison & Masson (2008), the mutation of *GSA-1/ARG1* results in larger amyloplast accumulation in roots relative to Col-0, verified by starch staining (Fig. 4a,b), probably by increasing cells with columella identity in the root cap (Harrison & Masson, 2008). As amyloplast degradation is well known to be involved in a variety of environmental stress responses (Takahashi *et al.*, 2003), we proceeded to test whether the sensitivity of *gsa-1* root gravitropism to NH_4^+ was associated with accelerated degradation of amyloplasts under NH_4^+ stress. At shorter NH_4^+ exposure times (< 24 h), no rapid degradation of amyloplasts was observed in the columella cells of Col-0. However, starch was gradually degraded with increasing treatment times and NH_4^+ concentrations, such as a 72-h treatment with 20 mM $(NH_4)_2SO_4$ or > 48-h treatment with 30 mM $(NH_4)_2SO_4$ (Figs 4c, S5). Interestingly, the g*sa-1* mutant showed a similar trend in amyloplast degradation as that seen in Col-0 under NH_4^+ stress (Figs 4d, S5). These results show that NH_4^+

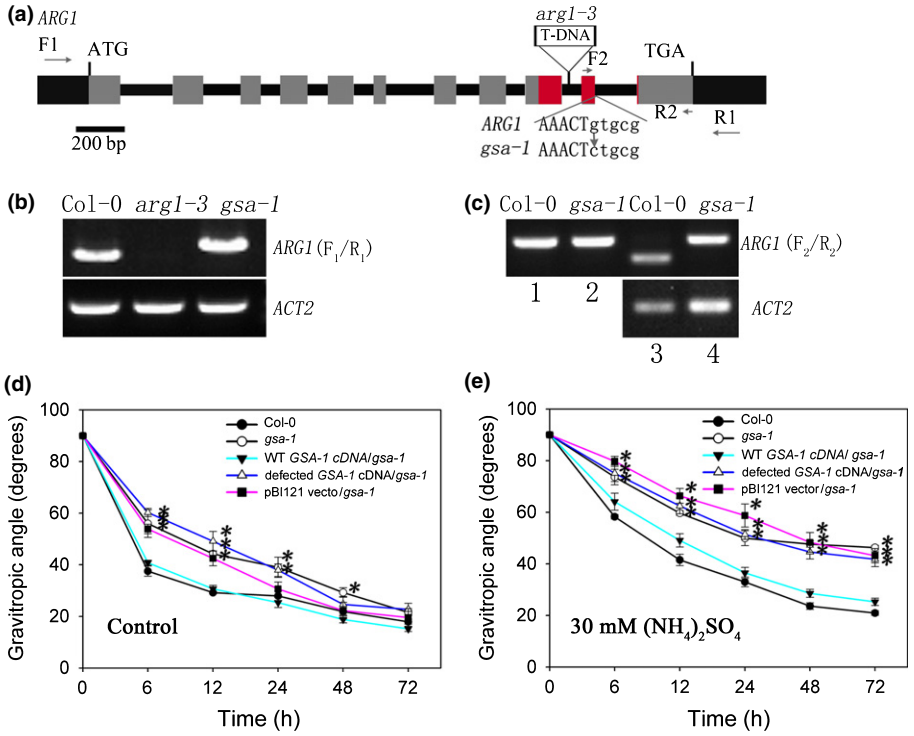

Fig. 3 Molecular characterization of *Arabidopsis thaliana gsa-1* mutant. (a) Genomic structure of *GSA-1/ARG1*, *arg1-3* and *gsa-1*. The diagram illustrates the positions of the start and stop codons, exons (boxes), introns (lines), T-DNA and regions coding for the coiled-coil domains (red) in *GSA-1/ARG1*. (b) Reverse-transcription polymerase chain reaction (RT-PCR) analyses using total RNA from seedlings of the indicated genotype with the F1/R1 primers displayed in (a), showing that the full-length product was undetectable in *arg1-3*, but larger in *gsa-1* relative to *ARG1*. Control reactions amplifying cDNA from *ACT2* are shown for quantitative comparison. (c) PCR amplifying DNA (1, 2) and cDNA (3, 4) from the indicated genotypes with the F2/R2 primers displayed in (a). Genomic DNA amplification products in *gsa-1* had the same length as in Col-0 and were also equal to cDNA amplification products in *gsa-1*, but larger than the cDNA amplification products in Col-0. (d, e) Gravitropic response of Col-0, *gsa-1*, T3 progeny of wild-type *ARG1 cDNA/gsa-1*-transformed homozygous plants, T3 progeny of defective *ARG1 cDNA/gsa-1*-transformed homozygous plants and T3 progeny of control expression vector pBI121-transformed homozygous plants on control plates (d) and plates supplied with 30 mM $(NH_4)_2SO_4$ (e). Data are from four independent experiments with 10–12 seedlings per experiment. Error bars indicate ± SE. *Significant differences ($P < 0.05$) compared with the controls.

treatments affect the levels of starch in columella cells, but that an altered degradation rate of amyloplasts is not involved in the NH_4^+-induced disruption of root gravitropism.

Ammonium increases the expression of *GSA-1/ARG1*

The expression of *GSA-1/ARG1* was studied with the GUS reporter gene under the control of the 1405-bp *GSA-1/ARG1* promoter (*ProARG1:GUS*). Histochemical analysis revealed GUS staining throughout the entire seedling, including the young leaves, vascular tissues of cotyledons, shoot apical meristem, hypocotyl and roots (Fig. 5a). The expression of *ProARG1:GUS* increased with seedling age (day 0–day 3) under control conditions, and GUS staining in the root tip was further intensified with prolonged NH_4^+ treatment (Fig. 5b). These results indicate that the expression of the *GSA-1/ARG1* gene is regulated by seedling age and is increasingly induced by extended exposure to elevated NH_4^+.

GSA-1/ARG1 regulates basipetal auxin transport in roots

DR5::GUS staining showed that, under vertical growth conditions, *gsa-1* seedlings accumulated higher levels of auxin in the expanded domain of the root cap relative to the wild-type, and there was no detectable lateral redistribution of auxin on gravistimulation (Figs 6, S6). In the presence of NH_4^+, the development of the lateral auxin gradient was both delayed and prolonged in Col-0 (Fig. 6), whereas epidermal staining was evident further away from the root tip in *gsa-1* after 24 h (Fig. 6).

Basipetal auxin transport of *gsa-1* was assessed by direct measurement with radiolabeled ^{3}[H]IAA. The mutation of *ARG1* reduced basipetal auxin transport by 38.96% ($P < 0.05$). However, there was no further reduction in basipetal auxin transport in *gsa-1* roots with NH_4^+ treatment (Fig. 7). In the wild-type, treatment with 30 mM $(NH_4)_2SO_4$ for 24 h decreased basipetal auxin transport by 28.68% ($P > 0.05$). These results show that *GSA-1/ARG1* is required for basipetal auxin transport in roots.

