Arabidopsis AZG2 transports cytokinins in vivo and regulates lateral root emergence

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Summary

- Cytokinin and auxin are key regulators of plant growth and development. During the last decade transport mechanisms have turned out to be the key for the control of local and long-distance hormone distributions. In contrast with auxin, cytokinin transport is poorly understood.
- Here, we show that Arabidopsis thaliana AZG2, a member of the AZG purine transporter family, acts as cytokinin transporter involved in root system architecture determination. Even though purines are substrates for both AZG1 and AZG2, we found distinct transport mechanisms.
- The expression of AZG2 is restricted to a small group of cells surrounding the lateral root (LR) primordia and induced by auxins. Compared to the wild-type (WT), mutants carrying loss-of-function alleles of AZG2 have higher LR density, suggesting that AZG2 is part of a regulatory pathway in LR emergence. Moreover, azg2 is partially insensitive to exogenous cytokinin, which is consistent with the observation that the cytokinin reporter TCSnpro:GFP showed lower fluorescence signal in the roots of azg2 compared to the WT.
- These results indicate a defective cytokinin signalling pathway in the region of LR primordia. The integration of AZG2 subcellular localization and cytokinin transport capacity data allowed us to propose a local cytokinin : auxin signalling model for the regulation of LR emergence.

Introduction

The shape of the plant body results from a fine-tuned interaction between genetic programming and environmental conditions. Root development is a great example of this: from an embryonic organ, plants modulate the root architecture in accordance to its requirement for anchorage and nutrients or water uptake. In eudicotyledons, root branching relies on the capability of secondarily developing lateral roots (LRs). This process begins with the mitotic activation of founder cells in the pericycle and the subsequent formation of LR primordia (LRP; De Smet et al., 2007). For LR emergence, LRP have to overcome the mechanical resistance offered by the external cell layers. Although the numerous contributions to the research field, there are still many questions to be answered about how all of these tissues interact with each other during LR organogenesis (Lucas et al., 2013; Vilches-Barro & Maizel, 2015).

Development of LR is tightly coordinated by different signals, with outstanding roles played by the morphogenic hormones auxins and cytokinins (CK) (Jing & Strader, 2019). Changes in their biosynthesis, transport, perception and cross-talk are necessary events for proper LR development (Nordstrom et al., 2004; Bishop et al., 2011; Marhavy et al., 2014).

A crucial event in LR initiation is the generation and maintenance of high auxin concentrations in the primed pericycle cells and primordial tip cells (Casimiro et al., 2001; Benkova et al., 2003). Auxin concentrations at cellular level are modulated by cell-to-cell transport through polar localized transporters (Grones & Friml, 2015). Their distribution at the plasma membranes is regulated by a complex signalling network, in which CK plays a crucial function (Laplaze et al., 2007; Marhavy et al., 2014). Auxin transport also is involved in remodelling of LR overlying tissues (OLT) enabling LR emergence. For instance, the AUXIN RESPONSE FACTORS (ARF)7 and 19 modulate the expression of several transcription factors of the LATERAL ORGAN BOUNDARIES DOMAIN (LBD) family in OLT. In turn, LBDs either transcriptionally regulate transporters such as the auxin importer LAX3 (LBD29) or act downstream of LAX3 regulating cell-wall remodelling enzymes (LBD16 and LBD18; Swarup et al., 2008; Lee et al., 2009, 2013, 2015; Porco et al., 2016). Consistently, the double arf7-1arf19-1 and the single lbd16, lbd18 and lbd29, as well as the lax3 loss-of-function
(LOF) mutant, show LR developmentally deficient phenotypes (Okushima et al., 2005; Wilmoth et al., 2005; Swarup et al., 2008; Feng et al., 2012; Lee et al., 2015). In contrast with auxin, the role of CK in OLT remodelling remains unclear. External addition of CK reduces the number of emerged LRs in a dose-dependent manner, whereas overexpression of CK-degrading enzymes as well as CK perception-deficient mutants show the opposite effect, indicating a potential restrictive function of CK in LR emergence (Bertell & Eliasson, 1992; Riefler et al., 2006; Werner et al., 2010). Nevertheless, it is not yet clear if CK restricts LR emergence at the OLT level and, if so, how this process is coordinated with auxin signalling and function.

In contrast with the abundant information on auxin transport, little is known about CK transport. Despite the described processes involving CK mobilization (Samuels et al., 1992; Takei et al., 2004; Kiba et al., 2013; De Rybel et al., 2014; Ohashi-Ito et al., 2014; Chang et al., 2015), for a long period only a few putative CK transporters were reported (Burkle et al., 2003; Hirose et al., 2005; Sun et al., 2005). However, no evidence was provided to link these transporters to physiological processes (Kudo et al., 2010). Lately, the ABC transporter AtABCG14 and the purine transporter PUP14 have been described as plasma membrane-localized CK transporters involved in long-distance transport and in morphogenesis, respectively (Ko et al., 2014; Zhang et al., 2014; Zürcher et al., 2016).

Here, we focused on the characterization of transporters belonging to the AZA-GUANINE RESISTANT (AZG) family. The first member of this transporter family has been described in Emerichella nidulans, EnAZGA, as a purine transporter (Cechetto et al., 2004). Some years ago, Arabidopsis thaliana AZG1 and AZG2 were identified and briefly characterized as adenine-guanine transporters (Mansfield et al., 2009). However, to date their in vivo function has not been studied. In this work, AZG2 was localized at the plasma membrane, but mainly in the ER, suggesting a role in CK distribution and signalling in internal cell compartments. Consistently, we found differences in the transport mechanisms of AZG1 and AZG2: although AZG1 is dependent on proton driving force and possibly acts as a cell importer, AZG2 is pH-independent and functions as a transport facilitator.

