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Allantoin accumulation mediated by allantoinase downregulation and transport by Ureide Permease 5 confers salt stress tolerance to *Arabidopsis* plants

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Abstract Allantoin, a metabolite generated in the purine degradation pathway, was primarily considered an intermediate for recycling of the abundant nitrogen assimilated in plant purines. More specifically, tropical legumes utilize allantoin and allantoic acid as major nodule-to-shoot nitrogen transport compounds. In other species, an increase in allantoin content was observed under different stress conditions, but the underlying molecular mechanisms remain poorly understood. In this work, *Arabidopsis thaliana* was used as a model system to investigate the effects of salt stress on allantoin metabolism and to know whether its accumulation results in plant protection. Plant seedlings treated with NaCl at different concentrations showed higher allantoin and lower allantoic acid contents. Treatments with NaCl favored the expression of genes involved in allantoin synthesis, but strongly repressed the unique gene encoding allantoinase (AtALN). Due to the potential regulatory role of this gene for allantoin accumulation, AtALN promoter activity was studied using a reporter system. GUS mediated coloration was found in specific plant tissues and was diminished with increasing salt concentrations. Phenotypic analysis of knockout, knockdown and stress-inducible mutants for AtALN revealed that allantoin accumulation is essential for salt stress tolerance. In addition, the possible role of allantoin transport was investigated. The Ureide Permease 5 (UPS5) is expressed in the cortex and endodermis of roots and its transcription is enhanced by salt treatment. Ups5 knockout plants under salt stress presented a susceptible phenotype and altered allantoin root-to-shoot content ratios. Possible roles of allantoin as a protectant compound in oxidative events or signaling are discussed.

Keywords Allantoin · Allantoinase · Ureide Permease · Salt stress tolerance · *Arabidopsis*

Introduction

One of the most limiting factors for crop production is the detrimental effect of soil salinity. High salt concentrations adversely affect plant growth and development due to water stress caused by lower osmotic potentials in the soil, ion toxicity and possible imbalances/deficiencies in mineral nutrition (Zhu 2001). The capacity of plant species to tolerate salt stress may be related to the constitutive expression of genes that encode salt-tolerance determinants or their better ability to regulate the expression of these genes (Hasegawa et al. 2000). Adaptive mechanisms of plants to saline environments include changes in the expression of genes involved in regulation of ionic transport and compartmentalization, biosynthesis of compatible osmolytes and scavenging systems for reactive oxygen species, as well as of genes participating in long distance signaling for systemic response (Han et al. 2014; Hasegawa et al. 2000). In addition to these mechanisms, other molecular components can potentially participate in plant response to salt stress, but its relevance has not been adequately addressed.

In several reports, variations in the content of purine degradation metabolites have been associated with plant

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responses to different stress conditions and leaf senescence, but the underlying molecular mechanisms remain poorly understood (Brychkova et al. 2008; Kanani et al. 2010; Nikiforova et al. 2005; Rose et al. 2012; Sagi et al. 1998; Ventura et al. 2014; Watanabe et al. 2014; Yobi et al. 2013). Plant purine metabolism has been extensively studied in tropical leguminous species, since it constitutes an essential component of translocation of symbiotically fixed atmospheric nitrogen (Schubert 1986; Smith and Atkins 2002). The pathway and enzymes involved in purine catabolism has been described in the model plant *Arabidopsis thaliana*. Xanthine is the first common intermediate for degradation of all purine bases (Zrenner et al. 2006). In the cytosol, xanthine dehydrogenase (AtXDH) converts xanthine to uric acid (Hesberg et al. 2004; Werner and Witte 2011), which is imported into the peroxisomes and oxidized by urate oxidase (AtUOX) to hydroxyisourate and subsequently converted to allantoin by allantoin synthase (AtAS) (Lamberto et al. 2010). Allantoin is hydrolyzed to allantoic acid through allantoinase (AtALN), which has been localized in the endoplasmic reticulum (Werner et al. 2013). Subsequent catabolism steps results in the complete decomposition of the purine ring, releasing carbon dioxide, glyoxylate, and four ammonium ions (Serventi et al. 2010; Werner et al. 2010, 2013). A family of transmembrane proteins able to transport allantoin has been identified in *Arabidopsis* (AtUPS1-5, suggesting that allantoin transport could be necessary in physiological processes (Desimone et al. 2002). AtUPS1, AtUPS2 and AtUPS5 have been characterized regarding substrate specificities and expression (Schmidt et al. 2004, 2006). Whereas AtUPS1 and AtUPS2 showed higher affinity for pyrimidines than allantoin, AtUPS5 transported allantoin with high affinity and did not use pyrimidines as substrates. Differently from other family members, AtUPS5 is expressed in cortical cells and endodermis of roots suggesting that this permease could be involved in long distance transport of allantoin from root to shoot.

The genes encoding the enzymes of this pathway are differentially regulated under stress conditions: while the expression of AtXDH, AtUOX and AtAS increase more or less under osmotic and salt stress, AtALN transcription is strongly repressed (Kilian et al. 2007). Since AtALN is the unique known enzyme able to catabolize allantoin in plants, an increase of the allantoin levels under these conditions may be expected. However, information about the physiological role of allantoin is scarce. Transgenic plants deficient in xanthine dehydrogenase showed a faster loss of chlorophylls during dark induced senescence, which could be reverted by addition of allantoin or allantoic acid, indicating a role of both compounds in leaf senescence (Brychkova et al. 2008). Moreover, allantoin and allantoic acid influenced antioxidant capacity, suggesting that these metabolites are potential scavengers of reactive oxygen species (ROS). Apart from that, a recent study demonstrated a causal relationship between physiological allantoin accumulation and plant tolerance to water stress analyzing the phenotype of AtALN knockout mutants (Watanabe et al. 2014). This work also suggests that allantoin accumulation induces water stress tolerance by de novo biosynthesis and deconjugation of the stress hormone abscisic acid (ABA).

A primary objective of this work was to know whether the concentrations of allantoin and/or allantoic acid increase in *Arabidopsis* plants subjected to salt stress and whether their accumulation results in plant protection. We comparatively analyzed the plant phenotype of different genotypes, in which allantoin levels were (1) constitutively elevated (aln-1, knockout), (2) moderately enhanced (aln-2, knockdown), (3) physiologically regulated wild-type [WT] or (4) strongly reduced by stress induction (RD29A::ALN/aln-2). The general conclusion that the transcriptional downregulation of AtALN is a critical and distinctive mechanism under stress conditions prompted us to study changes in the expression pattern at the cellular level during the life cycle and by the onset of stress conditions. In further experiments, we focused our attention on a potential transport of allantoin mediated by AtUPS5. We found changes in the expression pattern of this transporter under salt stress as well as differences in the root to shoot relative concentrations of allantoin by comparison of the WT with two independent T-DNA insertion lines in AtUPS5. The phenotype of these lines was sensitive to salt stress suggesting that the site of allantoin synthesis is different from the site of its protective action.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as WT and all mutant lines were in the Col-0 background. Homozygous seeds of Salk_146783 (designated aln-1), Salk_142607 (designated aln-2) and Salk_044810 (designated ups5-1) lines were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University) and confirmed by PCR as described below. T2 Heterozygous seeds of Salk_123120 line (designated ups5-2) were obtained from ABRC and placed on 0.5× MS (Murashige and Skoog 1962) plates (Sigma-Aldrich) plus kanamycin 50 μg/ml. Seeds obtained from different T3 resistant plants were sowed again in selective media, their homozygosis confirmed by PCR as described below and used for further experiments.

For plant growth, seeds were first stratified at 4 °C for 2 days. After imbibition, germinating seeds were placed in

a growth chamber (day 0 for experimental treatments) under a 16 h light/8 h dark photoperiod at 22 °C and a light intensity of 100–150 μE/m^2s on 0.5× MS plates (1 % agar) or on soil: vermiculite (1:1) mix.

DNA extraction

Leaves from WT and transgenic plants were ground with liquid nitrogen and transferred to Eppendorf tubes. 440 μl of SENT-Buffer (200 mM Tris–HCl pH 7.5, 250 mM NaCl, 75 mM EDTA, 0.5 % SDS) were added. Samples were incubated for 5 min at room temperature and centrifuged for 5 min. Next, 350 μl of supernatant were added to a new tube with 350 μl of isopropanol. Samples were incubated for 20 min at −20 °C, and then centrifuged for 15 min. Subsequently, supernatants were discarded and 200 μl of 70 % ethanol was added to the tubes. Samples were centrifuged for 15 min, and the supernatants were discarded. Pellets were dried for 15 min at 37 °C and resuspended in 50 μl of sterile water. All centrifugation steps were performed at 13,000 RPM at room temperature.