GSA-1/ARG1 regulates the expression of AUX1, but not PIN2, at the protein level

The appropriate expression and location of the auxin transporters AUX1 and PIN2 in lateral root caps and epidermal cells are required for normal basipetal auxin transport in roots (Friml, 2003). However, the expression and localization pattern of *proAUX1::AUX1-YFP* in lateral root caps of Col-0 were not

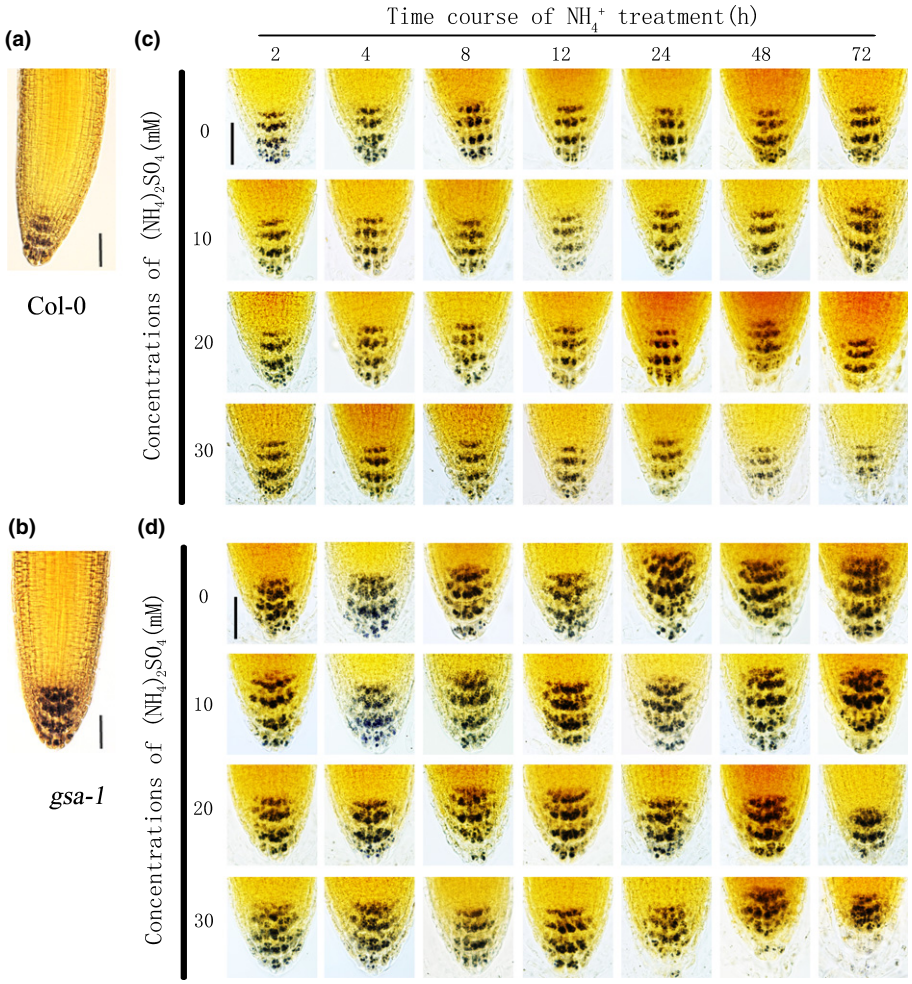

Fig. 4 Effects of ammonium on amyloplasts of columella cells in *Arabidopsis thaliana* Col-0 and *gsa-1*. Five-day-old seedlings were transferred to media with different concentrations of $(NH_4)_2SO_4$ and sampled at the indicated time points. Images are representative of 20–30 seedlings, stained in two to three separate experiments. Bars, 50 μm. (a, b) I_2-KI staining showed that *gsa-1* had more amyloplasts than Col-0 in the columella cells. (c, d) Concentration–time kinetics of $(NH_4)_2SO_4$ exposure and the degradation of amyloplasts in Col-0 (c) and *gsa-1* (d).

affected under 30 mM $(NH_4)_2SO_4$ stress on gravistimulation (Fig. 8a). By contrast, the fluorescence intensity of *proPIN2::PIN2-GFP* in whole-root apices of Col-0 decreased gradually with time, implying a relation of PIN2 during NH_4^+ stress (Fig. 8b).

To examine the distribution and levels of AUX1 and PIN2 proteins in the *gsa-1* background, the *proAUX1::AUX1-YFP* and *proPIN2::PIN2-GFP* constructs were introduced into the *gsa-1* mutant. The expression of *proAUX1::AUX1-YFP* resulted in a nearly undetectable AUX1-YFP fluorescence signal in the *gsa-1* root apices under the same conditions as Col-0. After extending the master gain of the YFP channel from 679 to 1017, a normal localization of the YFP signal (similar to that of Col-0) was seen in phloem tissue, columella cells, epidermal cells and the lateral root cap of the mutant primary roots (Fig. 9a). These results indicate that the AUX1 protein is distributed normally in the mutant, except for reduced expression in root apices. Nevertheless, NH_4^+ treatments did not affect the localization and expression intensity of AUX1-YFP in mutant root apices any further on gravistimulation, although an overall reduced root length was observed (Fig. 9b). By contrast, the mutation of *GSA-1/ARG1* had no effect on the expression and localization of PIN2 in root apices relative to Col-0 (Fig. 9c). Ammonium treatments decreased dramatically the PIN2-GFP fluorescence signal intensity in *gsa-1* root apices, as was also seen in Col-0 (Fig. 9c), implying that the expression and localization pattern of PIN2 in root apices are independent of *GSA-1/ARG1*.

eir1-1 and *aux1-22* enhance the gravitropic defect of *gsa-1*

To further explore the roles of AUX1 and PIN2 in NH_4^+-induced *gsa-1* root gravitropism, we constructed double mutants of *gsa-1aux1-22* and *gsa-1eir1-1*. Both *aux1-22* and *eir1-1* exacerbated the phenotype of *gsa-1* for seedlings germinated on normal growth medium, making the double mutants *gsa-1aux1-22* and *gsa-1eir1-1* grow in random directions (Fig. 10a). Root gravitropic angles relative to the gravity vector were measured and displayed for 5-d-old seedlings, with the system shown in Fig. 10(b). It was found that the root gravitropic angles of Col-0, *gsa-1*, *aux1-22* and *eir1-1* were distributed within 60° (−15° to 45°), 210° (−105° to 105°), 360° and 150° (−45° to 105°), respectively. Both *aux1-22* and *eir1-1* showed a wider distribution of *gsa-1* root gravitropic angles, with the double mutants *gsa-1aux1-22* and *gsa-1eir1-1* within 360° (Fig. 10c).