We provide evidence that AZG2 is expressed in a small number of cells surrounding the LRP. Furthermore, AZG2 is transporting not only purines with high efficiency, but also trans-zeatin (tZ) and other CKs, suggesting that the transporter is most probably involved in CK regulation of LR development. Transcriptional, phenotypic and CK reporter gene studies of AZG2 LOF mutants and plants ectopically expressing AZG2 revealed that the transporter plays a regulatory role within the CK-auxin hormonal cross-talk during LR emergence.

Materials and Methods

Plant material

Arabidopsis thaliana (L.) Heynh accession Columbia (Col-0) was used as wild-type (WT). AZA-GUANINE RESISTANT (AZG) 1 and AZG2 loss-of-function (LOF) lines were obtained from Nottingham Arabidopsis Stock Center (http://arabidopsis.info/). Two independent T-DNA insertion lines of AZG1, SAIL_114E03 (azg1-1) and GK-681A06 (azg1-2), and two independent T-DNA insertion lines of AZG2, SALK 000904 (azg2-1) and SAIL 658 G02 (azg2-2), were genotyped. The presence of T-DNA insertion was checked by PCR using the following primers couples: for azg1-1, Azg1-R and SAIL-LB3; for azg1-2, Azg1-R and GABI-Kat-LB; for azg2-1, SALK-LB61.3 and Azg2-R, and for azg2-2, Azg2-F and SAIL-LB1 (Supporting Information Table S1; Fig. S1b,c). The presence of the AZG1 and AZG2 WT alleles – by PCR – and the absence of mRNA – by reverse transcription (RT)-PCR – was checked with Azg1-F and Azg1-R; and GW-Azg2-F and GW-Azg2-R primers, respectively (Fig. S1d, e). AZG1 and AZG2 overexpression lines were obtained from the Dr Frommer laboratory. The arf7-1 and arf19-1 alleles were used in this study as knock-out mutants of ARF7 and ARF19. Both knock-out lines arf7 (N24607) and arf19 (N24617) were obtained from NASC.

Growth conditions

Plants were cultivated under 8 h : 16 h, light : dark photoperiod regime (at 23°C) for root phenotype analysis and 16 h : 8 h, light : dark for the rest. Imbibed seeds were stratified at 4°C for ≥48 h. Culture media and specific nutritional conditions are described below for each experiment.

Expression in yeast and uptake assays

The detailed methodology regarding transport experiments can be found in the Methods S1.

Constructs for AZG2 tissue and subcellular localization

The AZG2 1.5-kb promoter was cloned to drive β-glucuronidase (GUS) reporter (pGPTV-bar). To study the active length of AZG2, different lengths of AZG2 promoter (∼2149, −1388, −940, −169bp upstream from start codon) were chosen to drive green fluorescent protein (GFP) expression (Table S1). The four amplicon were cloned into pENTR®/D-TOPO® (Invitrogen) via BP reaction and the subsequent LR reaction into pPGG and pPGR gateway vectors with the mGFP5 and red fluorescent protein (RFP) markers, respectively. Also, to study subcellular localization, AZG2 fusions with GFP or RFP were generated. AZG2 CDS was cloned into pDONR207 entry vector and subcloned into pUBC-GW-GFP-DEST, pUBC-GFP-GW-DEST to express AZG2 constitutively with an N- and C-terminal reporter fusion (Greten et al., 2010). For overexpression with CMV35S promoter AZG2, CDS was cloned into pDONR221 and subcloned in pFRET-2in1-CC creating a C-terminal fusion with GFP. All constructs were sequenced to discard mutation. For all transformations, plasmids were introduced in Agrobacterium tumefaciens strain C58/ATCC33970. Five- to six-week-old plants were transformed using the floral dip method as described previously (Clough & Bent, 1998). Mutant plants were selected with the corresponding antibiotic and genotyped.
GUS staining

Histochemical analysis of the GUS reporter enzyme was performed as described by Martin et al. (1992), with minor modifications. For extensive expression description, 19 d after germination (dag) seedlings grown on Petri dishes and reproductive organs of adult plants growing in soil were stained. AZG2pro:GUS plants were incubated in staining solution overnight followed by three subsequent incubations with 80% ethanol. Stained material was mounted and analyzed and photographed under DIC microscopy with an Olympus BX61 microscope; an Olympus DP71 camera was used for image acquisition (Olympus, Tokyo, Japan).

Semiquantitative RT-PCR analysis

In order to evaluate the expression of the AZG2, total RNA from different organs of A. thaliana were isolated from 100 mg tissue using the TRIZol reagent (Gibco-BRL). RNA was converted into first strand cDNA using SuperScriptII Reverse Transcriptase (Invitrogen). PCR reactions were conducted in a final volume of 10 µl using 1 µl of the transcribed product and Taq DNA polymerase (Qiagen). The pairs of primers used were Azg2-Smal-F and Azg2-Xhol-R. Amplification conditions were as follows: 3 min denaturation at 94°C; 35 cycles at 94°C for 30 s, 53°C for 40 s and 72°C for 30 s, followed by 5 min at 72°C. As control, the Actin2 gene was amplified by 28 with the primers Actin2-F and Actin2-R.

