Characterization of the Arabidopsis family of Purine Permeases as candidates for a Cytokinin transport system

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Results PUP14

Characterization of *PUP14* functions in plant development
The TCSn reporter has allowed us to determine cytokinin signalling patterns in planta unravelling that signalling occurs in defined areas. It is however not clear how these distinct locales are specified. In this chapter, we report that PUP14 modulates the distribution of active cytokinins and thereby defines cytokinin signalling landscapes.

All of Chapter 3.1 is a manuscript draft.

Author Contributions
B.M. devised experiments, constructed the PUP14 reporter, inducible AHK3 and CKI1 constructs and the expression plasmids, did microscopic imaging and wrote the manuscript, E.Z. constructed all other constructs, generated transgenic plants, performed crosses, did transport experiments with protoplasts and seedlings, performed qRT-PCR analyses, and wrote the manuscript. J.L. performed the protoplast reporter assays, M.D. and M.G. performed transport experiments with microsomes. All authors contributed to the writing of the manuscript.

Please find the Extended Data attached as Appendix A1.
3.1 CYTOKININ IMPORTER PURINE PERMEASE 14 CONFINES THE CYTOKININ RESPONSES

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ABSTRACT

Multicellular organisms operate via the coordination of specialized cellular identities and functions dictated by signalling systems. However, the mechanisms that define the precise spatiotemporal cell domains and subcellular locales responding to specific signals are not well understood. In plants, cytokinins direct essential cell-to-cell signalling systems to initiate and maintain differential cell functions during gametophyte development, embryogenesis and postembryonic shoot and root development (Hwang et al., 2012; Kieber and Schaller, 2014). Guided by the live green fluorescent protein (GFP) imaging of the synthetic reporter, TCSn::GFP (Two Component signalling Sensor) (Zürcher et al., 2013), we illuminate precise cell populations activating conserved phosphorelay signalling circuitry despite much broader intracellular cytokinin signalling competence and potential. Here, we report a surprising finding that Arabidopsis PURINE PERMEASE 14 (PUP14) plays a pivotal role in confining the cytokinin signalling response throughout development by ligand sequestration. The expression patterns of PUP14 are inversely correlated with the cytokinin signalling readout, indicating an antagonistic relation. Indeed, the conditional PUP14 knock-down by an artificial microRNA (amiRPUP14) causes ectopic cytokinin signalling accompanied by aberrant morphogenesis in embryos, roots and the shoot apical meristem. PUP14 protein localizes to the plasma membrane and imports bioactive cytokinins as shown by analysing transport in mesophyll protoplasts, seedlings and microsomes. These activities of PUP14 reduce the apoplastic cytokinin pools and extracellular cytokinin perception by cytokinin sensors. The relevance of apoplastic cytokinins in initiating intracellular signalling is corroborated by our findings that signalling output is attenuated by secreted cytokinin-degrading enzyme CYTOKININ OXIDASE 2 (CKX2) (Werner et al., 2003), but not by intracellular CKX variants. These results uncover the existence of a previously unknown and dedicated transport system that patterns the cytokinin signalling landscape of vascular plants.
RESULTS AND DISCUSSION

