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Abstract

The Arabidopsis PARAQUAT RESISTANT1 (PAR1) was classified as L-TYPE AMINO ACID TRANSPORTER 4 (LAT4) based on a phylogenetic analysis of selected genes from *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Homo sapiens* that clustered LAT4 with four other members as a LAT family in Arabidopsis. *In-silico* analysis of the Arabidopsis LATs identified an amino acid permease domain and motifs that are common in amino acid transporters. However, their role in amino acid transport remained to be studied. A knockout mutant for *PAR1/LAT4* gene, reported here as *par1-5*, showed significantly altered growth compared to wild type on leucine-containing growth medium. Mutant *par1-5* seedlings showed reduced biomass compared to wild type on nitrate-containing Murashige and Skoog growth medium which was further reduced when grown on medium containing nitrate and leucine. Radio-labeled leucine uptake studies using leaf protoplasts and seedlings showed increased accumulation of leucine in *par1-5* mutants compared to wild type. Increased accumulation of leucine in *par1-5* was detected when seedlings or protoplasts were treated in the dark prior to isotopic feeding. These studies suggest that the PAR1/LAT4 protein, in addition to its ability to mediate paraquat and polyamine transport, possess leucine transport activity that is regulated by physiological conditions such as dark induction.

Keywords: amino acid; transporter, LAT4; L-type amino acid transporter; PAR1

Introduction

Plants take up nitrogen predominantly in the form of nitrate and ammonium that is assimilated into amino acids through the Glutamine Synthetase (GS)/ Glutamate Synthase (GOGAT) pathway [reviewed in (Lam et al. 1996)]. Some species also take up nitrogen in the form of amino acids and peptides [reviewed in (Nasholm et al. 2009)]. Assimilated nitrogen is transported from the source to sink tissues in the form of amino acids. The transport and distribution of these amino acids requires them to cross plasma and organellar membranes in various organs and tissues. Integral membrane transporter proteins, known as amino acid transporters, mediate the transport of amino acids across membranes. To date, over 60 genes in *Arabidopsis thaliana* have been annotated as amino acid transporters (Rentsch et al. 2007; Tegeder 2012). A phylogenetic analysis categorized these plant amino acid transporters into three super families: Amino acid Transporter Family1 (ATF1), Amino acid Polyamine Choline (APC), and Major Facilitator Superfamily (MFS) (Wipf et al. 2002). According to this classification, Arabidopsis LATs (LAT1, At5g05630; LAT2, At3g13620; LAT3, At1g31820; LAT4, At1g31830; and LAT5, At3g19553) were classified in the L-type Amino acid Transporter (LAT) family within the APC superfamily (Wipf et al. 2002). In a later phylogenetic study, these five members of the Arabidopsis LAT family were classified as Polyamine H⁺-Symporters (PHSs) (Okumoto and Pilot 2011).

Recent experimental studies renamed Arabidopsis LAT1 as Resistant to Methyl Viologen1 (RMV1) and Polyamine Uptake Transporter 3 (PUT3), and LAT4 as Paraquat Resistant1 (PAR1) and Paraquat-Resistant 2 (PQR2) (Dong et al. 2016; Fujita et al. 2012; Li et al. 2013; Mulangi et al. 2012). *In planta* studies suggested that Arabidopsis RMV1/LAT1/PUT3 mediates paraquat (a structural analog of polyamine) and cadaverine (a diamine derivative of lysine) import (Fujita et al. 2012; Sagor et al. 2016). Yeast complementation studies showed that this transporter also mediates polyamine and thiamine import (vitamin B1) (Martinis et al. 2016; Mulangi et al. 2012). Arabidopsis PAR1/LAT4/PQR2 was shown to mediate paraquat accumulation into chloroplast, and to have a role in anti-oxidative stress responses and ABA signaling (Dong et al. 2016; Li et al. 2013).

In this report, through *in-silico* and *in-planta* studies, we demonstrate that PAR1/LAT4/PQR2 mediates leucine transport and this activity is regulated by physiological conditions such as dark cycle.

Materials and methods

In silico analysis of PAR1/LAT4-family proteins

Amino acid sequences of the PAR1/LAT4-family proteins were taken from the plant membrane protein database (<http://aramemnon.botanik.unikoeln.de/>) and all other protein sequences used in alignment and motif search were taken from the NCBI protein database. Molecular weight and isoelectric point was calculated using Vector NTI 10 (supplementary table S1). Subcellular localization was predicted using AtSubp online prediction tool (Kaundal et al. 2010). InterPro scan was carried out using the InterPro protein sequence analysis and classification