Hormone treatments

For auxin induction, 10 dag seedlings of AZG2pro:GUS were transferred to liquid ½ Murashige & Skoog medium with 1 µM naphthalene acetic acid (NAA, PhytoTechnology labs, Shawnee mission, KS, USA) for 3, 6, 12 and 24 h. For seedlings carrying GFP or RFR reporter, 10 dag seedlings were incubated for 12 h and immediately observed under a Eclipse Ti confocal microscope (Nikon, Tokyo, Japan). For cytokinin (CK) 24 h treatments, plants were transferred from ½MS plates to liquid ½MS media with no tZ (MS control) and 0.2 µM tZ (Duchefa). For CK long-term treatment, plants were grown under ½MS without nitrogen + KNO3 5 mM + 0.2 µM tZ.

AZG2 subcellular localization and TCSn:GFP signal quantification

Seedlings (6 dag) of different lines growing in ½MS under short photoperiod were analyzed with a Nikon Eclipse Ti confocal microscope. Scans were performed with ×20 and ×60 oil immersion lens and 1024 × 1024 pixel resolution. U10pro:AZG2-GFP and U10pro:GFP-AZG2 samples were scanned with a 488 nm laser and emission detected with a 515/30 filter. For Brefeldin A (BFA) treatments, U10pro:GFP-AZG2 seedlings were incubated for 1 h with 10µM FM4-64 + 50 µM BFA. Shot FM4-64 incubations lasting 10 min were performed in order to stain the plasma membrane.

In order to study CK signalling we used the TCSnpro:GFP reporter. The plasmidic construction was kindly provided by the Dr Müller laboratory. Knock-out line azg2-1 was transformed as described above with the TCSnpro:GFP construct via floral dip and T3 plants were selected in homozygosis. Two independent insertional lines were selected and analyzed to discard insertional variation of the expression, and 9 dag plants were observed under a Nikon Eclipse Ti microscope. To quantify fluorescence intensity, lateral root primordia (LRP) of seedlings were imaged with a ×20 objective and 1024 × 1024 pixel resolution. Each sample image represents a z-stack of 12 steps throughout the root cylinder centred in the LRP. Stacks were z-projected (average intensity) and mean intensity was quantified. For the quantification of GFP signal in lateral root (LR) primordia, images of stages I and II primordia in the same position were considered. All images were processed and analyzed using ImageJ-Fiji.

Plant phenotype description

For the toxicity assay, A. thaliana seedlings were first sown on MS medium containing 1% sucrose (w/v) for 10 d and then alternatively transferred to controlled growth chamber conditions or to plates containing MS + 50 µM 8-aza-adenine, 8-aza-guanine or 0.2 µM tZ.

For root architecture studies, plants were grown in ½MS without nitrogen (PhytoTechnology) + 5 mM and 20 mM KNO3 for 19 d in square plates. Seeds of all lines belonged to the same bath to avoid any unwanted variation, as they also were grown side-by-side under uniform illumination. Plates were photographed with Power Shot SX150IS camera (Canon, Tokyo, Japan) and roots were measured using ImageJ. For phenotype analysis under exogenous CK exposition, the same procedure was carried out and plants were photographed at 19 dag.