Detection of T-DNA insertion

Genomic DNA was used for homozygous lines screening by two sets of PCR using DNA Polymerase I (Thermo Scientific) or KAPA3G Plant PCR Kit (Kapabiosystems). The first PCR was realized using gene-specific primers 5′-CGC TTA CGT GCT TTA TTC AGC-3′ and 5′-CAC TGT CTC CTT TGC CTT TTG'3 (Salk_142607), 5′-TGT TCA GTC CTT TAT GTG CCC'3 and 5′-GTT TGG AAG GCC TAT CAC TCC-3′ (Salk_146783), 5′-TTC AAT CCG TTG TCC AAA AAG-3′ and 5′-CTT ATG GAA TTA TTC CTG GCG-3′ (Salk_044810) or AAT GAA ACT TGG TGC CTC ATG and TGC AAG GAT TTT AAA ACC CTT G (Salk_123120). The second PCR was carried out with the T-DNA left border primer (LB1.3P) 5′-ATT TTG CCG ATT TCG GAA C-3′ and corresponding right primer for each line. Plants were considered homozygous if no PCR product was obtained in the mutants, but only in WT, by the first PCR reaction, and fragments of estimated size were amplified by the second PCR reaction. The T-DNA position in the genome was determined by fragment isolation, cloning in pJET1.2 and sequencing (Macrogen Inc., Korea). The sequence analysis indicated identical T-DNA locations as previously informed for each line (The *Arabidopsis* Information Resource).

DNA-cloning

DNAs for all constructs were amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) from genomic DNA or cDNA obtained from WT plants. Fragments were cloned into pJet1.2 using CloneJET PCR Cloning Kit (Thermo Scientific) and sequenced (Macrogen Inc., Korea). Restriction enzymes and T4 DNA ligase for cloning were supplied by Thermo Scientific.

The promoter region of AtALN comprising the 1996 bp genomic DNA extending from the 3′ untranslated region (UTR) of the preceding gene to the start codon was PCR amplified from genomic DNA with primers 5′-GAA TTC CAG CCT AAA CCC AAT AGA TGT CCC-3′ and 5′-AGG ATC CAT CTC TCT CTT GAT CTC TTC TGC G-3′ and cloned into pJet1.2. The promoter sequence was excised with EcoRI/BamHI and introduced into pCambia1381Xa (Cambia) to obtain pCAtALN::uida.

The coding region of AtALN was amplified by RT-PCR from WT RNA with primers 5′-GTC GAC ATG GAG AGA ACT TTG CTT CAA TGG-3′ and 5′-ACT AGT TTA AGT AGT TGC AAG TTG CAG AGA C-3′ and cloned into pJet1.2. The AtALN coding sequence was excised with SalI/SpeI and cloned into pCambia1380 (Cambia) to obtain pCALN. The promoter region of AtRD29A comprising the 1308 bp genomic DNA before the start codon was PCR amplified with primers 5′-GAA TTC TAG CGG CCC ATT AAT AAA GGT TAC-3′ and 5′-GGA TCC AGT AAA ACA GAG GAG GGT CTC AC-3′ and cloned into pJet1.2. The promoter was excised with EcoRI/BamHI and introduced into pCALN to obtain the pCRD29A::ALN construct.

Plant transformation and isolation of transgenic lines

pCALN::uida or pCRD29A::ALN constructs were introduced into *Agrobacterium* strain C58/ATCC33970 for transformation of WT or aln-2 plants, respectively. 8–10 weeks *Arabidopsis* plants were transformed using the floral dip method (Clough and Bent 1998) and transgenic plants (T1 to T3 generation) were selected using hygromycin B (InvivoGen). Genomic DNA was extracted as described above, and T-DNA insertion was confirmed using vector reverse primer 5′-GAC CGG CAA CAG GAT TCA ATC-3′ and forward primer 5′-CAT ACG TGT CCC TTT ATC TCT CTC AG-3′ (for pCALN::uida) or CTT AGT AGG CCC CGC TTC ATA G (for pCRD29A::ALN). For promoter analysis five pCALN:uida/WT T3 lines were used for further investigation. Five pCRD29A::ALN/aln-2 T2 lines were analyzed for allantoin levels and phenotypic alterations in stress experiments. Two pCRD29A::ALN/aln-2 T3 lines were selected for further stress experiments.

Stress experiments and assays

In all experiments, seeds were sowed on 0.5× MS vertical plates and stratified for 2 days at 4 °C. Then, plates were placed vertically in a growth chamber under a 16 h light/8 h dark photoperiod at 22 °C and a light intensity of 120 μE/m^2s.

Salt stress experiments were carried out in two different ways. In a first approach, plants were germinated and grown for 14 days on 0.5× MS vertical plates. Afterwards, plants were transferred to 0.5× MS vertical plates containing 0, 50, 100, 150, 200 or 250 NaCl concentrations for treatments. Material was frozen and stored at −80 °C for RNA extraction and RT-PCR. Fresh weight per plant was measured after 7 days of transplanting. Additional material was stored for chlorophylls and allantoin and/or allantoic acid quantification. In a second approach, plants were imbibed and germinated directly in 0.5× MS vertical plates plus 0 and 150 mM NaCl. After 8 days both leaf area and root length were measured.

To test the ability of plant mutants to use allantoin as sole nitrogen source, plants were germinated and grown for 8 days in vertical plates containing 30 mM total nitrogen. Standard 0.5× MS medium (Sigma-Aldrich) or 0.5× MS without nitrogen medium (M531, PhytoTechnology Laboratories) supplemented with 7.5 mM allantoin (Sigma-Aldrich) were used.

Determination of growing parameters

Leaf area and primary and lateral root length were measured with ImageJ (Schneider et al. 2012).

Determination of allantoin, allantoic acid and total chlorophylls

Plant material was dried overnight at 65 °C and the dry weight was measured. Allantoin and allantoic acid were obtained by incubation of plant samples in 6.25 mM K_2PO_4/KH_2PO_4 buffer pH 7 for 20 min at 100 °C and subsequent centrifugation for 5 min at 14,000 RPM. Allantoin and allantoic acid present in the supernatants were quantified using differential analysis of glyoxylate derivatives (Vogels and Van der Drift 1970).

Chlorophylls were measured from fresh material as described previously (Vernon 1960).

Reverse transcription-PCR analysis

Total RNA from plants was extracted with Tri Reagent according manufacturer protocol (Molecular research center, Inc.). cDNA was synthesized with RevertAid Premium M-MuLV Reverse Transcriptase (Thermo Scientific) and used for PCR reactions.

Semi-quantitative PCR was optimized using primers for specific coding regions. For AtALN, primers 5′-ACT ACA TTG GTT GAC ATG CCC-3′ and 5′-CTC CTT CCA TCA AAG CTT CCC-3′ were used to amplify a 682 bp fragment by PCR. For AtUOX (AtAS), primers 5′-ATC CAC AGG TAT TTA CTG CTA TCG-3′ (5′-CTT CTG CTT TAC AGG AGC TTG-3′) and 5′-CAT CTT TAA ACT TCA CCA TTG AAG GG-3′ (5′-CCC ATT TCT GTG ATT CTG TAA CC-3′) were used to amplify a 564 (645) bp fragment by PCR. For AtUPS5, primers 5′-GAG TGC CAG AGA CTA TTT GGA G-3′ and 5′-CGA TAC TAA AGG AAG TGC CTG AAC-3′ were used to amplify a 510 bp AtUPS5 fragment by PCR. In all cases, a 641 bp fragment of AcACT2 gene was amplified by PCR using primers 5′-CGT ACA ACC GGT ATT GTG CTG G-'3 and 5′-GGA CCT GCC TCA TCA TAC TCG-3′, and used as a control.

To identify allantoinase cDNAs in the knockdown line aln-2 and in pCRD29A::ALN/aln-2 transgenic lines, its full-length coding sequences were amplified by PCR using primers 5′-GTC GAC ATG GAG AGA ACT TTG CTT CAA TGG-3′ and 5′-ACT AGT TTA AGT AGT TGC AAG TTG CAG AGA C-3′, the fragments isolated and cloned into pJET1.2 for sequencing (Macrogen Inc., Korea). The sequences obtained matched exactly with the expected (GenBank, NM_116734).

Promoter GUS assays

Plants/organs harvested at different developmental stages or grown under stress conditions were analyzed for glucoronidase activity (Martin et al. 1992). Staining was performed overnight at 37 °C followed by 2–3 subsequent incubations in 80 % ethanol for 5 min at 80 °C for enzyme inactivation and cleaning. Stained material was observed with an Olympus SZX16 stereoscope (Olympus) and selected samples were mounted in slides with 50 % glycerol and used for observation with an Olympus BX61 microscope (Olympus). Olympus DP71 camera was used for images acquisitions.