Chemical hormones including cytokinins are key signals to govern pattern formation and morphogenesis throughout the plant life cycle. However, the mechanisms that define the precise spatio-temporal domains of cytokinin perception (Zürcher et al., 2013) are largely unknown. To address the question of how the localized signalling activities are established, we used heart-stage embryos as a model where the cytokinin response marks the provascular tissue (Fig. 1a). Formally, both the availability of bioactive cytokinins and the cellular competence to respond to a stimulus may control the signalling domains. To determine whether active cytokinins are limiting, heart-stage embryos were incubated for 16 h with an excess of the degradation-insensitive cytokinin benzyl adenine (BA) (Galuszka et al., 2007). This caused a stereotypical expansion of the TCSn::GFP response domain (Zürcher et al., 2013) (Fig. 1b). However, no TCSn::GFP induction was observed in the prospective cotyledons, despite the transcription of the cognate cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4 (AHK4) in these domains (Mähönen et al., 2000) (arrowheads Fig. 1c). Ethanol-induced expression (Roslan et al., 2001) of an AHK3 transgene encoding a redundantly acting cytokinin receptor (35S>ALC>AHK3) together with an excess of BA did not activate TCSn::GFP in prospective cotyledons either (arrowheads Fig. 1d), supporting the notion that failure to turn on signalling is not due to absence of the receptors. To test whether signalling downstream of receptors is functional, we expressed CYTOKININ INDEPENDENT 1 (CKI1) for 16 h using the ethanol-inducible system (35S>ALC>CKI1). CKI1 encodes a hybrid kinase with cytokinin-independent constitutive activity (Hwang et al., 2012) and caused ubiquitous TCSn::GFP activation (Fig. 1e). Together, these results suggest that cells of the prospective cotyledons fail to activate cytokinin signalling despite abundant active ligands and a functional signalling system. We hypothesized that productive ligand-receptor interactions in planta depend on cytokinin transporters that guide differential cellular localization of cytokinins. However, the genes that fulfil this postulated role have not been identified. Members of the vascular plant-specific family of transmembrane PURINE PERMEASES (PUPs) have been implicated in cytokinin translocation (Gillissen et al., 2000); yet, a link to cytokinin function in planta was not established. To test whether uncharacterized members of the Arabidopsis PUP family control the spatio-temporal landscape of cytokinin signalling, we first established a transcription profile of the family members based on our own analysis and published transcriptome data (Belmonte et al., 2013; Yadav et al., 2014; Adrian et al., 2015) (Extended Data Fig. 1). PUP14 expression prevailed in all organs and stages analysed, including embryos. To determine the PUP14 expression pattern, we analysed PUP14::PUP14-GFP transgenic plants. In heart-stage embryos, PUP14-GFP localized to cells of the prospective cotyledons (Fig. 1f) that failed to activate cytokinin signalling (Fig. 1b). We found PUP14 expression in the seedling’s main root (Fig. 1i), the lateral root primordia (LRP) (Fig. 1l), the shoot apical meristem (Fig. 2a), and in ovules and seeds (Extended Data Fig. 3a-b) exhibiting equivalent complementary patterns to those of cytokinin signalling (Fig. 1j,m, 2b and Extended Data Fig. 3a,b).
The near-exclusive nature of PUP14 expression and cytokinin signalling patterns is compatible with an inhibitory function of PUP14 in the cytokinin response. To test this hypothesis, we constructed an ethanol-inducible artificial microRNA (Schwab et al., 2006) targeting PUP14 (35S>ALC>amiRPUP14). Upon induction of the transgene, PUP14 mRNA levels significantly different: p<0.001 from unpaired t-test, (a) mock, (b,d) BA treatments, (d)16 h-induced AHK3 expression, or (e) CKI1 (see methods), arrowheads denote prospective cotyledons without TCSn::GFP expression. c, AHK4 mRNA detected by in situ hybridisation. f, PUP14::PUP14-GFP in heart stage embryo. g, Ectopic TCSn::GFP 16 h after amiRPUP14 induction (85% of embryos, n=53), h, Morphological defects 48h after amiRPUP14 induction (47% of embryos, n=96) including reduced size of prospective cotyledons (arrowhead) and cell division defects in the root meristem (cell boundaries outlined with white dotted lines). i, PUP14::PUP14-GFP in main root. j, TCSn::GFP in LRP. k, Ectopic TCSn::GFP in amiRPUP14 induced LRP (arrowheads). l, Relative changes of type-A ARR5, 6 and 7, and TCSn::GFP (as a group significantly different: p<0.001 from unpaired t-test, TCSn::GFP: n=4; TCSn::GFP, amiRPUP14: n=4 and PUP14 mRNA levels (significantly different: p<0.001 from unpaired t-test, TCSn::GFP: n=4; TCSn::GFP, amiRPUP14: n=7) after 16 h of amiRPUP14 induction in 7 d old seedlings of indicated genotype, assessed by quantitated real-time (qRT)-PCR, error bars represent s.e.m. p, Seedlings after 7 d on ethanol-containing medium. q, Growth curve of seedlings shown in (p). Scale bars (a-h) 20 µm, (i-k) 50 µm, (l-n) 10 µm, (p) 1 cm, +BA, 16 h treatment with 10µm BA.