Results

AZG2 transports purines and CK with high affinity

The AZG family has only two members in A. thaliana, AZG1 and AZG2. Both were described previously as purine transporters (Mansfield et al., 2009). To further investigate the transport capability of these two proteins, we transformed the yeast fly2 strain, which is deficient in purine transport, with pESC-AZG1 or pESC-AZG2. We studied the colony growth on medium containing 8-aza-guanine, a toxic purine analogue. Consistent with the data reported by Mansfield et al. (2009), AZG1 expressing yeast cells showed growth retardation. Surprisingly, such growth retardation was not observed for yeast expressing AZG2 (Fig. S1a). To substantiate the yeast growth phenotypes, we analyzed the growth performance of azg2-1 and 35Spro:AZG2 plant lines on growth medium containing 50 µM of either 8-aza-guanine or 8-aza-adenine. As a reference, we included azg1-1 and a 35Spro:AZG1 lines in the experiment (Fig. S1b–f). Contrary to the insensitive phenotype of azg1-1, we observed a developmental retardation and rapid accumulation of anthocyanins in the WT and azg2-1 seedlings growing with 8-aza-adenine. In addition,
uptake of the LOF showed slightly higher accumulation of 3H-8-aza-adenine competed with 14C-adenine, which is in line with paratively tested the uptake of 14C-adenine and 3H-tZ into sive in order to analyze CK transport (Ko transporter for CKs. New Phytologist/C6 value of 0.8 of 14C-adenine and even more than the unlabelled adenine itself benzyladenine (BA) and kinetin strongly inhibited the transport different CK species. Isopentenyl adenine (IP), trans-Zeatin (tZ), AZG1 and AZG2 have different substrate specificities AZG2 was investigated in 14C-adenine competition assays by adding a 10-fold excess of potential unlabelled substrates (Fig. 1a). As expected, unlabelled adenine inhibited 14C-adenine uptake to 10%, whereas hypoxanthine and guanine also interfered with the uptake significantly. Cytosine (a substrate for plant purine permeases (PUPs) and yeast FCY2), caffeine (transported by PUPs), adenosine (a substrate of ENTs) as well as, xanthine, uric acid, uracil and allantoin (substrates for nucleobase-Ascorbate Transporters (NATs; Maurino, et al., 2006; Niopек-Witz, et al., 2014) and ureide permeases (UPS)) inhibited transport of 14C-adenine only slightly. In addition, neither 8-aza-guanine nor 8-aza-adenine competed with 14C-adenine, which is in line with the previous toxicity experiments. The experimental evidence showing that purine transporters may have a role in CK transport (Burk et al., 2003; Zürcher et al., 2016) prompted us to evaluate AZG2 ability to transport 14C-adenine in competition with different CK species. Isopentenyl adenine (IP), trans-Zeatin (tZ), benzyladenine (BA) and kinetin strongly inhibited the transport of 14C-adenine and even more than the unlabelled adenine itself (Fig. 1a). This result indicates that AZG2 potentially functions as a transporter for Cks. As the yeast heterologous system has been reported to be elusive in order to analyze CK transport (Ko et al., 2014), we comparatively tested the uptake of 14C-adenine and 3H-tZ into Arabidopsis WT, azg2-1 and 35Spro:AZG2 seedlings. On the one hand, the LOF seedlings showed no significant differences in the 14C-adenine compared to the WT (Fig. 1b). On the other, the uptake of the LOF showed slightly higher accumulation of 3H-tZ uptake after 30 min compared to the WT, whereas 35Spro: AZG2 seedlings exhibited strong enhanced uptake capacity for both substances (Fig. 1c). The uptake rate for 3H-tZ by the 35Spro:AZG2 seedlings was three-fold higher than that of the WT (35Spro:AZG2: \( y = 1.98x + 0.18 \) and \( Wt: y = 0.64x + 0.08; P<0.001 \) and was not saturated even after 30 min of 3H-tZ feeding. These results demonstrate that AZG2 is able to transport adenine and tZ in vivo. In order to calculate the affinity of AZG2 for Cks avoiding the uptake complexity in complete seedlings, we established an uptake system using cells derived from Arabidopsis calli generated from 35Spro:AZG2 lines or WT controls. To test our system, we incubated calli cells with 0.25 μM 3H-tZ and measured the uptake after 4, 8 and 12 min. We observed equivalent results to the assays performed with seedlings (Fig. 1d). Using this system, we obtained substrate-saturating kinetics and calculated a \( K_m \) value of 0.8 ± 0.6 μM and a \( V_{max} \) value of 86.3 ± 15.8 pmol g⁻¹ FW min⁻¹ for tZ (Fig. 1e). We also were able to obtain kinetic parameters for adenine and hypoxanthine in the yeast system. The adenine and hypoxanthine transport rates were saturated by increasing substrate concentration and the deduced Michaelis–Menten parameters indicated \( K_m \) values of 4.8 ± 0.6 μM for adenine and 11.2 ± 1.3 μM for hypoxanthine (Fig. S2b,c). Thus, the \( K_m \) value for tZ was six- or 14-fold lower than that for adenine or hypoxanthine, respectively. This high affinity explains the strong competition effect observed for tZ in the yeast uptake system and, more importantly, indicates that CK are physiologi- cal relevant substrates. AZG2 facilitates substrate diffusion through membranes Our experiments using the yeast fcy2 mutant indicated that AZG1 transports 8-aza-guanine and 8-aza-adenine with higher efficiency than AZG2 (Fig. S1a,b), suggesting that the transporters have different transport features. We therefore measured the uptake of 3H-hypoxanthine, a purine transported by AZG2, into fcy2 cells at different extracellular pH values. AZG1 showed maximal uptake activity at pH4 which decreased with increasing pH (Fig. 2a). By contrast, AZG2-dependent purine uptake was pH-independent in the physiological range and reached a maximum at pH7 (Fig. 2b). In the absence of an energy source in the media (without Galac- tose) and in the presence of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or the ATPase inhibitor diethylstilbestrol (DES), the adenine uptake decreased in the yeast cells expressing AZG1 or AZG2 (Fig. 2c). Nevertheless, the dependence of purine uptake on an energy source and the existence of an electrochemical proton gradient over the plasma membrane were less pronounced for AZG2 than AZG1 (Fig. 2c). These data suggest that AZG1 mediates H⁺-driven purine transport in a simi- lar way to AZGA in Emerichella. However, AZG2 displays a dynamic of facilitated transport, being less dependent on the electrochemical proton gradient. Finally, to better understand the transport features of AZG1 and AZG2 even more, we fed yeast cells expressing AZG1 or AZG2 for 10 min with radiolabelled hypoxanthine. After several washings, the remaining radioactivity inside the cells was mea- sured at selected time points at optimal pH for each transporter: 4.5 for AZG1 and 7 for AZG2. We found that AZG2 lost the double of radioactivity compared to AZG1, with a drop of around 40% of the label (Fig. 2d). Taken altogether, these results suggest that whereas AZG1 relies on a proton coupled uptake, AZG2 behaves more as a diffusion facilitator. AZG2 localizes to the endoplasmic reticulum and plasma membrane The transport mechanism proposed for AZG2 is compatible with a protein that facilitates substrate diffusion between intracellular compartments independent of a pH gradient. As it was proposed that CK perception occurs in the lumen of endoplasmic reticulum (ER) (pH ~ 7.2; Caesar et al., 2011; Wulfetange et al., 2011), we studied the subcellular localization of AZG2. In silico analyses indicated that AZG2 is a membrane protein with 9–12
transmembrane domains and no specific signal peptides for subcellular trafficking (Fig. S3a–c). To trace AZG2 subcellular localization, we generated transgenic Arabidopsis lines expressing N- and C-terminal GFP fusions of AZG2 under the control of the *Ubiquitin 10* promoter (*U10*). After treating seedlings for a short period (10 min) with FM4-64, we observed a partial colocalization of AZG2 and the dye at the plasma membrane (PM) of root cells (Fig. 3a). The N-terminal and C-terminal GFP fusions of AZG2 showed similar localization patterns (Fig. 3a,b). When the cells were additionally treated with BFA, a drug that leads to the accumulation of endomembranes into the so-called ‘BFA compartments’, AZG2 and FM4-64 colocalized