The root gravitropic kinetics of these mutants under NH_4^+ stress were also explored (Fig. 11). In contrast with the NH_4^+

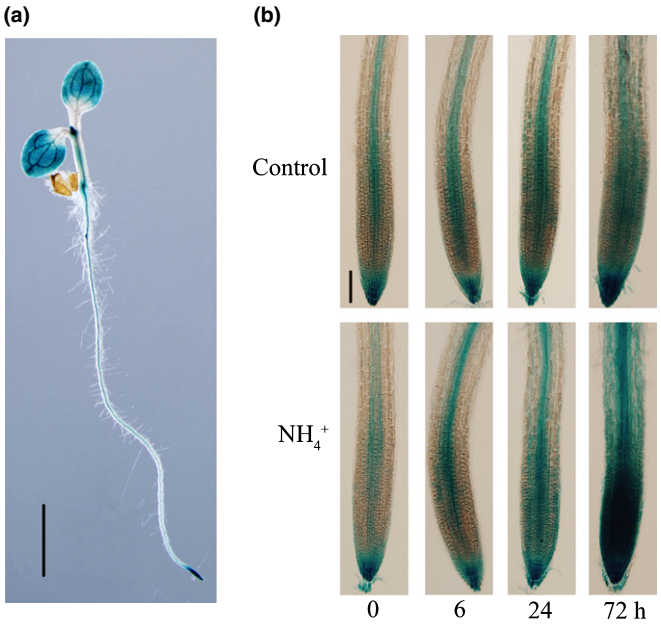

Fig. 5 Ammonium regulates the expression of *GSA-1/ARG1*. The images shown are representative of at least 10 plants for each treatment, and the experiments were repeated at least twice. (a) *GSA-1/ARG1* is expressed throughout the plants. Five-day-old *proARG1::GUS*-transformed homozygous *Arabidopsis thaliana* seedlings were subjected to histochemical analysis of β-glucuronidase (GUS) activity. Bar, 2 cm. (b) Expression of *GSA-1/ARG1* induced by extended NH_4^+ exposure and following gravistimulation. Bar, 100 μm.

effects on Col-0 and *gsa-1* root gravitropism, NH_4^+ partially restored the root agravitropism of *aux1-22*. In addition, *gsa-1aux1-22* roots had the same tendency of gravitropic kinetics as roots of the *aux1-22* mutant line under NH_4^+ treatments. Ammonium also partially restored the agravitropism of *eir1-1* and *gsa-1eir1-1*, but the gravitropic kinetics of *gsa-1eir1-1* roots under NH_4^+ stress were not consistent with that scored for *eir1-1* roots.

Discussion

Much recent interest has centered around the mechanisms governing the root gravitropic response, in particular under

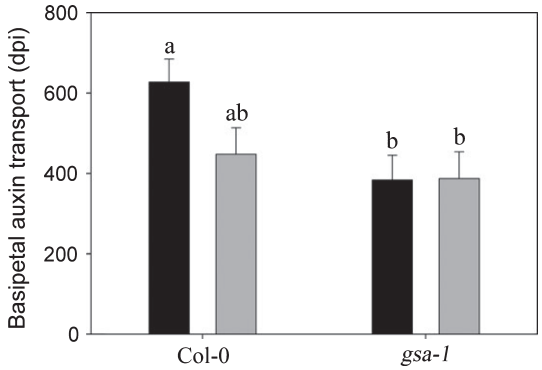

Fig. 7 Effects of *GSA-1/ARG1* and ammonium on basipetal auxin transport in 5 d after germination (DAG) seedlings of *Arabidopsis thaliana* Col-0 and *gsa-1* using ^{3}H-IAA-labelling assays. Control, black bars; NH_4^+, gray bars. Data are from three replicates with 10 seedlings each, and bars show +SEM. Letters above the bars indicate significantly different ($P < 0.05$) as determined by ANOVA.

environmental stress (Chavarría-Krauser *et al.*, 2008; Li & Zhang, 2008; Sun *et al.*, 2008; Rigas *et al.*, 2012). With respect to the important environmental stress of excess NH_4^+ (Britto & Kronzucker, 2002), however, very little is known about the specific targets and pathways that lead to impaired gravitropism (Zou *et al.*, 2012). To gain an insight into the mechanisms of the effects of NH_4^+ on root gravitropism, we employed a molecular genetics approach, based on a mutant screen for altered response to NH_4^+. Strongly enhanced NH_4^+ sensitivity of root gravitropism was found in *gsa-1*; this defect was rescued by transformation using wild-type *GSA-1cDNA* constructs, and wild-type *GSA-1cDNA/gsa-1* lines displayed similar root gravitropic dynamics to wild-type Col-0 under NH_4^+ stress (Fig. 3d,e). These results suggest that *GSA-1/ARG1* plays an important role in the NH_4^+-induced impairment of root gravitropism. It defines a novel genetic locus essential for NH_4^+ tolerance in *Arabidopsis*.

By examining the new mutant, we identified and characterized a new mutant allele in the *Arabidopsis ARG1* gene. Distinguished from the T-DNA insertional null mutant *arg1-3*, the agravitropism phenotype of the *gsa-1* allele was caused by a G to C mutation at the universally conserved 5′-GT dinucleotide splicing motif of intron 10, giving rise to aberrantly spliced mRNA

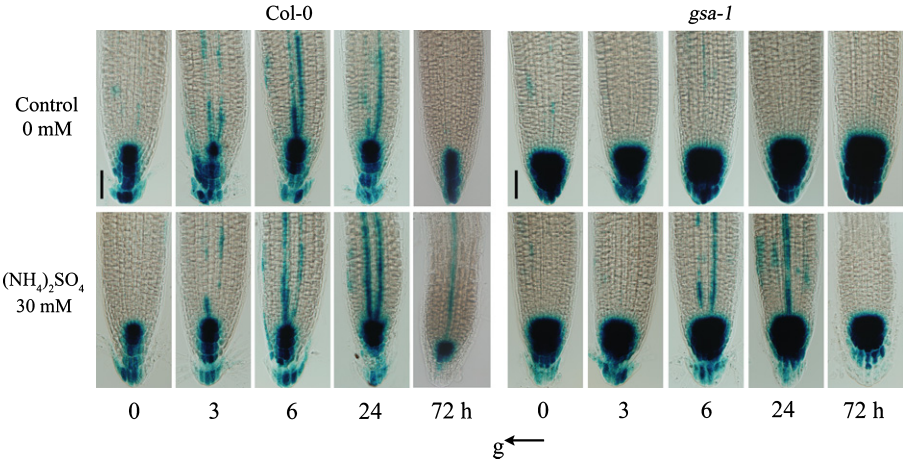

Fig. 6 Influence of ammonium on *DR5::GUS* expression in the root tip during the gravitropic response. Five-day-old *DR5::GUS* transgenic seedlings in *Arabidopsis thaliana* Col-0 and *gsa-1* backgrounds were transferred onto control medium or medium supplied with 30 mM $(NH_4)_2SO_4$ for the indicated times of gravistimulation, and then subjected to 4 h of staining and observation. The arrow indicates the direction of the gravity vector. Images are representative of 20–30 seedlings, stained in two to three separate experiments. Bar, 50 μm.