Figure 1 | PUP14 confines the cytokinin response in embryos and roots

a,b,d,e, Micrographs of TCSn::GFP in heart-stage embryos overlaid with transmitted light pictures subjected to 16 h (a) mock, (b,d) BA treatments, (d)16 h-induced AHK3 expression, or (e) CKI1 (see methods), arrowheads denote prospective cotyledons without TCSn::GFP expression. c, AHK4 mRNA detected by in situ hybridisation. f, PUP14::PUP14-GFP in heart stage embryo. g, Ectopic TCSn::GFP 16 h after amiRPUP14 induction (85% of embryos, n=53), h, Morphological defects 48h after amiRPUP14 induction (47% of embryos, n=96) including reduced size of prospective cotyledons (arrowhead) and cell division defects in the root meristem (cell boundaries outlined with white dotted lines). i, PUP14::PUP14-GFP in main root. j, TCSn::GFP in LRP. k, Ectopic TCSn::GFP in amiRPUP14 induced LRP (arrowheads). l, Relative changes of type-A ARR5, 6 and 7, and TCSn::GFP (as a group significantly different: p<0.001 from unpaired t-test, TCSn::GFP: n=4; TCSn::GFP, amiRPUP14: n=4 and PUP14 mRNA levels (significantly different: p<0.001 from unpaired t-test, TCSn::GFP: n=4; TCSn::GFP, amiRPUP14: n=7) after 16 h of amiRPUP14 induction in 7 d old seedlings of indicated genotype, assessed by quantitated real-time (qRT)-PCR, error bars represent s.e.m. p, Seedlings after 7 d on ethanol-containing medium. q, Growth curve of seedlings shown in (p). Scale bars (a-h) 20 µm, (i-k) 50 µm, (l-n) 10 µm, (p) 1 cm, +BA, 16 h treatment with 10µm BA.
and PUP14-GFP levels were strongly reduced within 24 hrs of induction (Extended Data Fig. 2a-c), demonstrating the efficacy of this approach. The phenotypes of amiRPUP14-induced embryos and seedlings were complemented by an amiRPUP14-resistant transgene (PUP14*) encompassing the PUP14 locus (Extended Data Fig. 2c-e), validating that the inducible amiRPUP14 acts specifically. Inducing amiRPUP14 expression for 16 h caused widespread ectopic cytokinin signalling in the embryo (Fig. 1g), also in cells of the prospective cotyledons that are non-responsive to treatments with exogenous cytokinins (arrowheads Fig. 1b), supporting the role of PUP14 in confining the cytokinin response. The same treatment did not affect the auxin response (Extended Data Fig. 2f), indicating that PUP14 acts specifically on cytokinin signalling.

Longer inductions caused morphological defects in prospective cotyledons and the nascent root meristem (Fig. 1h). Similarly, amiRPUP14 induction resulted in ectopic cytokinin signalling in the seedling root, particularly in the meristematic region of the root tip (Fig. 1k).