For the quantification of GUS staining in AtALN promoter studies during salt stress, digital images were processed using ImageJ. Blue image component was selected with the Threshold Colour plugin (http://www.dentistry.bham.ac.uk/landinig/software/software.html) and images were converted to 8 bit. Pixel values were calibrated to optical density units (OD) to which the mean OD value of five WT plants (non-transformed with the pCALN::uida contruct) were subtracted. Shoot and root sections of at least 5 plants were analyzed for each treatment and the

obtained values are expressed relative to control plants (0 mM NaCl treatment).

Results

Salt stress increase allantoin concentration in *Arabidopsis* seedlings

To know whether *Arabidopsis* plants accumulate allantoin under salt stress conditions, 14 day-old seedlings were transferred to growth medium containing different NaCl concentrations. After 7 day-treatments in media with 150, 200 and 250 mM NaCl, the seedlings showed visual stress symptoms such as chlorotic leaves and general growth reduction (Fig. 1a). The fresh weight of seedlings was significantly reduced beginning at a concentration of 150 mM NaCl (Fig. 1b), which is in accordance with previous studies showing salt stress symptoms in *Arabidopsis* plants (Claeys et al. 2014). A significant reduction of dry weight was only observed at 250 mM NaCl. The allantoin content was not significantly different between controls and plants exposed to 50 or 100 mM NaCl, but at higher salt concentrations, a decrease in allantoic acid together with an increase in allantoin content was observed (Fig. 1c). This result shows that the total allantoin content increase in *Arabidopsis* seedlings under salt stress similarly as recently reported for water stress (Watanabe et al. 2014). Since a 150 mM NaCl treatment was able to induce significant allantoin accumulation, but moderate stress symptoms, this concentration was used for further salt stress experiments.

Expression of genes for allantoin synthesis and degradation are oppositely regulated during salt stress

At least, two ways could be expected to influence allantoin accumulation in the whole plant: an increase in its biosynthesis or a decrease in its degradation. Therefore, expression studies were focused on genes encoding the two enzymes involved in the sequential allantoin synthesis

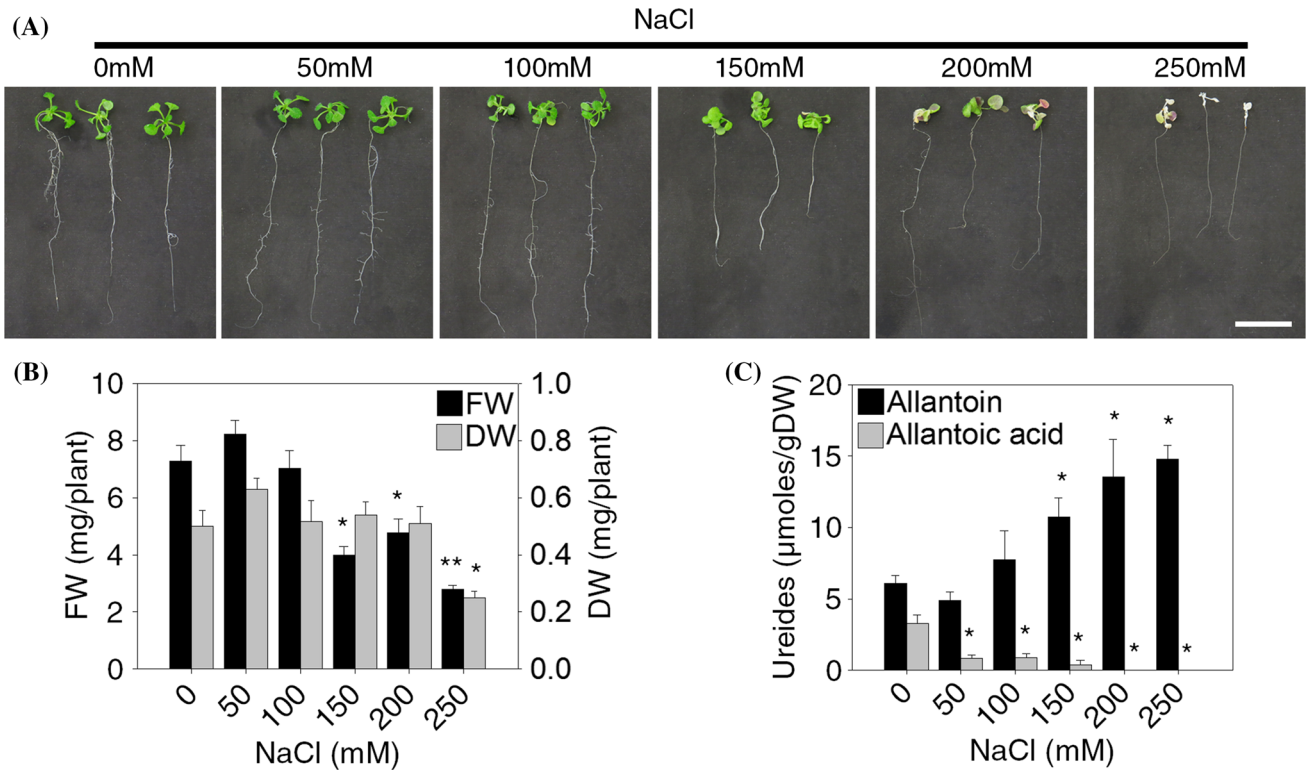

Fig. 1 Response of WT seedlings to different concentrations of NaCl. 14 day-old seedlings were transferred to solid 0.5× MS medium supplemented with 0–250 mM NaCl, and grown for another 7 days. **a** Representative seedlings grown on vertical plates (*bar* 1 cm), **b** fresh weight (FW) and dry weight (DW) per seedling and **c** ureide concentration are shown. *Bars* represent the means and standard errors of at least 3 independent measurements. *Asterisks* indicates significant differences between control (0 mM) and NaCl treatments (N = 27 and 36 for **b** and **c**, respectively, $*P < 0.05$, DGC test)

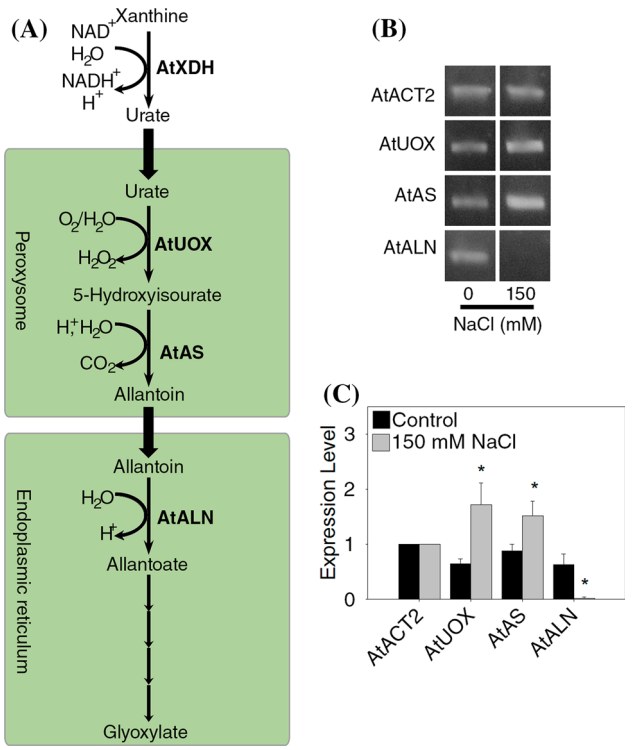

Fig. 2 Gene expression of the *Arabidopsis* purine degradation pathway enzymes under salt stress conditions. **a** The purine degradation pathway. Enzymes involved in the allantoin synthesis (AtXDH, xanthine dehydrogenase; AtUOX, urate oxidase/uricase; AtAS, allantoin synthase) and catabolism (AtALN, allantoinase) are shown. **b** RT-PCR of transcripts present in seedlings with or without salt stress. 14 day-old WT plants were transferred to 0.5× MS vertical plates supplemented with 0 or 150 mM NaCl. After 24 h, four independent samples for each treatment were harvested. After reverse transcription, a 564 bp AtUOX, a 645 bp AtAS and a 682 bp AtALN fragment were amplified by PCR (30 cycles). A 641 bp AtACT2 fragment was used as control (30 cycles). **c** Relative expression of AtUOX, AtAS and AtALN in *Arabidopsis* plants in response to salt stress. AtACT2 values were used as internal control. *Asterisks* indicates significant differences between control (0 mM) and NaCl treatments (N = 8. $*P < 0.05$, DGC test)

(urate oxidase, AtUOX and allantoin synthase, AtAS) and the allantoin degrading enzyme (allantoinase, AtALN) (Fig. 2a). All three enzymes are encoded by unique *loci* in the *Arabidopsis* genome and there is no evidence for an alternative pathway in plants. The expression of these genes was first assessed using semi-quantitative RT-PCR analysis of two week-old seedlings subjected for additional 24 h to salt stress or control conditions (Fig. 2b). Salt stress treatment increased AtOUX and AtAS mRNA levels and strongly decreased AtALN expression (Fig. 2c). These results fit consistently with previous studies using microarrays (Kilian et al. 2007). In particular, the strong down-regulation of AtALN expression suggests that the regulation of this gene is mainly responsible for allantoin accumulation observed under salt stress.