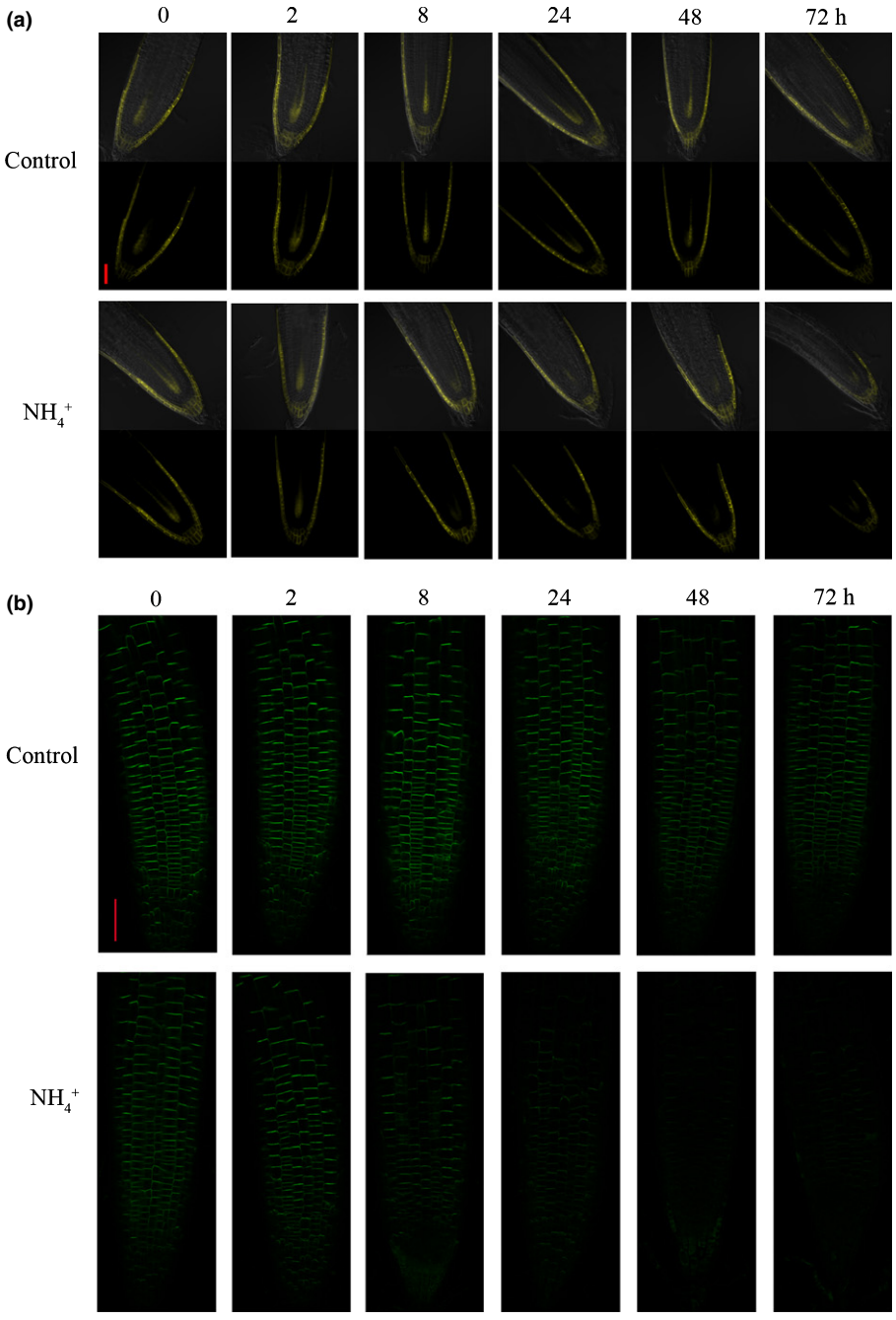

Fig. 8 Effects of ammonium on AUX1 and PIN2 in Col-0 roots. Five-d-old *Arabidopsis thaliana* wild-type seedlings, containing *proAUX1::AUX1-YFP* or *proPIN2::PIN2-GFP* constructs, were transferred onto control medium or medium supplemented with 30 mM $(NH_4)_2SO_4$ for gravistimulation. The fluorescence signal was detected with a Zeiss LSM710 confocal laser scanning microscope at the specified time points following gravistimulation. One representative image for each experiment is shown. Bars, 50 μm. (a) Effects of ammonium on the expression of *proAUX1::AUX1-YFP* in roots. The images were captured using the same confocal setting and are representative of at least 30 roots obtained from at least three independent experiments. (b) Effects of ammonium on the expression of *proPIN2::PIN2-GFP* in roots. The images were captured using the same confocal setting and are representative of 30 roots obtained from three independent experiments.

(Fig. 3a–c). Previously, a mutation has been described at the *aux1-22* allele, caused by a T to A mutation at the 5′-splice site of intron 5, thus abolishing the GT splice site consensus (Marchant & Bennett, 1998). With *gsa-1*, the mutant had a similar phenotype to that of the null mutant *arg1-3*. It is plausible that the larger mRNAs in *gsa-1* were removed to prevent the accumulation of potentially harmful proteins. As a result, *gsa-1* also presented as a null mutant at the protein level. The selective degradation of nonsense-containing transcripts has been reported in yeast, in the nematode *Caenorhabiditis elegans* and in plants (Marchant & Bennett, 1998). Further, the C-terminal coiled-coil region might be essential for the function of *GSA-1*/*ARG1*, or the function of *GSA-1*/*ARG1* in root gravitropism may require a joint action of the N-terminal J domain and the C-terminal coiled-coil region, as a consequence of which the disruption of the C-terminal coiled-coil region would lead to a loss of function in *GSA-1*/*ARG1* (Boonsirichai *et al.*, 2003). Therefore, the identification of new mutant alleles of the *ARG1* gene of *Arabidopsis thaliana* provides a useful system to examine novel mutations affecting mRNA stability and pre-mRNA splicing or functional protein stability.

We also explored the function of *GSA-1*/*ARG1* in the regulation of basipetal auxin transport, and the expression of AUX1 at the protein level. The *ARG1* mutation reduced basipetal auxin