Figure 2 | PUP14 confines the cytokinin response in the SAM
a, PUP14::PUP14-GFP, and b, TCSn::GFP in floral SAM. Arrows indicate peak PUP14-GFP levels at organ-organ boundaries. c, Ectopic TCSn::GFP (arrowheads) after amiRPUP14 induction. Longitudinal optical sections in lower panels (a-c) at cyan-coloured brackets, dotted lines mark organ boundaries. d-h, Comparisons of ethanol-treated TCSn::GFP, and TCSn::GFP, amiRPUP14 phenotypes. d, Inflorescences. e, Numbers of flower primordia at stages 6-12 on the main apex (Smyth et al., 1990), n=6. f, Shoot architecture. Red dots denote primary rosette branching (RI), yellow dots primary cauline branching (CI). g, Number of RI and CI, n=6. h, Inflorescence stems, arrowheads indicate perturbations. Data represent mean value, error bars represent s.d. **p < 0.01 unpaired t-test. Scale bars (a-c) 20 µm, (d) 1 mm, (f) 5 cm, (h) 1 cm.
and LRP (Fig. 1n). Accordingly, transcription of the immediate-early cytokinin target genes type-A ARABIDOPSIS RESPONSE REGULATORS (ARR) ARR5, 6 and 7 (D’Agostino et al., 2000) was induced in seedlings (Fig. 1o). Continuous induction of amiRPUP14 led to a strong growth retardation of the seedling root, abnormal cotyledons, and a suppression of lateral roots (Fig. 1p,q), consistent with increased cytokinin activities (To et al., 2004). In the shoot, cytokinin controls the homeostasis of the shoot apical meristem (SAM) (Gordon et al., 2009), where increased cytokinin causes a more active meristem with more primordial (Bartrina et al., 2011). Reduction of PUP14 levels after amiRPUP14 induction caused ectopic cytokinin output in the SAM (Fig. 2c), which was accompanied by a higher number of primordia (Fig. 2d,e), increased shoot branching and disturbed phyllotaxis (Fig. 2f-h).

Similar phenotypes have been observed in plants mutant for CYTOKININ OXIDASE (CKX) 3 and 5 (Bartrina et al., 2011), ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (Besnard et al., 2014), and ARR3-9 (Leibfried et al., 2005), which also display ectopic cytokinin activities. Together, these findings strongly support that PUP14 functions to limit the cytokinin response domains throughout development.

Next, we addressed the cellular function of PUP14. PUP14-GFP fusion proteins localize to the plasma membrane (Fig. 1f,l). To test PUP14’s cytokinin transport capacity, we conducted uptake experiments using labelled trans-zeatin (tZ), an abundant natural cytokinin (Werner et al., 2003). Transient expression of PUP14 in mesophyll protoplasts or tobacco microsomes stimulated the uptake of labelled tZ (Fig. 3a,b). The PUP14 transport activity was ATP-dependent and higher compared to PUP1 (Gillissen et al., 2000) (Fig. 3b). Uptake was inhibited by unlabelled tZ, by the common natural cytokinin isopentenyl adenine (iP), by the aromatic cytokinin BA, and also by adenine, but not by tZ riboside, the major cytokinin transport form (Beveridge et al., 1997), nor auxin (IAA), nor allantoin, which is an unrelated substrate (Fig. 3c,d). Energy-dependent cytokinin uptake into a microsomal cell-free system further excludes that uptake is dependent on cytoplasmic metabolisation.

Conversely, seedlings with decreased PUP14 levels exhibited a reduced uptake rate for exogenously added tZ compared to control seedlings (Fig. 3e).

Our data show that plasma membrane localized PUP14 imports bioactive cytokinins, implying that PUP14 activity depletes ligands from the apoplast, which leads to a suppression of the cytokinin response. In this scenario, extracellular cytokinins binding to the sensing domains of plasma-membrane localized receptors (Kim et al., 2006; Wulfetange et al., 2011) (Extended Data Fig. 3c,d) are important to initiate the signalling response, while intracellular cytokinins binding to endoplasmic reticulum (ER)-localized receptors (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011) would contribute less for activation of the pathway. To test this hypothesis, we devised experiments that compare the effects of differentially targeted cytokinin-degrading enzymes on the cytokinin signalling response. Mesophyll protoplast cells responded to as little as 100 pM of exogenously added tZ by activating cytokinin signalling (Müller and Sheen, 2008), suggesting they depend on exogenous cytokinins, and thus serve as a suitable model to study cytokinin perception independent of production (Fig. 3f). Transient transfection with plasma membrane-localized
PUP14 (Fig. 3h) caused a marked reduction of cytokinin-dependent TCS::LUCIFERASE (LUC) activity, in agreement with loss of PUP14 function that caused ectopic cytokinin responses (Fig. 1g,k,n,o, 2c). Similarly, transient expression of a wild-type CKX2 that is targeted for secretion to the apoplast (Werner et al., 2003) (Fig. 3i) attenuated the cytokinin response. In contrast, a variant of CKX2 that lacks the N-terminal signal peptide (CKX2∆SP) and localizes to the cytoplasm (Fig. 3j) did not affect the cytokinin response. Neither did CKX7, which has been reported to localize to the cytoplasm (Köllmer et al., 2014) (Fig. 3k), Finally, a CKX2 variant that is targeted to the lumen of the ER (CKX2-ER) (Fig. 3l) did not significantly reduce the signalling response either (Fig. 3f). The outcomes of these experiments support the dominant role of apoplastic cytokinins to trigger signalling in target cells via plasma membrane-localized AHK receptors (Kim et al., 2006; Wulfetange et al., 2011) (Extended Data Fig. 3c,d).