online tool with the default settings (<http://129.175.105.74/genomics/lbmgeiprscan.html>). The transmembrane domain prediction was performed using the TMHMM v.2.0 online tool (<http://www.cbs.dtu.dk/services/TMHMM/>). The cartoon representation of PAR1/LAT4 (Fig. 1a) was produced using an online tool TMRPres2D (<http://bioinformatics.biol.uoa.gr/TMRPres2D/>). The conserved domain search was performed using the NCBI online conserved domain search tool (<http://www.ncbi.nlm.nih.gov/>). Amino acid sequence alignment featuring sequence identity, PFAM analysis, and the amino acid permease domain cartoons (Fig. 1b) were produced using the Vector NTI 10 AlignX tool. A motif search in the 26 related proteins including *Arabidopsis thaliana*, *Oryza sativa*, *Homo sapiens*, and *Mus musculus* LATs (Fig. 1c) was carried out using the online tool Multiple Em for Motif Elicitation (<http://meme-suite.org/tools/meme>) (Bailey and Elkan 1994), with the default settings except the number of motif which was changed from 3 to 6. Unknown motifs identified in the LAT proteins were searched against *Arabidopsis thaliana*, *Oryza sativa*, *Homo sapiens*, and *Saccharomyces cerevisiae* protein databases using online Motif Alignment and Search Tool (<http://meme-suite.org/tools/mast>) (Bailey and Gribskov 1998). The C-terminal FWC motif search in the Arabidopsis LAT protein sequences was carried out by aligning them with yeast general amino acid permease ScGAP1(CAA82113) sequence using Vector NTI 10 AlignX tool.

Plant growth and analysis

The Columbia-0 ecotype of *Arabidopsis thaliana* was used in all studies. All plants (unless otherwise mentioned) were grown on well-aerated fertilized soil in the growth chamber under a 16/8 hours of light/dark regime under $\sim 120 \mu\text{E m}^{-2} \text{sec}^{-1}$ light intensity at 23°C day time temperature. Soil was a commercially available Sunshine Mix formulated with peat moss, coarse perlite, organic nutrient, gypsum, and dolomitic limestone (Terralink Horticulture Inc., Abbotsford, BC, Canada). Plant Prod 20-20-20 fertilizer (Master Plant-Prod Inc., Brampton, ON, Canada) was dissolved in water as per manufacturer's recommended dose and applied to soil during pot preparation.

For phenotypic studies nitrogen-free half-strength MS medium (Murashige and Skoog 1962) was prepared using stock solutions of macronutrients, micronutrients, and vitamin mixture at a molar concentration described in the commercially available MS basal salt mixture (Catalog number ICN2623120, Fisher Scientific, ON, Canada) without ammonium nitrate. To obtain desired nitrogen concentration (3mM nitrate and 1mM or 2mM leucine, Fig. 2), potassium nitrate and L-leucine (Sigma-Aldrich, Oakville, ON, Canada) were added to the MS-agar medium. For each growth condition, both genotypes were grown on the same plate with 3-5 plate replications. Due to poor growth of the *par1-5* seedlings on 2mM leucine, for each growth condition two seedlings per genotype were weighed per unit sample to minimize technical error. Data analysis and significance tests (two-way t-test) were performed using the software PRISMv.5. The relative percentage of growth in *par1-5* seedlings was calculated based on setting the biomass obtained by the corresponding WT at 100%.

Isolation of the mutant par1-5

An *Arabidopsis* line (SALK_119707C) with a T-DNA insertion in the *PAR1/LAT4* locus (supplementary figure. S1a) was obtained from the Arabidopsis Biological Resource Centre (Alonso et al. 2003). This line was previously confirmed for being resistant to paraquat, lacking detectable *PAR1/LAT4* transcripts, and was named *par1-5* (Li et al. 2013). We used the *par1-5* line in all studies after confirming by PCR and Reverse Transcriptase (RT)-PCR that it was a homozygous mutant that lacked detectable *PAR1/LAT4* transcripts (supplementary figure. S1b). Primers used for RT-PCR were specific to the 5' and 3'-untranslated regions of the *PAR1/LAT4* locus with sequences as follows: forward 5'-CCGTTTAAAATCTGGTTGACG-3' and reverse 5'-GACGAGGGAAATAACTAAG-3'. For RT-PCR, total RNA was extracted, using the RNeasy Plant Mini Kit (Qiagen, Darmstadt, Germany), from 100mg of flash frozen leaf tissues as per manufacturer's instruction. To remove contaminating DNA, RNA was treated using Ambion's DNA-free kit. The quantity and quality of RNA was checked by using Nanodrop and Bioanalyzer (Agilent 2100 Bioanalyzer). One-step RT-PCR using *PAR1/LAT4* and *Actin2* specific primers in the same reaction vial was performed using SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity (Catalog No. 12574-030). *Actin2* was used as a technical control and wild type plant tissues were used as a positive control. The reactions were set up as per manufacturer's instruction and the program for the thermal cycler was as follows: cDNA synthesis at 55°C for 30 minutes, pre-denature at 94°C for 2 minutes, 40 cycles of: denature at 94°C

for 15 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 1 minute, final extension at 68°C for 5 minutes.