Fig. 1 AZA-GUANINE RESISTANT (AZG2) transports purine with high affinity in yeasts (*Saccharomyces cerevisiae*) and cytokinins (CK) *in planta* (*Arabidopsis thaliana*). (a) A yeast strain deficient in adenine transport (*fcy2*) was transformed with AZG2 expressed under the control of the *GAL10* promoter in pESC-Ura. Yeast AZG2-mediated uptake of 14C-adenine (20 μM) was determined in the presence of 10-fold excess (200 μM) of unlabelled compounds. Values are expressed relative to uptake rates obtained without additions (100 %). Background uptake rates (empty vector) were subtracted (*n* = 3). (b, c) Uptake of 14C-adenine (b) and 3H-tZ (c) into 19 d after germination (dag) Arabidopsis seedlings. 35Spro:AZG2 (closed circles), azg2-1 (open circles) and wild-type (WT) (triangles) seedlings were incubated with 25 μM 14C-adenine or 3H-tZ for the indicated time. Values represent the mean ± SD of six independent experiments. Lines and shadows represent the estimated linear regression and a 0.95 confidence interval respectively. Mean significant differences at 30 min were determined by Student’s *t*-test (*, *P* < 0.01; **, *P* < 0.001; ***, *P* < 0.0001). (d) Uptake of 3H-tZ in Arabidopsis calli of 35Spro:AZG2 (closed circles) and WT (open circles) incubated with 0.25 μM tZ for 4, 8 and 12 min. (e) AZG2-mediated 3H-tZ uptake rates (pmol adenine (106 cells)–1 min–1) were measured in 35Spro:AZG2 calli at different substrate concentrations. Uptake rates in WT calli were subtracted (*n* = 3). Values represent the mean ± SEM of three independent experiments.
AZG2 gets transported via the trans-Golgi network on the secretory route to the plasma membrane. Interestingly, besides the PM localization, a GFP signal also was detected around the nucleus (Fig. 3b; white arrows). Such a protein pattern has been reported as ER localization in roots (Lomin et al., 2017) and corresponds to the signal of ER-targeted GFP (Fig. 3a; Ottenschlager et al., 2003; Müller & Sheen, 2008). To verify that the observed pattern corresponds to the ER, we analyzed a transgenic Arabidopsis line expressing AZG2-GFP under the control of the CaMV 35S promoter. This overexpression line exhibited a GFP signal in leaf epidermal cells that is characteristic for an ER pattern (Fig. 3c). It has been argued that gene expression under 35S promoter results in experimental artefacts by leading protein retention into the secretory pathway (Campanoni et al., 2007; Grefen et al., 2010). However, the root signal pattern (Fig. 3d) was coincident with that observed in the lower expression lines U10pro:AZG2-GFP and U10pro:GFP-AZG2 (Fig. 3a,b) suggesting that the observed pattern is not the product of an overexpression artefact. The presence of AZG2 in the ER membrane indicates that this transporter connects cytosol and the ER lumen for CK signalling.

AZG2 is expressed around the LRP in the overlying tissues

Our RT-PCR analysis showed that AZG2 mRNA mainly was present in roots and in reproductive tissues (Fig. 4a), results that were supported by transcriptomic data (Arabidopsis eFP Browser; Winter et al., 2007). To analyze the tissue specific expression pattern of AZG2 in more detail, we generated AZG2pro:GUS and AZG2pro:GFP stable transgenic Arabidopsis lines. We detected strong signals in a few cells of the root’s overlying tissues (OLT) throughout LR development (Fig. 4b,c), but not in reproductive organs (Fig. S4a–d). Nevertheless, weak GUS staining was visible in micropylar endosperm cells of germinating seeds (Fig. S4e–g). To gain more insights on AZG2 expression in the root, we performed 3D reconstructions of cross-sections of AZGpro:GFP expressing root tissue. GFP fluorescence was detected along the primordia at the region where cells became separated (17 µm) to allow the emergence of the new LR (Fig. 4d ii; Video S1). Thereby, we observed reporter activity in cortical and epidermal cells (Fig. 4d iii; segmented lines). This distinctive expression pattern in LRP OLT suggests that AZG2 may play a role in the regulation of LR development.

Auxin induces AZG2 expression via ARF7 transcription factor

Auxin is one of the major hormones involved in the transcriptional regulation of LR development genes. Interestingly, public expression data revealed that AZG2 is induced 3–6 h after auxin treatment (Fig. S5a–d; Redman et al., 2004; Goda et al., 2008; De Rybel et al., 2012; Bargmann et al., 2013; Lewis et al., 2013).
To characterize the response of the AZG2 promoter to auxins, we studied AZG2prom:GUS and AZG2prom:GFP activities after treatment with 1 \( \mu M \) NAA. A time-course experiment showed that, after 12 h of auxin supply, GUS staining was no longer restricted to the LRP environment but instead enhanced and spread throughout the root (Fig. 5a). A similar result was obtained using the AZG2prom:GFP line (Fig. 5b). This remarkable change in expression distribution may reflect an alteration in local auxin concentrations generated by NAA treatment and suggests that additional unknown factors regulated by auxins are repressing AZG2 expression. We further analyzed AZG2 promoter regions of different lengths, from 2200 to 170 bp, for their auxin responsiveness. We did not observe any differences in the expression patterns, indicating that 170 bp upstream of the AZG2 start codon is sufficient for the auxin-dependent induction of AZG2 (Figs 5b, S5g,h).