Figure 3 | PUP14 cellular function
a-e, Relative 14C-IZ uptake rates on y-axis. a, Transfected mesophyll protoplasts. b, Transport in microsomes derived from 35S::GFP, 35S::PUP1, or 35S::PUP14 transfected N. benthamiana. c, Competition by indicated substances in PUP14-transfected protoplasts. d, Competition in microsomes of 35S::PUP14 transfected N. benthamiana. e, amiRPUP14 vs. Col-0 seedlings. f, Relative TCSn::LUC activities with 10 nM IZ in mesophyll protoplasts co-transfected with transgenes as indicated, normalised to empty vector control. g-i, Subcellular localization of transfected gene products as shown in (f). Data represent mean value, error bars represent s.d. (a) or s.e.m. (b,c,d,e,f). **p < 0.01, ANOVA with Tukey’s HSD post hoc test. Scale bars 10 µm.
Based on our findings, we propose a model (Extended Data Fig. 4) where PUP14 activity inversely correlates with the capacity of a cell to sense cytokinins. PUP14’s cytokinin import activity leads to a translocation of cytokinins from the apoplast to the cytosol, away from sensing domains of plasma membrane-localized receptors, which causes a reduction in cytokinin signalling. Feeding experiments with radiolabeled bioactive cytokinin suggested that the bulk of imported cytokinins are inactivated by conversion to monophosphates by ADENINE PHOSPHORIBOSYL TRANSFERASE enzymes (Moffatt et al., 1991; Zhang et al., 2013). Furthermore, N- or O-glycosylation, oxidative cleavage, or transport to other cells may contribute to clearance of intracellular cytokinins (Kieber and Schaller, 2014). In addition to PUP14, other members of the family are likely to control cytokinin signalling in defined developmental contexts. The fact that PUP genes are specific to vascular plants (Hildreth et al., 2011) may suggest their function to support more complex cytokinin signalling patterns associated with the bauplan of land plants.

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MATERIALS AND METHODS

Microscopy and live imaging
RNA in situ hybridization and embryo clearings were observed with a transmission microscope under bright field or differential interference contrast with a 40x oil immersion lens. For GFP imaging, live embryos or seedlings were mounted in 0.5 strength Murashige and Skoog (MS) dissolved in water. SAMs were mounted in warm 0.8% low-gelling agar dissolved in 0.5 strength liquid MS. Confocal microscope observations were done on a SP2 or SP5 spectral detection confocal microscope (Leica) equipped with a 20x glycerol immersion (seedling root, seeds), 40x oil immersion (SAM) or 63 glycerol immersion lens (female gametophytes, embryos). Images were processed using Imaris (Bitplane, Zurich). Maximum partial projections of equivalent serial sections are shown. Adult plants were recorded with a DP3 Merrill digital camera (Sigma), and flower primordia with a MZFLII fluorescence stereomicroscope equipped with a DFC 420C digital camera (Leica).