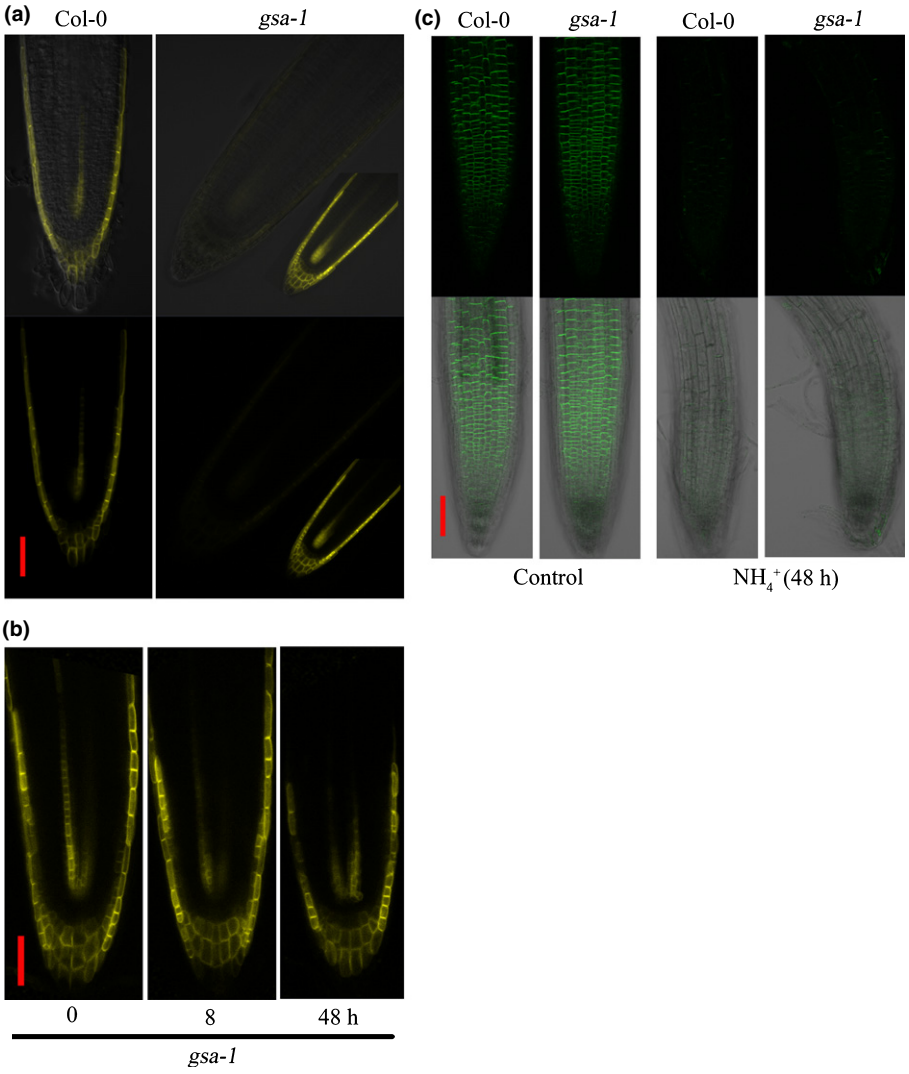

Fig. 9 Expression and localization of AUX1 and PIN2 in *gsa-1* roots in response to ammonium exposure. Five-day-old seedlings containing *proAUX1::AUX1-YFP* or *proPIN2::PIN2-GFP* constructs in the *Arabidopsis thaliana* Col-0 or *gsa-1* background were used. (a) Levels of *proAUX1::AUX1-YFP* were attenuated, but the localization was not affected, in *gsa-1* roots relative to Col-0. Col-0 and *gsa-1* were maintained under the same confocal setting, and the insert shows *gsa-1* roots, but extending the master gain of the yellow fluorescent protein (YFP) channel from 679 to 1017. Upper columns show the single YFP images; lower columns show the integration images of YFP and bright field. (b) Five-day-old *gsa-1* seedlings containing *proAUX1::AUX1-YFP* constructs were transferred onto medium supplemented with 30 mM $(NH_4)_2SO_4$ for gravistimulation. The fluorescence signal was detected with a Zeiss LSM710 confocal laser scanning microscope at the specified time points following gravistimulation, with the master gain of the YFP channel set to 1017. (c) Expression and localization of *proPIN2::PIN2-GFP* in *gsa-1* roots relative to Col-0 under control and NH_4^+ stress. Top panel shows single confocal sections; bottom panel shows integration with bright field. Bars, 100 μm.

transport by 38.96% (Fig. 7), which is in good agreement with the greater amount of auxin accumulated in an expanded domain of the root apex relative to the wild-type (Boonsirichai *et al.*, 2003; Harrison & Masson, 2008; Fig. 6). These findings suggest that the reduction in basipetally transported auxin results in auxin accumulation in the root cap. A similar phenomenon has also been described in the agravitropic mutant *pin2/eir1/wav6/agr1*, where a reduction in basipetally transported auxin was compensated for increased auxin accumulation in root tips, as indicated by *DR5::GUS*, *DR5::GFP* and *IAA2:uidA*, and by direct chemical measurement of the apical IAA content (Rashotte *et al.*, 2000; Ottenschläger *et al.*, 2003; Shin *et al.*, 2005; Swarup *et al.*, 2005).

ARG1 and PIN3 are engaged in the same gravity signal transduction pathway in root statocytes, and ARG1 is required for PIN3 relocalization and the asymmetrical redistribution of auxin (Harrison & Masson, 2008). The *arg1* mutant shows a more severe root gravitropic defect relative to that of *pin3* (Harrison & Masson, 2008), which may be associated with other established roles of *GSA-1/ARG1*, such as basipetal auxin transport and the expression of AUX1 protein (Figs 7, 9a). The specific AUX1 expression in the lateral root cap and in epidermal cells is required for the establishment of auxin gradients and root curvature following gravistimulation (Bainbridge *et al.*, 2008). AUX1 probably functions by facilitating the uptake of auxin into the lateral root cap and the epidermal region, and PIN2 by mediating its directional translocation towards EZ in the gravitropic response (Friml, 2003). This interpretation is supported by direct basipetal auxin transport measurements and the higher accumulation of auxin in the root cap (Figs 6, 7). However, the mechanisms by which GSA-1/ARG1 regulates AUX1 expression have yet to be elucidated.

The double mutants *aux1-22gsa-1* and *eir1-1gsa-1* exacerbated the phenotype of *gsa-1*, indicating that the disruption of either *aux1-22* or *eir1-1* in the *gsa-1* background may enhance the gravitropic defect of *gsa-1* (Figs 10a,c, 11). Although it was found that the expression and localization of AUX1 were not affected, the PIN2 protein was decreased gradually under NH_4^+ stress (Figs 8, 9). This suggests a tight relationship for PIN2 in the NH_4^+-induced inhibition of root gravitropism. In addition to