AtALN promoter activity is present in specific plant tissues during plant development and is strongly repressed by salt stress

Although the allantoin content in the complete plant increased up to threefold under salt stress conditions (Fig. 1c), the relative concentrations in each organ, tissue or cell type could be more pronounced. Since AtALN gene regulation seems to play a crucial role in allantoin accumulation, the transcriptional activity of this gene was analyzed in transgenic plants expressing the *uida* gene under the control of AtALN promoter. Promoter activity was not observed in both dry- and in 24 h imbibed seeds (data not shown). Seedlings grown on 0.5× MS vertical plates showed GUS activity in roots and leaves at day 7 (Fig. 3a, b). In 21-day-old plants, GUS staining was strongly observed in all tissues of young leaves, whereas it was mainly present in the vasculature of older leaves and in the central cylinder of roots (Fig. 3c–e). In 7- to 8 week-old plants grown on soil, GUS staining was present in rosette leaves and stipules, but also in flowers and fruits (Fig. 3f, i). Expression was higher in young leaves and stipules than in old leaves (Fig. 3g). In closed flowers expression was higher in the vasculature of sepals. However, in open flowers staining was observed in pedicels as well as in petals (Fig. 3h, i). This expression pattern indicates that AtALN is required at different stages of plant development for unknown functions.

AtALN promoter activity was also analyzed under salt stress conditions. A reduction of GUS staining was noticeable in the whole plant by increasing NaCl concentration (Fig. 4a). After 7 days of 100 mM NaCl exposure, GUS staining disappeared totally in roots, while it was partially reduced in shoots. The expression was drastically reduced in shoots in the 150 mM NaCl treatment, remaining only in some leaf spots. Higher NaCl concentrations reduced totally GUS staining in the whole plant (Fig. 4a). Remarkably, gene repression occurs earlier in root cells even at low salt concentrations compared to shoots, suggesting a role in plant response to moderate stress conditions (Fig. 4b, c).

Transcriptional regulation of AtALN is a key factor for salt stress tolerance mediated by allantoin accumulation

To point out the physiological relevance of downregulation of AtALN during salt stress, the phenotypes of knockout, knockdown and stress-inducible AtALN transgenic lines were compared. Two lines (aln-1 and aln-2) carrying independent T-DNA insertions in the AtALN gene served as experimental genotypes with potentially increased allantoin concentrations (Fig. S1a, b). Whereas aln-1 was

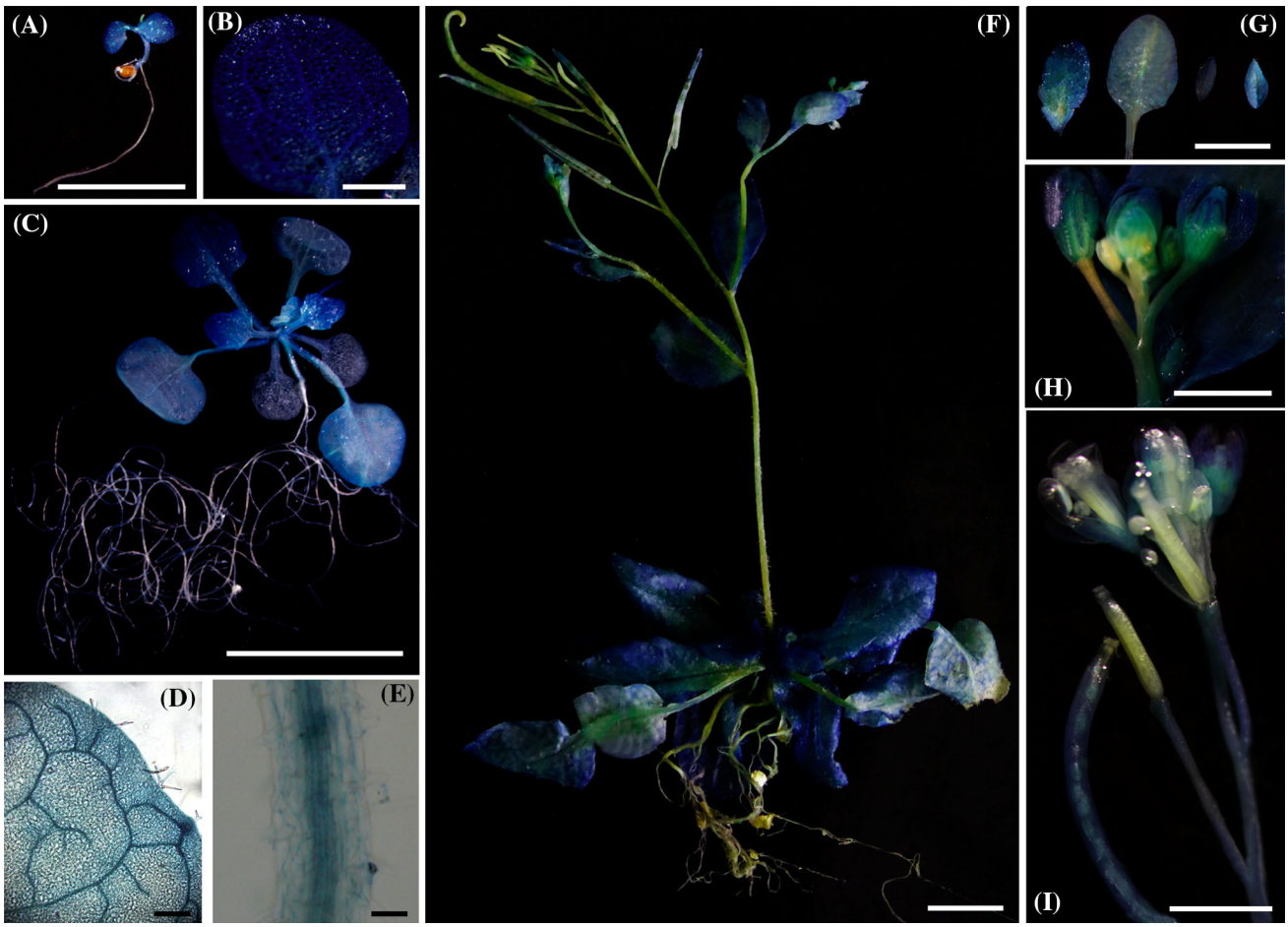

Fig. 3 Expression of the *uida* gene under the control of the AtALN promoter in *Arabidopsis*. Seedlings were grown on solid 0.5× MS vertical plates for **a**, **b** 7 and **c**, **d**, **e** 21 days and **f**, **g**, **h**, **i** plants were grown in vermiculite:soil mix (1:1) for 8 weeks prior harvesting and GUS-activity analysis. 5 independent lines were analyzed and shown a similar staining pattern. Scale *bar* 0.5 cm (**a**, **c**, **f**, **g**), 0.05 cm (**b**), 100 µm (**d**, **e**) or 0.25 cm (**h**, **i**)

established as a genuine knockout (Fig. S1c, see also Watanabe et al. 2014), the presence of transcripts in aln-2 could still be detected by RT-PCR and identified by sequencing as full length AtALN cDNAs (Fig. S1c). However, the expression was strongly reduced in aln-2 compared with WT as indicated in semi-quantitative analysis of the RT-PCR at different number of PCR cycles (Fig. S1c). The T-DNA insertion in aln-2 occurred in the ninth intron of the gene, which was confirmed by sequencing in this study, probably explaining gene knockdown. In addition, transgenic lines carrying the AtALN coding sequence under the control of the stress inducible AtRD29A promoter (Yamaguchi-Shinozaki and Shinozaki 1994) on the aln-2 genetic background (RD29A::ALN/aln-2) were generated (Fig. S1d). The purpose of using the latter transgenic plants was to take advantage of a genetic background able to respond to salt stress in a manner opposite to the WT: namely inducing allantoinase and potentially reducing allantoin content. The insertion of RD29A::ALN construct in aln-2 genome was verified by PCR using specific primers. RT-PCR analysis of RD29A::ALN/aln-2 plants confirmed the functionality of the construct since expression was strongly induced under salt stress conditions in contrast with its genetic background (aln-2) and the WT (Fig. S1e). The full length cDNA obtained from RD29A::ALN/aln-2 plants under salt stress was sequenced and confirmed as AtALN. To analyze the response to salt stress of these genotypes, plants were grown for 14 days in standard media and then transferred to medium without or with 150 mM NaCl for 7 days (Fig. 5). Comparative analyses of allantoin content in plants grown under standard conditions showed that the aln-1 knockout accumulated about 5-times more allantoin than WT plants (Fig. 5d). Without stress conditions, no significant differences were found in allantoin content between the knockdown aln-2 and RD29A::ALN/aln-2 lines, although both lines presented ~ twofold the WT content. Under salt stress, the allantoin content in WT and