As auxin-responsive genes are often induced by transcription factors of the AUXIN RESPONSE FACTOR (ARF) family (Guilfoyle et al., 1998), we analyzed the expression profiles of AZG2 in the arf7 and arf19 mutant backgrounds. ARF7 and ARF19 were both linked to LR development (Wilmoth et al., 2005). Consistently, AZG2 transcription seems to depend on ARF7 or both, ARF7 and ARF19 (Fig. S5b; supplemental table 4 in Okushima et al., 2005). Moreover, in plants carrying the gain-on-function srl1/iaa14 allele, which constitutively inhibits ARF7 and ARF19, the auxin-dependent induction of AZG2 was impeded (Fig. S5c; Vanneste et al., 2005). To investigate the relationship among ARF7, ARF19 and AZG2 in more detail, we transformed arf7-1, arf19-1 and WT plants with AZG2prom:RFP. Although no RFP signal was observed in the roots of arf7-1 (with or without NAA treatment), roots of arf19-1 showed the same RFP signal as the WT (Fig. 5c). We discarded a mechanistic ARF-independent induction because treatment with naxillin, a nonauxin molecule that promotes root branching, does not induce AZG2 expression (Fig. S5d; De Rybel et al., 2012). Taken together, these results demonstrate that the AZG2 gene is a downstream target of auxin-mediated signalling via ARF7 transcriptional regulation during LR development.

AZG2 is part of an LR emergence inhibitory pathway

The restricted AZG2 expression pattern and its dependence on auxin signalling suggest that AZG2 may play a physiological role during LR development. To address this issue, we studied the root phenotype of different AZG2 LOF lines (azg2-1, azg2-2) and a transgenic line ectopically expressing AZG2 (35Sprom:AZG2) in the WT background. The LOF and overexpressing line showed no obvious phenotype under standard growth conditions. However, a differential phenotype was observed by varying the nitrate concentration of the growth media. Compared to the WT, at 5 mM and 20 mM KNO3, azg2-1 and azg2-2 showed higher LR density (Fig. 6a,b). The 35Sprom:AZG2 plants exhibited lower LR density at 5 mM KNO3. This result together with the more stable phenotype observed in the LOF lines led us to select

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**Fig. 3** AZA-GUANINE RESISTANT (AZG)2 is localized in plasma membrane and in endoplasmic reticulum (ER). (a, b) Carboxyl (a) and amino (b) terminal fusion of AZG2 with GFP (green fluorescent protein) expressed under the control of Ubiquitin-10 promoter in the main root tip of 7 d after germination (dag) Arabidopsis thaliana seedlings. ER-GFP corresponds to a GFP-tagged ER for retention under the control of TCSnpro. White arrows, perinuclear signal. FM4-64, short treatment label PM. (c, d) Overexpression of AZG2-GFP using 35S promoter showing AZG2 signal in (c) leaf epidermal cell and (d) in the root tip. (e) Seedlings of U10prom:GFP-AZG2 after 1 h BFA 1 mM treatment followed by a 10-min incubation with FM4-64. (f) Fluorescence quantification in GFP (cyan) and FM4-64 (magenta) channels within the highlighted region in yellow in (e) (PM, plasma membrane; BFAC, Brefeldin-A compartment; EM, endomembrane). (g) Detail of a cell in the root elongation zone treated with BFA. Bars: (a–e) 50 \( \mu m \); (g) 25 \( \mu m \).
the 5 mM KNO₃ condition for subsequent experiments. We did not find differences regarding the primary root length (Fig. S6a).

It is well-known that nitrate (NO₃⁻) availability modulates root architecture (Krouk et al., 2010; Gruber et al., 2013) and CK biosynthesis (Takei et al., 2004). Therefore, we investigated whether AZG2 expression can be modulated by NO₃⁻. We observed no differences in AZG2 expression level in a wide range of NO₃⁻ concentrations (S6b). This is in accordance with publically available expression data (Fig. S5e–f; Vidal et al., 2013).

In order to address more precisely the activity of AZG2 along primordia formation, we studied if their development was altered during LRP emergence. LRP s were grouped according to the mechanical barriers that they have to overcome to emerge as a new LR (Malamy & Benfey, 1997; Lucas et al., 2013; Stoeckle et al., 2018). Comparing azg2-1 and the WT, we found that LRP density was only unbalanced at the latest stages (VI–VIII; Fig. S6c). Although the difference in seedlings was not statistically significant (P = 0.090), a higher LRP density of primordia near emergence in azg2-1 suggests a permissive progression in this line and helps to understand the cause of the higher LR density phenotype on plants (Fig. 6a,b). We discarded a retention phenotype of the LRP because we did not observe arrested primordia, and due to the higher LR density phenotype described previously. Remarkably, during stages VI to VIII, the primordia has already passed through the endodermis and is growing across the cortex and epidermal layers where AZG2 is present, and is most likely playing its regulatory role.

Because CKs are known to have a negative impact on LR density (Werner et al., 2010), we analysed the root phenotype of the WT, azg2 and the 35Spro:AZG2 growing on 5 mM KNO₃ supplemented media containing in addition 0.2 µM tZ. Under this condition the WT and 35Spro:AZG2 seedlings showed retarded development and chlorosis; in addition, the WT developed nearly no LRs, whereas 35Spro:AZG2 seedlings had no LRs (Fig. 6c,e). Even though azg2-1 and azg2-2 also displayed developmental retardation, they produced a complex root system with several emerged LR. Interestingly, 35Spro:AZG2 plants showed a shorter total root system than the WT and LOF mutant seedlings (Fig. 6d). Only in the 35Spro:AZG2 line AZG2 is expressed in the root apical meristem (RAM), most likely leading to a higher concentration of CK in the meristem, with a consequent disordered RAM activity. Taken together, the CK insensitivity of the azg2 mutants, the AZG2 expression pattern, and the fact that AZG2 is a purine transporter indicate that AZG2 most likely functions in the local distribution of CKs.