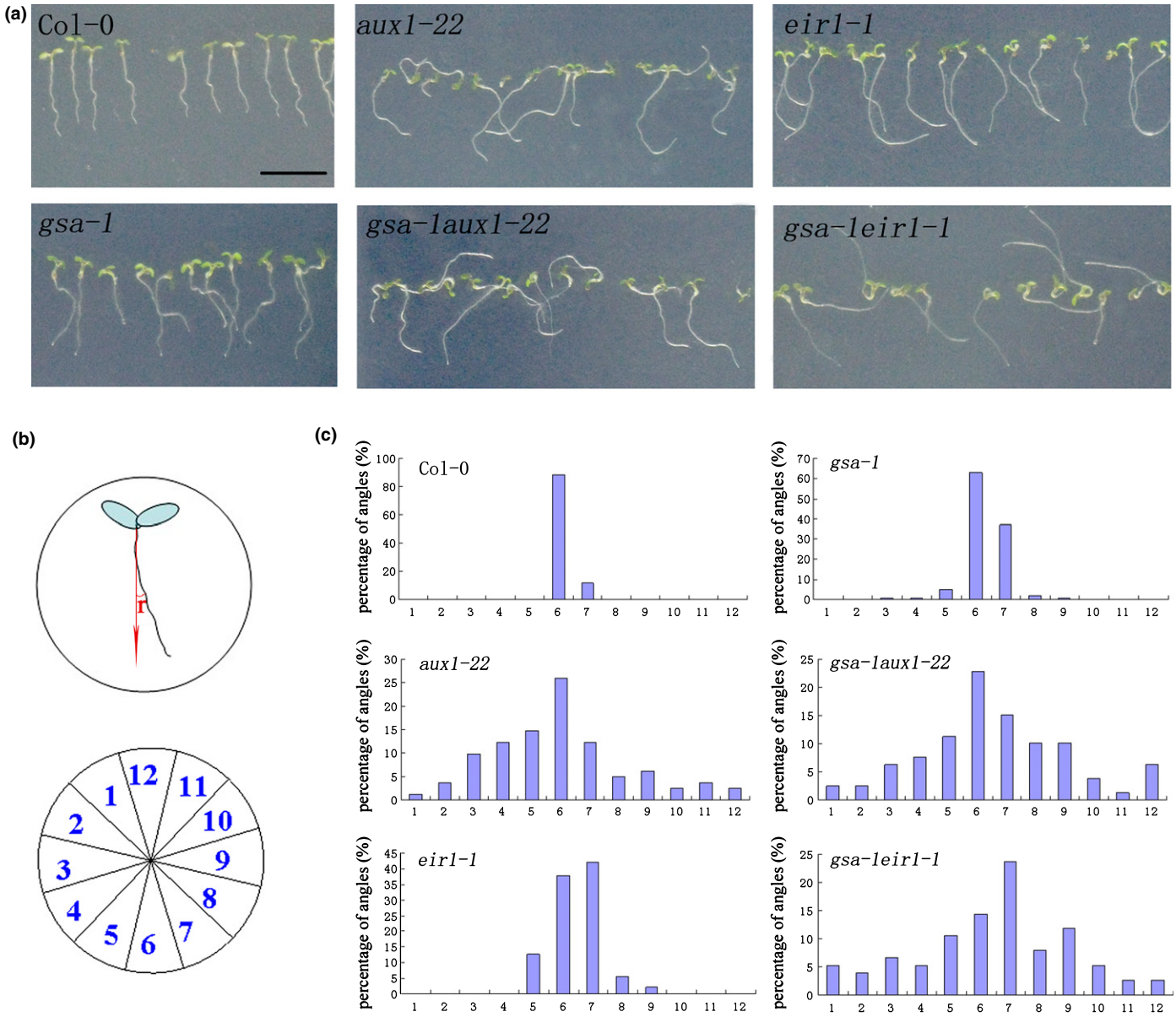

Fig. 10 Mutations of *aux1-22* and *eir1-1* enhance the phenotype of *gsa-1*. (a) Seedlings of *Arabidopsis thaliana* Col-0, *gsa-1*, *aux1-22*, *eir1-1*, *gsa-1aux1-22* and *gsa-1eir1-1* germinated on normal growth medium for 5 d. Bar, 1 cm. (b) Schematic diagram showing measurement of the primary root gravitropic angle (r). Root angles relative to the gravity vector (r) were measured and placed into one of the 12 bins, set at 30° intervals. (c) Distribution of the root gravitropic angle in Col-0, *gsa-1*, *aux1-22*, *eir1-1*, *gsa-1aux1-22* and *gsa-1eir1-1* within 12 bins covering 360°. $n = 50–60$ from three independent experiments.

participating in AUX1-mediated basipetal auxin transport, PIN2 has been shown to be involved in the gravitropic response, and the *pin2* mutant lacks the classic TZ bending response (Chavarría-Krauser *et al.*, 2008; Baluška *et al.*, 2010). This effect on TZ in relation to PIN2 was seen as a general stress target on account of the pronounced sensitivity to multiple adverse environmental stresses, such as cold, dark, salt and aluminum toxicity (Baluška *et al.*, 2010). For example, under salt stress, PIN2 was targeted to endomembrane compartments and selectively degraded to disassemble the PIN2-based gravisensitive network and to allow roots to deviate from the gravity vector and avoid salt-enriched areas of soil (Li & Zhang, 2008; Sun *et al.*, 2008). PIN2 is also degraded and basipetal auxin transport is inhibited if roots are kept in the dark (Laxmi *et al.*, 2008; Wan *et al.*, 2012), which is the natural root environment (Yokawa *et al.*, 2011, 2013).

Two distinct bending regions have been reported in the literature, corresponding to EZ and TZ, and these drive the differential growth of root gravitropism (Verbelen *et al.*, 2006; Baluška *et al.*, 2010). The auxin concentration gradient-dependent EZ bending and the auxin concentration gradient-independent (but PIN2-related) TZ/DEZ bending are partially uncoupled from each other, and it has been shown that the mutation of *PIN3* affects the bending of EZ, but not TZ/DEZ curvature (Chavarría-Krauser *et al.*, 2008). We speculate that, similar to the *pin3* mutant, the *gsa-1* mutant lacks the auxin concentration gradient-

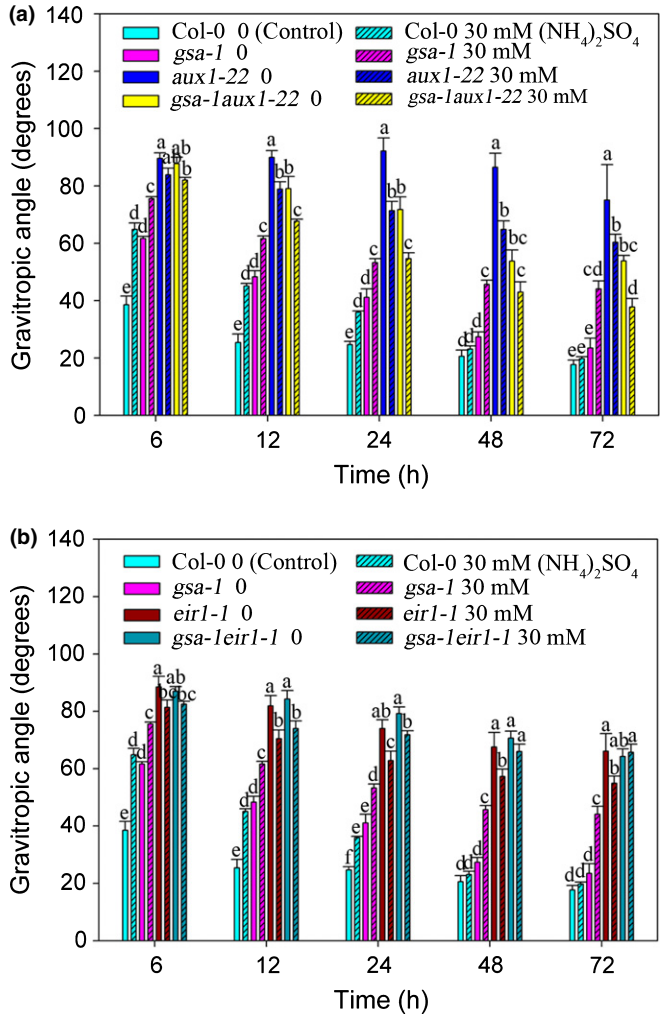

Fig. 11 Ammonium enhances the phenotype of *gsa-1* and partially rescues the agravitropic responses of *aux1-22*, *eir1-1*, *gsa-1aux1-22* and *gsa-1eir1-1*. Root gravitropic kinetics of 5-d-old seedlings of *Arabidopsis thaliana* Col-0, *gsa-1*, *aux1-22*, *eir1-1*, *gsa-1aux1-22* and *gsa-1eir1-1* as shown in Fig. 10. Data are from three independent experiments with 10–12 seedlings per experiment. Error bars indicate + SE. Letters above the bars indicate whether the different treatments are significantly different ($P < 0.05$), as determined by ANOVA followed by least-significant difference (LSD) test.

dependent EZ curvature, whilst possessing normal TZ/DEZ bending. First, this is supported by the distinctly positive gravitropic response of *gsa-1* in the absence of lateral auxin redistribution following gravistimulation (Figs 1–3, 6). Second, NH_4^+ decreased significantly the root gravitropism of *gsa-1*, whilst not further reducing basipetal auxin transport (Fig. 7).

The analysis of the root gravitropism kinetics of *gsa-1*, *aux1-22*, *eir1-1*, *gsa-1aux1-22* and *gsa-1eir1-1* under NH_4^+ stress (Fig. 10d) supports the model in which *AUX1* and *GSA-1/ARG1* participate in the same pathway, whereas *PIN2* and *GSA-1/ARG1* function independently. Lateral auxin gradient-dependent EZ bending, which involves PIN3, regulates the lateral auxin gradient across the root cap, and AUX1- and PIN2-mediated basipetal auxin transport into EZ. Therefore, we speculate that GSA-1/ARG1 functions in this process by direct or indirect regulation of PIN3 and AUX1, consistent with the observed increased *GSA-1/ARG1* expression, the delayed lateral auxin gradient and the prolonged root gravitropic response of Col-0 under NH_4^+ stress (Fig. 6).