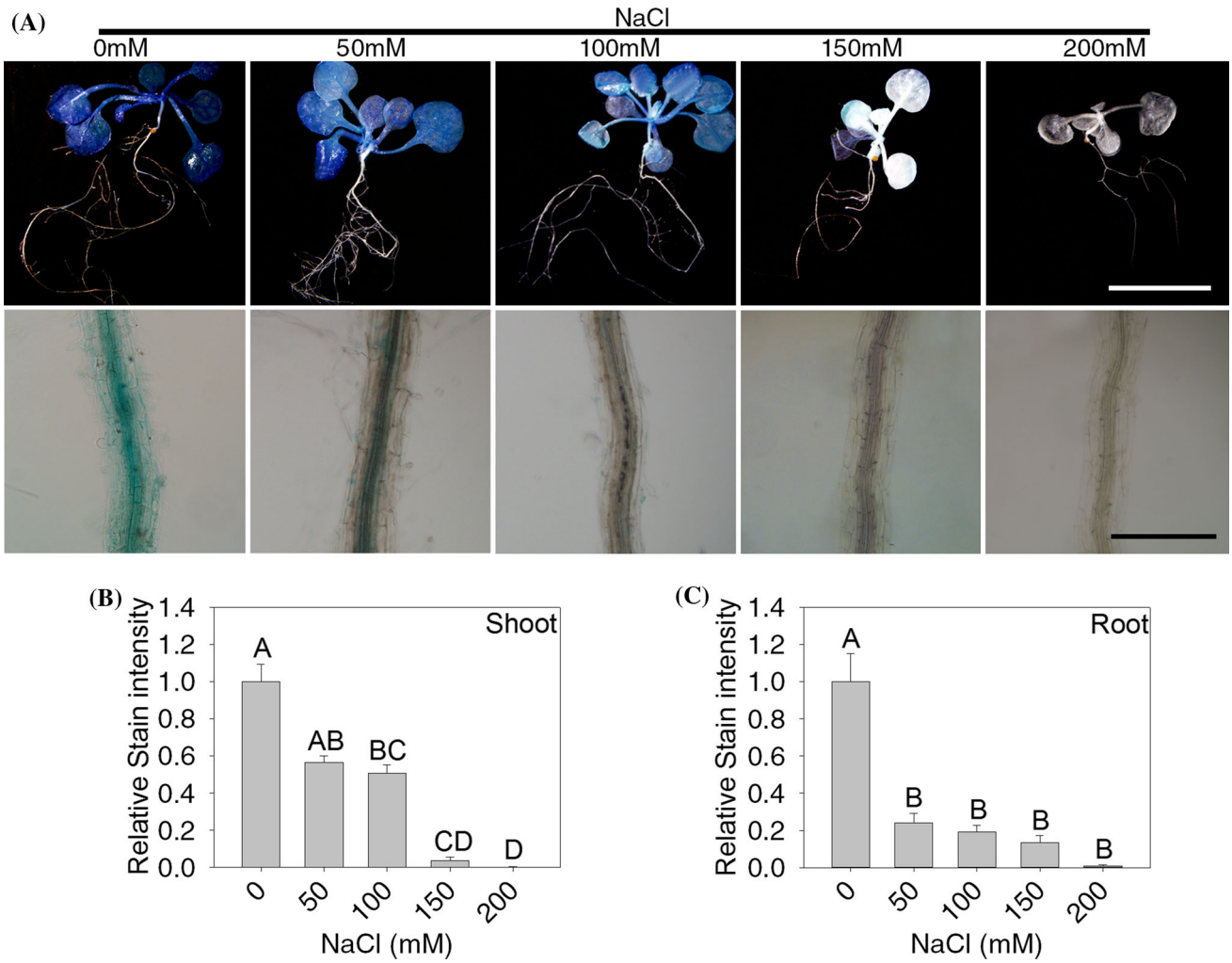

Fig. 4 Response of the uida gene under the control the AtALN promoter in seedlings subjected to salt stress. 14 day-old seedlings were transferred to solid 0.5× MS medium supplemented with 0–200 mM NaCl, and grown for another 7 days. **a** GUS staining of total plants (*top*) and root details (*bottom*) are shown. *Scale bar* 0.5 cm (*top*) or 100 µm (*bottom*). GUS Staining intensity values of **b** shoots and **c** roots are relative to control plants (0 mM NaCl treatment). n = 5 for 100 and 200 mM NaCl treatments, n = 6 for 0, 50 and 150 NaCl treatments. *Bars* represent the means and standard errors of independent measurements. *Different letters* indicates significant differences between treatments (N = 28, $^*P < 0.05$, Kruskal–Wallis test)

aln-2 plants was 90 and 40 % higher than the controls, respectively, whereas aln-1 plants presented similarly high values as control plants without stress. Since aln-1 plants have transcriptionally blocked the allantoin degradation pathway, but not its biosynthesis, the lack of a further increase in allantoin level in this mutant under salt stress suggest that allantoinase is the key enzyme controlling allantoin accumulation. As expected, RD29A::ALN/aln-2 plants showed ~58 % allantoin content of controls under stress conditions (Fig. 5d). The capacity of these genotypes to respond to salt stress was analyzed. Aln-1 and aln-2 mutants showed a better growth under salt stress compared to WT plants (Fig. 5a). Furthermore, both fresh weight and chlorophyll content were higher in these mutants than in WT lines (Fig. 5b, c). On the contrary, RD29A:ALN/aln-2 plants were severely damaged by salt as shown by its reduced growth and chlorophyll content with respect to WT plants (Fig. 5a–c). Taken together, these results suggest a positive relationship between allantoin content and plant tolerance to salt stress.

To determine the importance of AtALN regulation in early developmental stages, plants of each transgenic line were germinated and grown for 8 days in 0.5× MS medium supplemented with or without 150 mM NaCl and their respective phenotypes were compared (Fig. 6). No significant differences in growth were observed between the WT, aln-1, aln-2 and stress inducible lines without salt, whereas all lines showed growth reduction under salt stress