Plant material and growth conditions
The ecotype Col-0 was used as wild type. Seeds were surface sterilized for 18 min in 5 % (v/v) bleach and 0.1 % (v/v) triton-X, washed three times in sterile ddH$_2$O and kept in the dark at 4 °C for a minimum of 2 days for stratification. Seeds were sown on 0.5 strength MS medium with 2 % (w/v) sucrose, 0.8 % (w/v) phytagar and 2 mM MES pH 5.6 containing the appropriate antibiotic or herbicide for selection. Seeds on selection plates were placed into a Percival plant incubator (CU-36L6/D Percival Scientific Inc., Perry IA, USA) with 22°C and a 14/10 h light/dark regime with 120 mmol m$^{-2}$ s$^{-1}$. To phenotype seedlings, plants were grown vertically on 12 cm square plates containing 10 mL of medium. To prevent desiccation of the plates, 1 mL of ddH$_2$O was added and the plates were sealed with parafilm. Seeds on vertical plates were placed into a Percival plant incubator (CU-36L6 Percival Scientific Inc., Perry IA, USA) with 22°C and 12/12 h light/dark cycles with 90 mmol m$^{-2}$ s$^{-1}$. For long-term growth, seedlings were transferred to soil and grown at 22 °C during the day, 20 °C at night with a 16/8 h photoperiod.

Plant transformation
Plant transformation was performed using *Agrobacterium tumefaciens* of the GV3101 strain (Clough and Bent, 1998). Inducible expression constructs were super transformed into TCSn::GFP or PUP14::PUP14-GFP transgenic lines.

Ethanol induction of transgenes
Ethanol was applied as follows to induce expression of amiRPUP14, CKI1, or AHK3 from the ethanol-inducible two-component system (Roslan et al., 2001). For phenotype assessments of seedlings, 1 mL of 1 % (v/v) ethanol was added to the bottom of the vertical plates 4 days after germination. For expression analyses, 7 day old seedlings grown on vertical plates were transferred to 6-well culture dishes with 3 mL of liquid medium (0.5-strength MS, 2 % (w/v) sucrose, 2 mM MES pH 5.7) with and without 1 % (v/v) ethanol) for 16 - 24 h.
Dishes were sealed with parafilm. Induction in adult plants was by watering with 1 % (v/v) ethanol every 4 days starting from bolting stage as described (Roslan et al., 2001). Embryo inductions were performed as described (Müller and Sheen, 2008). Controls shown are TCSn::GFP treated with ethanol in parallel to the experimental genotypes. Similar results were obtained with untreated TCSn::GFP, amiRPUP14 plants (see Fig 1o).

**Constructs**

For PUP14::PUP14-GFP, PUP14 encompassing the locus including the 2.3 kb upstream fragment was amplified from Col0 genomic DNA by PCR and cloned into the binary vector pCB302(Xiang et al., 1999) with the enhanced GFP coding sequence for C-terminal fusion, nopaline synthase 3’ untranslated region and ligation-independent cloning (LIC) (Aslanidis and de Jong, 1990) adaptors. For protoplast studies, PUP14, CKX2, CKX7, AHK4 genomic regions from translational start to stop were amplified from Col0 genomic DNA and annealed to LIC-modified expression vectors to yield 2HA or GFP C-terminal translational fusions. The artificial microRNA (amiR) constructs were designed with the Web MicroRNA Designer (http://wmd3.weigelworld.org), assembled by PCR amplification on the pRS300 as template (Schwab et al., 2006). Sequences of the amiRPUP14_1 and amiRPUP14_2 are TTATTTGCACAAAGTGTTCTG and TGTTGATAGGTATTTGCACGA, respectively. Both amiRPUP14 constructs caused similar phenotypes upon induction. Corresponding target sites in PUP14 are CAGAACAATTTGTGCAAATAC and TTGTGCAAATACCTATCAACA. The PCR amplicons were cloned into the LIC-modified DM7 vector (Zürcher et al., 2013). amiR-resistant versions of PUP14 (PUP14*) were constructed by site-directed mutagenesis of the amiRPUP14_2 target site to change all codons within the amiR target site to synonymous codons with overall comparable codon usage frequency. The PUP14* encompasses the PUP14 genomic region and was cloned into pCB302 by LIC. 35S::renillaLUC was used to normalise for transfection efficiency (Bielach et al., 2012) of TCS::LUC reporter assays, and W1 solution was supplemented with 15 mM sucrose. For reporter assays, transfected protoplasts were incubated over night, tZ or solvent at indicated concentrations were added, and protoplasts were harvested 90 min later for LUC measurements. For transport assays, transfections were scaled up according to needs and purified plasmids were transfected in 1:1 ratio between effector and empty plasmid. Transfected protoplasts were cultivated between 12 and 24 hrs at 22 °C in light (120 mmol m⁻² s⁻¹). Means and standard error of means of at least three independent experiments with three technical replications each are represented.
**Transport assays**