In summary, our molecular genetic and physiological results identify a gene locus essential for NH_4^+ tolerance in the specific context of root gravitropism. The *gsa-1* mutant isolated displays distinct root gravitropic characteristics relative to those of the Col-0 wild-type under NH_4^+ stress. The effects of NH_4^+ on root gravitropism were independent of the classic amyloplast-involving gravity-sensing pathway. In addition to PIN3 (Harrison & Masson, 2008), GSA-1/ARG1 can be shown to be further required for appropriate AUX1 expression and basipetal auxin transport in root apices. Moreover, the expression of PIN2 was dramatically reduced, independent of GSA-1/ARG1 function, in root apices under NH_4^+ stress. Taken together, we propose that, during NH_4^+ stress, GSA-1/ARG1 expression is increased and is essential for the establishment of the PIN3-mediated lateral auxin gradient across the root cap. AUX1-related basipetal auxin transport on gravistimulation can partially antagonize the reduction in PIN2-mediated root gravitropism in the wild-type. However, the NH_4^+-induced inhibition of PIN2 could also cause the reduction of the root gravitropic response via disruption of the GSA-1/ARG1-dependent pathway. Thus, PIN2 in TZ emerges as a general stress target during multiple adverse environmental stresses, including NH_4^+ stress. Our working model provides new insights into the acclimation of root gravitropism to environmental stress.

Acknowledgements

We thank Professors Malcolm Bennett (University of Nottingham), Ben Scheres (Utrecht University) and Tom Guilfoyle (University of Missouri) for providing the transgenic lines of *Arabidopsis*, and the Arabidopsis Biological Resource Center for the mutant seeds. We are grateful to Dr Jianru Zuo (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for kind provision of *Arabidopsis* seeds mutagenized with T-DNA transformation, Feifei Sun (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for assistance with amyloplast staining and Dr Guangjie Li (Institute of Soil Science, Chinese Academy of Sciences) for assistance with *DR5::GUS* staining. We also thank other members of our team and members of the laboratory of Professor Yanhua Su (Institute of Soil Science, Chinese Academy of Sciences) for their assistance. This work was supported by the National Natural Science Foundation of China (31200189 and 30771285), the Chinese Academy Sciences Innovation Program (ISSASIP1103) and the Natural Sciences and Engineering Research Council of Canada (NSERC, Discovery Grant 217277-2009).

References

Bainbridge K, Guyomarc'h S, Bayer E, Swarup R, Bennett M, Mandel T, Kuhlemeier C. 2008. Auxin influx carriers stabilize phyllotactic patterning. *Genes & Development* **22**: 810–823.

Baluška F, Mancuso S, Volkmann D, Barlow PW. 2010. Root apex transition zone: a signalling-response nexus in the root. *Trends in Plant Science* **15**: 402–408.

Blancaflor EB, Fasano JM, Gilroy S. 1998. Mapping the functional roles of cap cells in the response of *Arabidopsis* primary roots to gravity. *Plant Physiology* **116**: 213–222.

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B. 2005. The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* **433**: 39–44.

Bonser AM, Lynch J, Snapp S. 1996. Effect of phosphorus deficiency on growth angle of basal roots in *Phaseolus vulgaris*. *New Phytologist* **132**: 281–288.

Boonsirichai K, Sedbrook JC, Chen R, Gilroy S, Masson PH. 2003. ALTERED RESPONSE TO GRAVITY is a peripheral membrane protein that modulates gravity-induced cytoplasmic alkalinization and lateral auxin transport in plant statocytes. *Plant Cell* **15**: 2612–2625.

Britto DT, Kronzucker HJ. 2002. NH_4^+ toxicity in higher plants: a critical review. *Journal of Plant Physiology* **159**: 567–584.

Caspar T, Pickard BG. 1989. Gravitropism in a starchless mutant of *Arabidopsis*. *Planta* **177**: 185–197.

Chavarría-Krauser A, Nagel KA, Palme K, Schurr U, Walter A, Scharr H. 2008. Spatio-temporal quantification of differential growth processes in root growth zones based on a novel combination of image sequence processing and refined concepts describing curvature production. *New Phytologist* **177**: 811–821.

Chen R, Guan C, Boonsirichai K, Masson PH. 2002. Complex physiological and molecular processes underlying root gravitropism. *Plant Molecular Biology* **49**: 305–317.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**: 735–743.

Forde B, Lorenzo H. 2001. The nutritional control of root development. *Plant and Soil* **232**: 51–68.

Friml J. 2003. Auxin transport – shaping the plant. *Current Opinion in Plant Biology* **6**: 7–12.

Friml J, Wiśniewska J, Benková E, Mendgen K, Palme K. 2002. Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* **415**: 806–809.

Ge L, Peer W, Robert S, Swarup R, Ye S, Prigge M, Cohen J, Friml J, Murphy A, Tang D. 2010. *Arabidopsis* ROOT UVB SENSITIVE2/WEAK AUXIN RESPONSE1 is required for polar auxin transport. *Plant Cell* **22**: 1749–1761.

Gerendás J, Zhu Z, Bendixen R, Ratcliffe RG, Sattelmacher B. 1997. Physiological and biochemical processes related to ammonium toxicity in higher plants. *Zeitschrift für Pflanzenernaehrung und Bodenkunde* **160**: 239–251.

Harrison BR, Masson PH. 2008. ARL2, ARG1 and PIN3 define a gravity signal transduction pathway in root statocytes. *Plant Journal* **53**: 380–392.

Kempinski CF, Haffar R, Barth C. 2011. Toward the mechanism of NH_4^+ sensitivity mediated by *Arabidopsis* GDP-mannose pyrophosphorylase. *Plant, Cell & Environment* **34**: 847–858.

Kiss JZ, Hertel R, Sack FD. 1989. Amyloplasts are necessary for full gravitropic sensitivity in roots of *Arabidopsis thaliana*. *Planta* **177**: 198–206.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1997. Conifer root discrimination against soil nitrate and the ecology of forest succession. *Nature* **385**: 59–61.

Laxmi A, Pan J, Morsy M, Chen R. 2008. Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in *Arabidopsis thaliana*. *PLoS ONE* **3**: e1510.

Lewis DR, Muday GK. 2009. Measurement of auxin transport in *Arabidopsis thaliana*. *Nature Protocols* **4**: 437–451.

Li BH, Li Q, Kronzucker HJ, Shi W. 2011b. Roles of abscisic acid and auxin in shoot-supplied ammonium inhibition of root system development. *Plant Signaling & Behavior* **6**: 1451–1453.

Li BH, Li Q, Su YH, Chen H, Xiong LM, Mi GH, Kronzucker HJ, Shi WM. 2011a. Shoot-supplied ammonium targets the root auxin influx carrier AUX1 and inhibits lateral root emergence in *Arabidopsis*. *Plant, Cell & Environment* **34**: 933–946.

Li BH, Li Q, Xiong LM, Kronzucker HJ, Krämer U, Shi WM. 2012. *Arabidopsis* plastid AMOS1/EGY1 integrates abscisic acid signaling to regulate global gene expression response to ammonium stress. *Plant Physiology* **160**: 2040–2051.