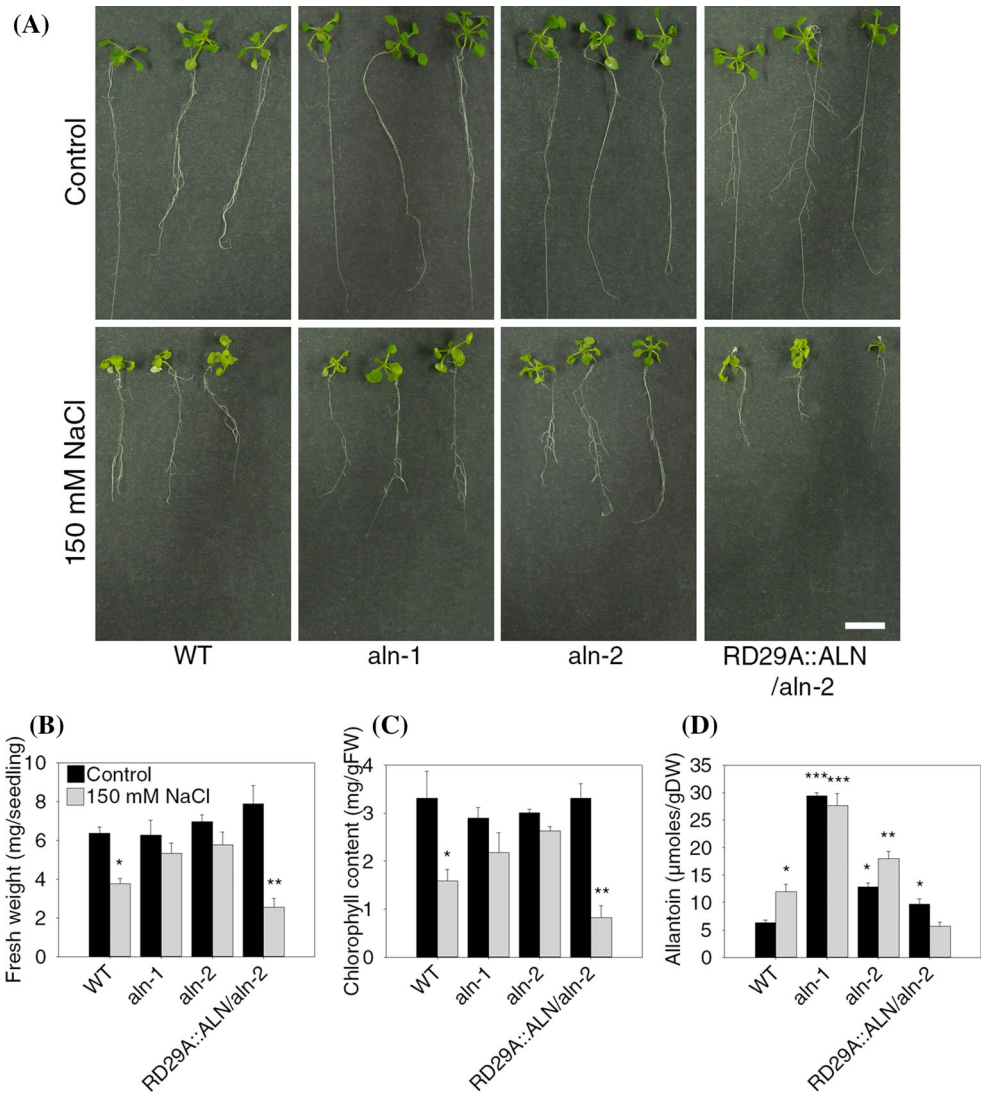

Fig. 5 Response of WT, aln T-DNA insertion lines and AtALN stress-inducible lines to salt stress. 14 day-old seedlings were transferred to solid 0.5× MS medium supplemented with 0 or 150 mM NaCl, and grown for another 7 days. **a** Representative seedlings grown on vertical plates (*bar* 1 cm), **b** fresh weight, **c** chlorophyll content and **d** allantoin concentration are shown. *DW* dry weight. *Bars* represent the means and standard errors of at least 3 independent measurements (*$P < 0.05$). *Asterisks* indicates significant differences between genotypes and treatments (N = 38, 43 and 50 for **b**, **c** and **d**, respectively, *$P < 0.05$, DGC test)

(Fig. 6a–c). However, both aln-1 and aln-2 showed a significantly higher plant growth than WT plants and a minor, but significant, difference in root length between aln-1 and aln-2 plants was observed (Fig. 6a–c). In contrast, RD29A:ALN/aln-2 lines showed a significant reduction in both leaf area and root length when compared to WT, aln-1 and aln-2 plants (Fig. 6b–c). Moreover, there was a clear disparity in the overall plant growth between RD29A:ALN/aln-2 compared to its genetic background line (aln-2). Indeed, RD29A:ALN/aln-2 lines showed a reduction of 83 % in leaf area and 80 % in root length under salt stress compared with controls without salt, while aln-2 lines presented a reduction of only 40 % in leaf area and 58 % in root length (Fig. 6b, c). Taken together, these data show that AtALN down-regulation at transcriptional level confers plant tolerance to growth in saline medium at different developmental stages.

Ups5 knockout plants under salt stress present a susceptible phenotype and altered root-to-shoot allantoin content ratios

In tropical legumes, allantoin is typically synthesized in the root nodules and subsequently transported to the shoot (Schubert 1986). This observation led to speculate whether the protective effects of allantoin during salt stress occur in *Arabidopsis* at the site of synthesis or its transport to another organ/tissue is required. Since AtUPS5 is a candidate for either allantoin reallocation in the organ itself or long distance movement to the shoot, its relevance in response to salt stress was studied.

To investigate a potential role of AtUPS5 in allantoin mediated salt stress response, two independent T-DNA insertion lines (ups5-1 and ups5-2) were characterized. Using PCR amplification and sequencing, the T-DNA

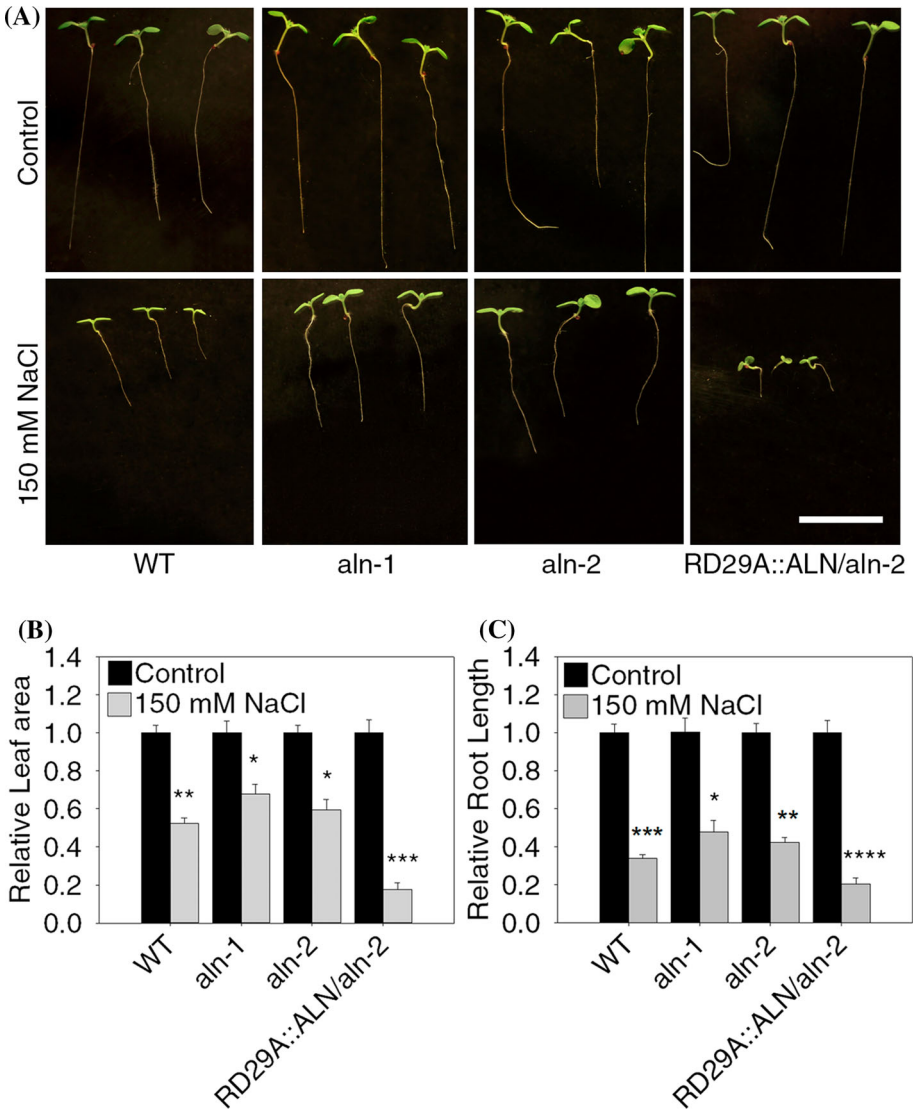

Fig. 6 Phenotype analysis of WT, aln-1, aln-2 and AtALN stress-inducible lines grown in salt stress conditions. Seedlings were germinated and grown vertically on plates with solid 0.5× MS supplemented with 0 (control) or 150 mM NaCl for 8 days. **a** Representative seedlings grown on vertical plates (*bar* 1 cm), **b** leaf area and **c** root length are shown. Values are relative to control plants and *bars* represent the means and standard deviations of at least 14 independent measurements. *Asterisks* indicates significant differences between genotypes and treatments (N = 152 and 241 for **b** and **c**, respectively, $*P < 0.05$, Student test)

insertion was localized in the 5′ UTR or in the promoter region, 149 bp or 386 bp upstream of the start codon, respectively (Fig. S2a, b). By RT-PCR analysis, AtUPS5 transcripts were not detected in ups5-1 lines, but products could still be distinguished in ups5-2 lines. The identity of these products as AtUPS5 was confirmed by sequencing. However, AtUPS5 expression in ups5-2 lines was strongly decreased in comparison to WT lines (Fig. S2c). To test the functionality of AtUPS5 in vivo, both ups5 T-DNA insertion lines were analyzed in their capacity for utilization of external supplied allantoin for seedling growth. Aln-1 and aln-2 lines, which are deficient in allantoin degradation, and WT plants served as controls. No phenotypic differences could be observed under control conditions in 0.5× MS medium containing inorganic nitrogen sources (Fig. S3a–c). When allantoin was used as sole nitrogen source, the growth performance of both ups5-1 and ups5-2 seedlings was not different from aln-1 and aln-2 seedlings, but was significantly decreased in comparison with WT plants (Fig. S3a–c). This result demonstrates that AtUPS5 is involved in allantoin cell uptake for utilization as nitrogen source. To determine possible changes in gene expression under salt stress conditions, AtUPS5 promoter activity was studied in seedlings using previously described transgenic lines (Schmidt et al. 2006). GUS activity was in general higher under salt stress, although the expression pattern in the root tissues remained invariable. Just one conspicuous difference in color distribution was noticeable: roots in saline medium showed a strong coloration in the distal portion of primary roots as well as in lateral roots (Fig. 7).