For protoplast transport assays, protoplasts were harvested at 100 rcf for 2 min and resuspended in percoll solution (0.5 M Sorbitol, 1 mM CaCl₂, 20 mM MES NaOH pH 5.6, 25% (v/v) percoll) and mixed with the same volume of glycine betaine solution (0.5 M glycine betaine, 1 mM CaCl₂, 20 mM MES NaOH pH 5.6) containing ¹⁴C-labelled tZ and ³H₂O. The final concentration of labelled tZ was 1 or 2 µM. For competition studies, unlabelled cold substrate was added in a 100-fold excess. Transport was stopped by centrifugation of samples on a percoll cushion after indicated time points. For scintillation counting, pelleted protoplasts were transferred into 3 mL of Ultima Gold™ (PerkinElmer AG, Schwerzenbach, Switzerland) and subjected to 10 min of disintegration counting of ¹⁴C and ³H. Three independent replicates of the uptake experiment were conducted with similar results, and means with standard deviations from one representative experiment with four technical replications are shown. For competition assays, mean values from three independent experiments with each four technical replications are shown. Indicated relative uptake was calculated as the radioactivity of ¹⁴C per radioactivity of ³H₂O normalized to the first time point (30 s). For seedling transport assays, twelve-day old induced seedlings were transferred to 0.5 x MS, 2% (w/v) sucrose, 2 mM MES and vacuum infiltrated for 5 min and twice 3 min. For each replicate >10 mg of plant material was used. Radiolabelled tZ was added to a final concentration of 2 µM in 2 mL. Seedlings were washed after indicated time points with excess volumes of cold 0.5 MS, 2% (w/v) sucrose, 2 mM MES on a Büchner funnel. Seedlings were dried on filter paper and transferred to 1.5 ml tubes containing 800 mL of 80% (v/v) ethanol and heated for 5 min at 95 °C. Samples were transferred into scintillation vials containing 3 mL Ultima Gold™ (PerkinElmer) and subjected to 2 min disintegration counting of ¹⁴C. Indicated relative uptake was calculated as the radioactivity per fresh weight normalized to the radioactivity per fresh weight at the first time point (1 min). Mean values from 3 independent experiments with each 4 technical replications are shown. For microsomal uptake experiments, 35S::PUP1, 35S::PUP14 and 35S::GFP were transiently expressed in N. benthamiana leaf tissue by Agrobacterium tumefaciens-mediated transfection and microsomes were prepared as described (Henrichs et al., 2012). For tZ-uptake experiments, ¹⁴C-labelled tZ was diluted into transport buffer (10 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10% sucrose, pH 7.6 with or without 5 mM ATP) and added to 300 µg of microsomes to yield a final concentration of 1µM labelled tZ. For substrate competition assays unlabelled substrate was included in the transport buffer at a 100-fold excess. After 10 s and 4 min of incubation at 20°C, aliquots of 100 µL were vacuum-filtered on Whatman™ NC45 filters (GE Healthcare, Little Chalfont, UK) and washed 3 times with 1 mL cold ddH₂O. Air-dried filters were objected to scintillation counting as described below. Indicated relative uptake was calculated as the radioactivity normalized to the first time point (10 s). Means and standard error of means of at least four independent experiments with three technical replications each are represented.

**qRT PCR analysis**

Quantification of relative gene expression was done by qRT-PCR on an Applied Biosystems 7500 Fast Real-Time PCR System using SYBR® Green PCR Master Mix (Applied
Biosystems, Life Technologies Europe B.V., Zug, Switzerland) or SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories AG, 1785 Cressier, Switzerland) according to manufacturer’s recommendation. Final primer concentrations were 400nM in a total volume of 20μl. The relative values of the transcripts were normalized to *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (eIF4A, At3G13920) levels. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Means and standard error of means of at least four independent experiments with three technical replications each are represented.
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