Li GJ, Li BH, Dong GQ, Feng XY, Kronzucker HJ, Shi WM. 2013. Ammonium-induced shoot ethylene production is associated with the inhibition of lateral root formation in *Arabidopsis*. *Journal of Experimental Botany* **64**: 1413–1425.

Li Q, Li BH, Kronzucker HJ, Shi WM. 2010. Root growth inhibition by NH_4^+ in *Arabidopsis* is mediated by the root tip and is linked to NH_4^+ efflux and GMPase activity. *Plant, Cell & Environment* **33**: 1529–1542.

Li X, Zhang W. 2008. Salt-avoidance tropism in *Arabidopsis thaliana*. *Plant Signaling & Behavior* **3**: 351–353.

Liao H, Rubio G, Yan X, Cao A, Brown KM, Lynch JP. 2001. Effect of phosphorus availability on basal root shallowness in common bean. *Plant and Soil* **232**: 69–79.

Luschnig C, Gaxiola RA, Grisafi P, Fink GR. 1998. EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes & Development* **12**: 2175–2187.

Marchant A, Bennett MJ. 1998. The *Arabidopsis* AUX1 gene: a model system to study mRNA processing in plants. *Plant Molecular Biology* **36**: 463–471.

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ. 1999. AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO Journal* **18**: 2066–2073.

Ottenschläger I, Wolff P, Wolverton C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K. 2003. Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proceedings of the National Academy of Sciences, USA* **100**: 2987–2991.

Perrin RM, Young LS, Murthy UMN, Harrison BR, Wang Y, Will JL, Masson PH. 2005. Gravity signal transduction in primary roots. *Annals of Botany (London)* **96**: 737–743.

Qin C, Qian W, Wang W, Wu Y, Yu C, Jiang X, Wang D, Wu P. 2008. GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **105**: 18308–18313.

Rashotte AM, Brady SR, Reed RC, Ante SJ, Muday GK. 2000. Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis*. *Plant Physiology* **122**: 481–490.

Rigas S, Ditengou FA, Ljung K, Daras G, Tietz O, Palme K, Hatzopoulos P. 2012. Root gravitropism and root hair development constitute coupled developmental responses regulated by auxin homeostasis in the *Arabidopsis* root apex. *New Phytologist* **197**: 1130–1141.

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR. 1995. Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. *Genetics* **139**: 1393–1409.

Salathia N, Lee HN, Sangster TA, Morneau K, Landry CR, Schellenberg K, Behere AS, Gunderson KL, Cavalieri D, Jander G *et al.* 2007. Indel arrays: an affordable alternative for genotyping. *Plant Journal* **51**: 727–737.

Sedbrook JC, Chen R, Masson PH. 1999. ARG1 (altered response to gravity) encodes a DnaJ-like protein that potentially interacts with the cytoskeleton. *Proceedings of the National Academy of Sciences, USA* **96**: 1140–1145.

Shin H, Shin HS, Guo Z, Blancaflor EB, Masson PH, Chen R. 2005. Complex regulation of *Arabidopsis* AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. *Plant Journal* **42**: 188–200.

Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X. 2008. Salt modulates gravity signaling pathway to regulate growth direction of primary roots in *Arabidopsis*. *Plant Physiology* **146**: 178–188.

Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M. 2001. Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. *Genes & Development* **15**: 2648–2653.

Swarup R, Kargul J, Marchant A, Zadika D, Rahman A, Mills R, Yemm A, May S, Williams L, Millner P *et al.* 2004. Structure–function analysis of the presumptive *Arabidopsis* auxin permease AUX1. *Plant Cell* **16**: 3069–3083.

Swarup R, Kramer EM, Perry P, Knox K, Leyser HMO, Haseloff J, Beemster GTS, Bhalerao R, Bennett MJ. 2005. Root gravitropism requires lateral root

cap and epidermal cells for transport and response to a mobile auxin signal. *Nature Cell Biology* **7**: 1057–1065.

Takahashi N, Yamazaki Y, Kobayashi A, Higashitani A, Takahashi H. 2003. Hydrotropism interacts with gravitropism by degrading amyloplasts in seedling roots of *Arabidopsis* and radish. *Plant Physiology* **132**: 805–810.

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ. 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**: 1963–1971.

Verbelen J-P, De Cnodder T, Le J, Vissenberg K, Baluška F. 2006. The root apex of *Arabidopsis thaliana* consists of four distinct zones of cellular activities: meristematic zone, transition zone, fast elongation zone, and growth terminating zone. *Plant Signaling & Behavior* **1**: 296–304.

Vicente-Agullo F, Rigas S, Desbrosses G, Dolan L, Hatzopoulos P, Grabov A. 2004. Potassium carrier TRH1 is required for auxin transport in *Arabidopsis* roots. *Plant Journal* **40**: 523–535.

Wan Y, Jasik J, Wang L, Hao H, Volkmann D, Menzel D, Mancuso S, Baluška F, Lin J. 2012. The signal transducer NPH3 integrates the phototropin1 photosensor with PIN2-based polar auxin transport in *Arabidopsis* root phototropism. *Plant Cell* **24**: 551–565.

Weigel D, Glazebrook J. 2002. *Arabidopsis: a laboratory manual*. New York, NY, USA: Cold Spring Harbor Laboratory Press, 243–245.

Wisniewska J, Xu J, Seifertová D, Brewer PB, Ruzicka K, Blilou I, Benková E, Scheres B, Friml J. 2006. Polar PIN localization directs auxin flow in plants. *Science* **312**: 883.

Wolverton C, Mullen JL, Ishikawa H, Evans ML. 2002. Root gravitropism in response to a signal originating outside of the cap. *Planta* **215**: 153–157.

Yokawa K, Kagenishi T, Baluška F. 2013. Root photomorphogenesis in laboratory-maintained *Arabidopsis* seedlings. *Trends in Plant Science* **18**: 117–119.

Yokawa K, Kagenishi T, Kawano T, Mancuso S, Baluška F. 2011. Illumination of *Arabidopsis* roots induces immediate burst of ROS production. *Plant Signaling & Behavior* **6**: 1460–1464.

Zhang J, Xu JX, Kong YZ, Ji ZD, Wang XC, An FY, Li C, Sun JQ, Zhang SZ, Yang XH *et al.* 2005. Generation of chemical-inducible activation tagging T-DNA insertion lines of *Arabidopsis thaliana*. *Acta Genetica Sinica* **32**: 1082–1088.

Zou N, Li BH, Dong GQ, Kronzucker HJ, Shi WM. 2012. Ammonium-induced loss of root gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from the inhibition of root elongation in *Arabidopsis*. *Journal of Experimental Botany* **63**: 3777–3788.

Zuo J, Niu QW, Chua NH. 2000. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant Journal* **24**: 265–273.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 F2 segregation analysis.

Fig. S2 Gravitropic response of *Arabidopsis thaliana* Col-0 and *gsa-1* seedlings to NH_4Cl, K_2SO_4, KNO_3 and mannitol exposure.

Fig. S3 The crossed F1 progeny of *gsa-1* and *arg1-3* do not complement the gravitropism-defect phenotype of each other.

Fig. S4 Wild-type *AtARG1* cDNA rescues the gravitropic defect of *gsa-1*.

Fig. S5 Changes in amyloplasts of the columella cells of Col-0 and *gsa-1* roots in response to ammonium treatment.

Fig. S6 Ammonium does not affect the auxin response in the root tip of *gsa-1* during the gravitropic response.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.