Taking into account these results, it became of interest to analyze the phenotype and the allantoin content in roots and shoots separately of both WT and ups5 plants subjected to salt stress (Fig. 8). In general, roots evidenced a higher allantoin content than shoots in all studied conditions (Fig. 8b). Under control conditions, no significant

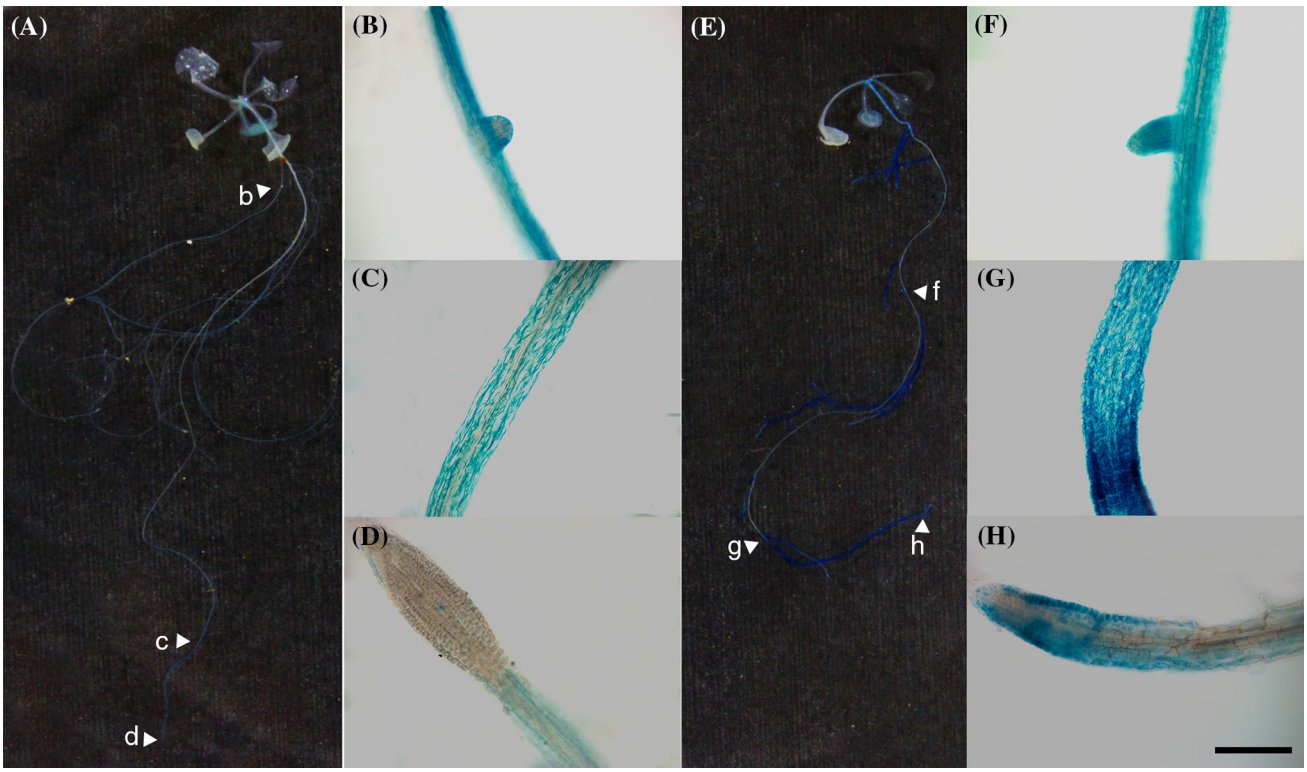

Fig. 7 Expression of the uida gene under the control the AtUPS5 promoter subjected to salt stress. 14 day-old seedlings were transferred to solid 0.5× MS medium supplemented with **a–d** 0 or **e–h** 150 mM NaCl, and grown for another 7 days, prior to GUS staining. GUS staining of **a**, **e** whole plant and details of **b**, **f** lateral roots, **c**, **g** primary root final segment and **d**, **h** tip are shown. *Scale bar* 100 µm

differences were found in either shoot or root allantoin content, root length, total lateral root length and leaf area in WT and both ups5-1 and ups5-2 T-DNA insertion lines (Fig. 8b–e). However, WT and ups5 seedlings responded differentially to salt stress (Fig. 8a). Although primary root length of ups5 plants was not significantly different from WT plants, total lateral root length was significantly reduced (Fig. 8d). Moreover, ups5 seedlings showed a reduced leaf area compared to WT (Fig. 8e). The allantoin content in shoots was significantly lower in ups5 than in WT plants, while the allantoin content in roots was ~t-wofold higher in ups5 than in WT plants (Fig. 8b). Thus, salt stress conditions lowered the root-to-shoot allantoin content ratio in WT plants, whereas this ratio was clearly increased in ups5 plants (Fig. 8c). The susceptible phenotype of the ups5 mutants under salt stress suggests that allantoin transport mediated by AtUPS5 is an important event in salt stress alleviation.

Discussion

Allantoin is a metabolite generated in the oxidative degradation pathway of purines and has primarily been considered an intermediate for recycling of the abundant nitrogen assimilated in these compounds. A myriad of studies on tropical legumes, however, demonstrated that allantoin and allantoic acid serve as major nodule-to-shoot nitrogen transport compounds representing in e.g. nodulated soybean (*Glycine max*) up to 80 % of the transported nitrogen (McClure and Israel 1979). In other plant species, important variations in allantoin concentrations have been observed (1) during the plant seasonal cycle suggesting a transient nitrogen accumulation in organic molecules by conditions that generate low C/N relationships (Bollard 1957), (2) by nitrogen remobilization during leaf senescence (Brychkova et al. 2008) and (3) in the context of environmental adverse conditions as a potential protective factor during physiological stress (Watanabe et al. 2014). In this work, increased concentrations of allantoin were measured in response to salt stress in *Arabidopsis* seedlings. In contrast, the concentrations of allantoic acid, the next metabolite in the pathway, decreased with the intensity of salt stress (Fig. 1c). Interestingly, a previous study showed a simultaneous increase of both metabolites in ryegrass under salt stress (Sagi et al. 1998), suggesting that the regulation of the metabolic pathway or transport varies in different plant groups (Monocots vs. Dicots) and/or under distinct nutritional conditions (e.g. Carbon to Nitrogen ratios). Comparable changes in allantoin content

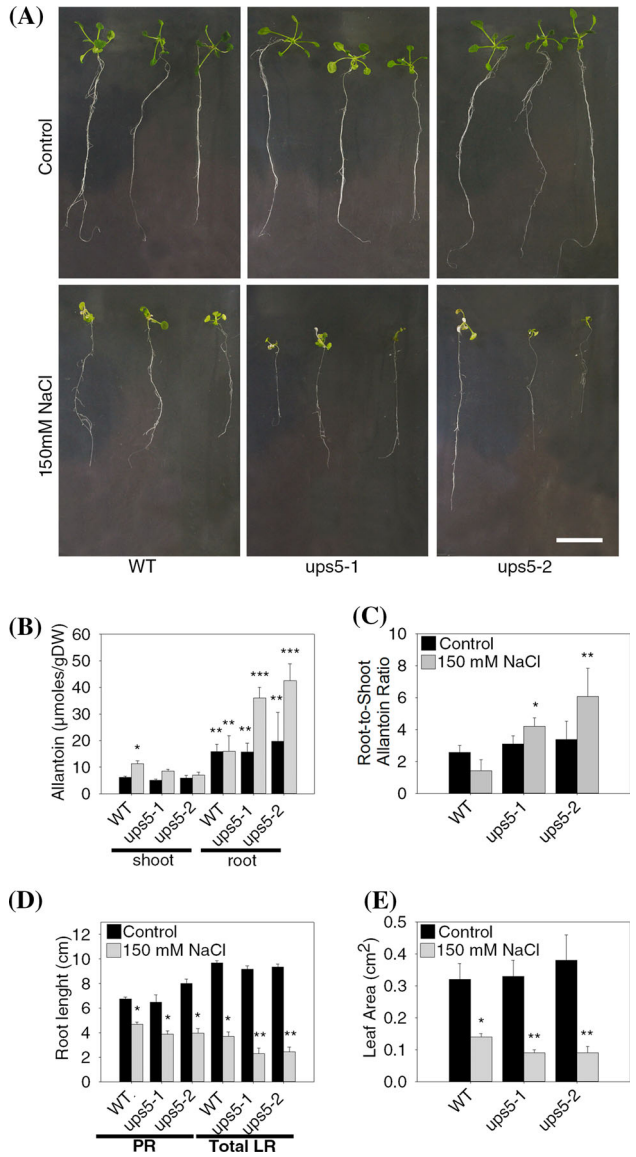

Fig. 8 Response of T-DNA ups5 lines to salt stress. 14 day-old WT and ups5 seedlings were transferred to solid 0.5× MS medium supplemented with 0 (control) or 150 mM NaCl for 7 days. **a** Representative seedlings grown on vertical plates (*bar* 1 cm), **b** shoot and root allantoin content, **c** root-to-shoot allantoin content ratio, **d** total length of Primary root (PR) and Lateral roots (LR), and **e** leaf area are shown. *DW* dry weight. *Bars* represent the means and standard errors of at least 3 independent measurements. *Asterisks* indicates significant differences between organs, genotypes and treatments in (**b**) and significant differences between genotypes and treatments in (**c**), (**d**) and (**e**) (N = 43, $*P < 0.05$, DGC test)

were recently informed for water stress (Watanabe et al. 2014), suggesting that the osmotic component of saline conditions could explain the plant response described here. However, it cannot be excluded that ion toxicity can additionally influence plant response in other ways. Clearly different is the accumulation pattern of allantoin and allantoic acid found in senescent leaves, where both rise together (Brychkova et al. 2008). This may indicate that the regulation of purine catabolism is different in the context of senescence or physiological stress.