The absence of AZG2 compromises CK signalling in roots

In order to gain deeper insights into AZG2 function in CK signalling, we studied the activity of the CK signalling reporter TCSnpro:GFP (Zürcher et al., 2013) in the WT and azg2-1. As shown in Fig. 7(a), the TCSnpro:GFP reporter gene was active in both genetic backgrounds under control conditions. To confirm the inducibility of the reporter, both lines were exposed to tZ for 24 h. The GFP signal strength effectively increased in both genotypes, but a differential GFP pattern was noticed in the expression domain of AZG2 in OLTs (Fig. 7b). In order to study if this differential pattern of TCSnpro activity has an impact on overall CK signalling, we analyzed the GFP signal of two independent insertion events of TCSnpro:GFP in the azg2-1 mutant background and compared it to the signal in the WT. The GFP signal was significantly lower in the vicinity of azg2-1 LRPs compared...
to the signal of WT roots (Fig. 7c,d). To analyze whether the absence of AZG2 affected CK signalling within the LRP cells, we quantified TCSnpro:GFP signals in this specific region of the WT and azg2-1 seedlings. No significant differences were found in the GFP signal intensity of primordia cells between both genetic backgrounds (Fig. 7e). These results reinforce the hypothesis that AZG2 functions as a CK transporter involved in root hormone signalling in the OLTs.

Discussion

The root system architecture is determined by root branching, which is highly regulated by hormones, mainly auxins and cytokines (CKs) (Bishopp et al., 2011). During the last decade, hormone transport has become a key factor in the understanding of plant hormone homeostasis. To date two CK transporters, ABCG14 and PUP14, have been identified as being involved in the modulation of plant development. On the one hand, ABCG14 is expressed in the endodermis and stellar cells, acting as an efflux carrier for long distance CK transport (Ko et al., 2014; Zhang et al., 2014). On the other, PUP14 is expressed throughout the plant and has been proposed as a transporter that modulates the efficiency of CK signalling at plasma membrane level (Zürcher et al., 2016). In this study, we introduce AZA-GUANINE RESISTANT (AZG)2 as a novel CK transporter with a very restricted expression domain in few cells covering the lateral root primordia (LRP) (Fig. 4; Video S1). As overlying tissues (OLTs) play a key regulatory role on primordia development (Peret et al., 2012; Lucas et al., 2013; Vilches-Barro & Maizel, 2015), AZG2 arises as a suitable candidate for the control of local CK gradients in the lateral root (LR) environment.

AZG2 may supply endoplasmic reticulum (ER) membrane-localized receptors with CK

Purine transporters such as PUP1 and PUP14 are able to transport not only adenine, but also CK (Burkle et al., 2003; Zürcher et al., 2016). Mansfield et al. (2009) showed that AZG transporters from Arabidopsis are able to take up adenine and guanine in yeast and Arabidopsis seedlings. In this work, we demonstrated that AZG2 additionally transports CK in Arabidopsis with high affinity. When we determined AZG2-dependent CK uptake in Arabidopsis seedlings, we found slight differences between azg2 and wild-type (WT) seedlings only after 30 min. This is probably a consequence of the spatially very restricted activity of AZG2, as the minor addition of CK to a few cells is weakened against the background of the entire seedling. By contrast, the 35Spro:AZG2 line took up significantly more labelled tZ than the WT, showing that AZG2 is able to transport CK in vivo. To understand a potential role of AZG2 in the CK signalling pathway, it is necessary to determine which cell compartments become connected by its activity. Therefore, the subcellular localization of AZG2 was traced in transgenic Arabidopsis plants by using different fusions to green fluorescent protein (GFP). All fusion proteins located to both the plasma membrane and the ER. Based on these observations, we questioned whether AZG2 is functional under the conditions given at both membrane sides. Studies in yeast indicated that AZG2 has maximal transport activity in media with pH between 5 and 8, contrasting with AZG1 which shows clear transport dependence on an electrochemical proton gradient...
In particular, the capability of AZG2 to efficiently transport substrates even at slight alkaline pH fits very well with a functional transporter at the ER. Additionally, export studies in yeast suggest that AZG2 is able to move its substrates in both directions (Fig. 2d). Thus, AZG2 most probably functions in vivo as a diffusion facilitator, whereas AZG1 most probably performs secondary active transport. Taken together, these results support the presence of functional AZG2 in both the plasma membrane (PM) and ER of cells expressing the transporter. The latter observation is relevant regarding the ongoing discussion about the subcellular localization of the functional CK receptors (Caesar et al., 2011; Romanov et al., 2018).