Analysis of the AtALN promoter activity using GUS as a reporter revealed that even at moderate salt stress conditions (50 mM NaCl), gene transcription is sequentially repressed in root tissues: first in the external cell layers and later in the vasculature (Fig. 4, data not shown). Thus, the local regulation of AtALN expression caused by salt stress and the consequent allantoin accumulation may provide plants an adaptive mechanism for either root cell protection and/or plant signaling. Moreover, its promoter is totally inactivated in the root, but also in the shoot at higher salt concentrations (Fig. 4). AtALN downregulation in the shoot could be alternatively caused either by perception of low water potentials in shoot cells, a possible increase of systemic NaCl concentrations or other unknown signals originated in the root. Allantoin has been recently proposed as a compound that induces abscisic acid de novo biosynthesis and deconjugation, suggesting that this hormone acts downstream in osmotic stress signaling (Watanabe et al. 2014). On the other hand, abscisic acid negatively influences AtALN gene expression, particularly in guard cells, which may indicate allantoin accumulation in this specific cell type (Pandey et al. 2010). As AtALN transcription represents a key factor for allantoin accumulation in plant response to stress, further attempts should be done to better understand the complexity of allantoinase gene regulation.

To evaluate whether allantoin accumulation is a causal factor of plant stress tolerance or it is an accompanying phenomenon derived from stress conditions, the phenotype of transgenic plants with different capacities to accumulate allantoin was analyzed in depth. AtALN knockout plants (aln-1) with a constitutive high level of allantoin clearly showed a phenotype tolerant to salt stress (Figs. 5 and 6). Interestingly, no additional accumulation of allantoin could be measured after onset of salt stress conditions, suggesting that the biosynthesis pathway is feedback inhibited by the maximal allantoin observed in this line (Fig. 5d). The knockdown mutant aln-2 presented similar stress tolerance as aln-1, although the levels of allantoin ranged between those of aln-1 and the WT and increased in salt stress (Figs. 5 and 6). These findings are in line with a previous report showing the tolerant phenotype of the aln-1 mutant against water stress (Watanabe et al. 2014). The protective role of allantoin during salt stress was further tested using transgenic plants, in which allantoin is degraded (RD29A::ALN/aln-2) instead of accumulated (WT or aln-2) under stress conditions. We favored the strategy of conditional control of gene expression over typical constitutive gene overexpression (e.g. CaMV 35S promoter) in order to avoid a possible strong phenotype during plant

development and to start salt stress experiments with plants containing comparable levels of allantoin in RD29A::ALN/aln-2 and in its genetic background aln-2 (data not shown). The conditional depletion of allantoin in RD29A::ALN/aln-2 caused a very susceptible phenotype under salt stress (Figs. 5 and 6). This finding reinforces the notion that the elevation of allantoin levels is essential for the development of plant tolerance to salt stress.

Although AtALN was found widely distributed in various organs of *Arabidopsis* plants, analysis of the vegetative organs separately showed that allantoin concentrations were basically higher in roots than in shoots (Fig. 8b, data not shown). Allantoin concentration was ~2-times higher in shoots of plants subjected to salt stress compared with controls, but remained similar in roots (Fig. 8b). These results together with the observation that AtALN is downregulated in root tissues at moderate salt concentrations (Fig. 4) strongly suggest long distance transport of this compound. The identification of a membrane protein family (UPS) with five members in *Arabidopsis* able to transport allantoin (Desimone et al. 2002) served for the characterization of homologue transporters in nodulating legumes, which are crucial for long distance transport of ureides (Collier and Tegeder 2012; Pélissier et al. 2004). However, the physiological role of these genes in *Arabidopsis* (AtUPS) remained elusive. Due to its expression in cortical cells and endodermis of plant roots and its high affinity for allantoin transport, AtUPS5 constitutes a candidate transporter for allantoin long distance movement (Schmidt et al. 2006). Moreover, the transcription of this gene is enhanced by salt stress (Fig. 7). Two mutants with reduced expression of AtUPS5 presented a sensitive phenotype against salt stress (Fig. 8a, d, e). These mutant lines were also defective in the use of externally supplied allantoin as sole nitrogen source, indicating that the expression of AtUPS5 in the root can serve for allantoin transport in vivo (Fig. S3). Moreover, the allantoin concentrations were higher in the roots, but not in the shoots of ups5 mutants under salt stress, contrasting with the relative concentrations in WT plants (Fig. 8b, c). These results are compatible with a model with ups5 acting as a key component in allantoin transport necessary for endodermis crossing, root xylem loading and subsequent export to the shoots. Consistently, AtALN downregulation in central cylinder cells under salt stress may favor allantoin stability during long distance transport. A similar model has been proposed for legumes species in which GmUPS1 serves for ureide translocation from nodules to aerial organs of soybean plants (Collier and Tegeder 2012). So far, there is no evidence for cell exporters of ureides explaining how allantoin can be extruded from endodermis cells. Interestingly, a splicing variant of AtUPS5 encoding a potential truncated protein has been identified and expression experiments determined that both variants are expressed simultaneously. Whereas the longer protein (AtUPS5l) with ten predicted transmembrane domains (TMDs) functions as an cell importer, the function of the shorter protein with eight TMDs (AtUPS5s) could not be determined so far (Schmidt et al. 2006). Preliminary experiments, however, showed that co-expression of both proteins in yeast cells strongly decreased the capacity of AtUPS5l to take up allantoin, suggesting an inhibitory effect of AtUPS5s or a simultaneous substrate export by this isoform (data not shown). Present ongoing studies should clarify the subcellular fate of each transporter isoform and determine whether AtUPS5s is an allantoin exporter or has another unknown function. As long distance transport of allantoin has not been measured in the present work and it is only inferred from the relative concentrations in roots and shoots, it cannot be excluded that the variations observed here, occurred separately by synthesis and accumulation in each organ.

In an alternative scenario, AtUPS5 could be involved in the generation of a long distance signal for plant protection. As mentioned before, recent evidences argue for an involvement of allantoin in ABA de novo biosynthesis or deconjugation (Watanabe et al. 2014). In line with this concept, the expression of AtUPS5 under salt stress was particularly high in the terminal portion of the primary root (Fig. 7), where a major stress-induced biosynthesis of ABA has been suggested (Dinneny et al. 2008; Tan et al. 2003). Interestingly, ups5 mutants with increased levels of allantoin in the roots during salt stress showed a retardation of lateral roots growth (Fig. 8d). Similar root architectures were observed in WT plants grown in medium with allantoin or aln-1 mutants with constitutively elevated allantoin concentrations (data not shown). Furthermore, it has been recently proposed that reduction of lateral root growth induced by salt stress could be mediated by ABA (Duan et al. 2013).

The cellular accumulation of allantoin could potentially have additional functions. Whereas the precursor uric acid has been reported as an efficient scavenger of ROS in several biological systems, there is only limited information about the reactivity of allantoin (Glantzounis et al. 2005). Indirect evidence in plants indicates a protective effect of allantoin against ROS, but the mechanisms implied remain unknown (Brychkova et al. 2008). The cellular levels of ROS rise in plants subjected to salt stress at least by mechanisms involving NADP-oxidase in the apoplast and light driven O_2 reduction in chloroplasts (Miller et al. 2010). However, the subcellular distribution of allantoin has not been determined so far. The localization of AtUOX, AtAS, AtALN and allantoicase suggests that allantoin is produced in peroxysomes and loaded to vesicles for export via the secretory pathway, although the

transport system remains still unidentified (Lamberto et al. 2010; Werner et al. 2013). Specific studies on oxidative stress in plants should be helpful to clarify a potential function of allantoin as a ROS scavenger and, in general, the role of the purine degradation pathway in stress signaling mediated by ROS.

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Author contributions C.I.L. did the cloning work, generation of transgenic plants and expression studies. Plant phenotyping was carried out by C.I.L., C.M. and C.A.G. The writing of the manuscript was performed by C.I.L., C.A.G. and M.D.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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