AZG2 is part of an LR emergence inhibitory pathway

We found that a promoter region 170 bp upstream of the AZG2 start codon was sufficient for auxin inducibility of the gene (Fig. 5b). AZG2 expression dependence on auxin previously was reported by Goda et al., (2008), Lewis et al. (2013), De Rybel et al. (2012) and Okushima et al. (2005). The microarray data showed an induction time of 3–6 h, whereas our β-glucuronidase (GUS) and GFP reporters were detectable after 12h (Fig. 5a). This discrepancy could be a consequence of different detection systems or additional regulatory mechanisms not revealed in this work.
It has been additionally found by Okushima et al. (2005) that AZG2 is induced by AUXIN RESPONSE FACTORS (ARF)7, but not ARF19. Here, we confirmed this by studying AZG2 promoter:RFP expression in the arf7-1 and arf19-1 mutant backgrounds (Fig. 5c). However, canonical auxin response elements (AuxRE; Ballas et al., 1993) are absent within these 170 bp, suggesting that either another, ARF7-dependent, transcription factor or a noncanonical AuxRE might be involved in AZG2 regulation. Interestingly, we found a G-box (5'-CACGTG-3'; Fig. S5i) in the AZG2 promoter sequence, 165 bp upstream of the start codon of the coding sequence. This sequence has been reported as a target sequence for the LBD29 transcription factor (Xu et al., 2018), a primary auxin response gene which acts downstream of ARF7 (Okushima et al., 2007). A role for LBD29 or other auxin
signalling mediators are predicted by the relative long incubation times needed to induce AZG2 expression (Fig. 5a), which positions AZG2 more probably as an auxins secondary induced gene, likewise LAX3 (Porco et al., 2016). Nevertheless, other LBDs cannot be excluded in AZG2 gene regulation, because they modulate expression of many genes involved in root architecture (Feng et al., 2012; Lee et al., 2015). Indeed, LBDs induce the expression of several genes in OLT such as those encoding the cell-wall remodelling enzymes EXP14 and EXP17, which are required for LRP emergence (Lee & Kim, 2013; Lee et al., 2013). Here, it is important to highlight that AZG2 is expressed exclusively in the OLT and the micropylar endosperm (Fig. S4e–g), both tissues with a high cell-wall remodelling activity. Moreover, LBD29 is responsible for LAX3 expression induction in OLTs, which is, in turn, involved in LR emergence promotion via cell wall remodelling (Swarup et al., 2008). Although it is known that CK regulates several genes involved in cell wall remodelling, its physiological relevance during LR development has not been studied yet (Brenner et al., 2012). Thus, these genes represent strong candidates to explain the processes downstream of AZG2 that influence LR emergence.

Both auxin-dependent induction and CK transport activity of AZG2 suggest that this transporter is part of the CK: auxin hormonal cross-talk system. The direct relevance of AZG2 function in CK signalling is evident as exogenous supply of CK fails to inhibit LR emergence in azg2 LOF and TCSnpro-GFP/azg2-1 roots showed less CK signalling activity around LRP s than the WT (Fig. 7). Within this regulatory loop, auxins release ARF7 from AUX/IAA14 inhibition (Fukaki & Tasaka, 2009). Then, ARF7 upregulates LBD transcription factors, which in turn influence a set of genes related to lateral organ regulation, and among them the CK transporter AZG2 (Fig. 8b). In summary, LR emergence regulation is the outcome of a diversity of integrated endogenous and environmental signals.

We observed that the differential LR phenotype of AZG2 mutants is detectable within a specific nitrate concentration range (Fig. 6a,b), although AZG2 expression is not modulated by nitrate (Figs 8b, S5e,f, S6b). It is well known that high nitrate concentration regulated root branching by IPT3 expression induction and consequently CK biosynthesis (Zhang et al., 1999; Takei et al., 2004). This systemic increase of CK: auxin balance negatively influences LR development at different levels including emergence. Consistently, the AZG2 mutant phenotype can be strongly enhanced by the addition of exogenous Cks (Fig. 6c,e). However, the expression of the auxin transporter LAX3 is regulated by nitrate via NRT1.1 and facilitates the emergence of LR when even mild concentrations are available (Maghiaoui et al., 2020). We propose that AZG2 and LAX3, both specifically expressed in OLT, might be involved in a regulatory feedback loop in order to integrate internal signals (systemic balance between CK and Auxins) and environmental local signals (nitrate). LR emergence may be impeded both by low nitrate due to lack of LAX3 expression in the OLTs or by high nitrate-induced CK synthesis and perception in cells expressing AZG2. By intermediate nitrate concentrations LR emergence will progress by the fine-tuning of permissive signals (local nitrate, LAX3 expression, auxins increase in OLT cells, induction of remodelling genes) and
repressive signals (systemic nitrate, CK increase, AZG2 transport, AHKs perception, repression of remodelling genes).

The auxin induction of a member of the CK signalling pathway may be against the classic reported antagonistic role of these two hormones (Müller & Sheen, 2008; Bishopp et al., 2011; Birnbaum, 2016). However, local activation of CK signalling by auxins has been reported previously (De Rybel et al., 2014; Ohashi-Ito et al., 2019). To confirm whether AZG2 is part of a negative regulatory feedback loop with auxins, or if auxins act only as positional cues to determine the AZG2 restricted expression pattern, further experiments are required. Finally, despite the fact that downstream events of AZG2 transport activity are unknown, future studies of cell-wall remodelling in azg2 mutants could lead to better understanding of AZG2 functionality during LR emergence. This could help to explain how AZG2 along with other positive and negative regulators comprises a fine-tuned LR emergence regulatory network.

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Author contributions

TMT, IL and VM carried out the genotyping of transgenic lines, phenotype characterization, expression studies and the hormonal response; GP carried out the LR, primordia quantification; TMT, SB and DW participated in cloning of GFP-fusions and subcellular localization; BS, EW and MD performed transport experiments; and TMT, CAG, KH and MD conceived the project and wrote the article. All authors contributed to the final shape of the manuscript and figures.

Accession numbers

Sequence data for genes in this article can be found in the GenBank/EMBL database under the following accession numbers: AtAZG1 (At3g10960), AtAZG2 (At5g50300), ARF7 (At5g20730), ARF19 (At1g19220).

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

*Fig. S1* Purine analogue resistance phenotype and LOF line genotyping.

*Fig. S2* Purine transport characterization in yeasts.

*Fig. S3* AZG2 transmembrane domains analysis and signal peptide prediction.

*Fig. S4* AZG2 expression in reproductive tissues and seeds.

*Fig. S5* AZG2 expression characterization and auxin induction.

*Fig. S6* AZG2 mutants phenotype and nitrate inducibility.

**Methods S1** Expression in yeast and uptake assays.

**Table S1** List of primer used in this work.

**Video S1** AZG2 expression domain.