Quantitative RT-PCR for organ specific expression analysis

For the organ specific quantitative RT-PCR (Fig. 3), RNA was extracted from roots, stems, rosette leaves, cauline leaves, senescing leaves, flowers, siliques, and whole plant tissues separately. Total RNA extraction, *DNase* treatment, quantification, and quality check was carried out as described above. All samples were adjusted to a final concentration of 50ng.µl⁻¹. For each sample, 2.5µg of total RNA was used in the cDNA synthesis with oligo-dT and random primers (Invitrogen). SuperScript II *Reverse Transcriptase* (Invitrogen) was used in the reverse transcription reaction for 50 minutes at 42°C. *Elongation Factor 1α* (*EF1α*) was used as an endogenous control. A TaqMan Gene Expression Assay (assay ID: At02351062_sH for *PAR1/LAT4*; assay ID: At02337969_g1 for *EF1α*) was used in the PCR amplification with the following program: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantitation was calculated using a 7500 Fast System SDS software (Applied Biosystems). Each replication is an average of three reactions and each data point is an average of three biological replications.

In vivo ¹⁴C-leucine and ³H-leucine uptake study

Radio-labelled L-leucine was used in all studies. The ¹⁴C-leucine and ³H-leucine uptake studies were carried out using homozygous *par1-5* seedlings compared to wild type as biological control. Three-week old seedlings grown on fertilized peat-based growth medium were used in the uptake studies. For dark treatment,

seedlings in the growth tray were covered with air-permeable black cover and kept in the dark for 27 hours prior to use. In all studies, a mixture of ^{14}C -leucine and ^3H -leucine was used with the isotopic label in the alpha-carbon and amino group, respectively. Radio-labelled leucine was added to an uptake medium (liquid MS basal salts without sucrose and nitrogen supplement) with cold leucine to a final concentration of $10\mu\text{M}$. A total of 15ml uptake medium was used for each replication sample. Seedlings were removed from the uptake medium at two-hour time point. Roots were rinsed three times in sterile water for a total of 10 minutes. Roots and shoots were separated immediately after washing and stored directly in glass scintillation vials. Samples were prepared for counting radioactivity using a protocol similar to the one described in (Pratelli et al. 2010). Tissues were dried at 70°C overnight and weighed. Samples were digested with 1ml of 5% NaClO overnight at room temperature. Vials were kept open for at least one hour under a fume hood before adding 5ml Hionic-Fluor (Ultima Gold XR, Perkin Elmer Inc., Waltham, MA, USA) to each vial. Radioactivity in roots and shoots of the same plant were combined to obtain total radioactivity in each sample. Data was presented as leucine concentration in $\text{pmol.mg}^{-1}\text{DW.hr}^{-1}$ (Fig. 4a-d).

To prepare leaf protoplasts, rosette leaves of similar size and age were collected from two-month old plants. For each genotype, 30 leaves were used from 10 plants. Plants were kept in the dark for 24 hours before leaves were collected. Protoplast preparation was performed in the dark using a protocol described by (Yoo et al. 2007). For size analysis, photos of protoplasts were taken using a

ZEISS Scope A1 light microscope attached to an optronics camera. All photos were taken under the 20x magnification. For each genotype, 100 cells were counted from five photographs from three individual plants. Cell size was measured using PictureFrame (TM) Software Application version 2.3. The program was calibrated using a stage micrometer under the 20x magnification prior to measuring the diameter of the protoplasts. Data was presented as cell size in $\mu\text{m}.\text{cell}^{-1}$ (Fig. 4e).

For the ^{14}C -leucine uptake study, cells of each genotype were counted with a haemocytometer and the final concentration was adjusted to $4 \times 10^5 \text{ cells.ml}^{-1}$. The uptake medium was a MMG solution (4mM Mes, 0.4M Mannitol, and 15mM MgCl_2 , pH 5.6) (Yoo et al. 2007), with added ^{14}C -leucine (618 pmol.ml^{-1}) and cold leucine to a final concentration of 10 μM . After one hour of uptake under light, cells were rinsed three times with MMG solution before radioactivity was counted in DPM in 4×10^5 cells in each replication vial. Data was presented as leucine concentration in $\text{pmol}.10^5 \text{ cells.hour}^{-1}$ (Fig.4f)

Free amino acid extraction and HPLC

Free amino acids were extracted using a protocol described in (Tuin and Shelp 1994), from the roots and shoots of WT and *par1-5* seedlings, grown under 16/8 light/dark cycle with or without sucrose in the growth medium (Fig. 5a). For the HPLC analysis of free amino acids, freshly weighed samples were ground in 1.5 ml extraction buffer (methanol: chloroform: water at 65:25:10 with 20 nmol.ml^{-1} internal standard (norvaline and sarcosine) added to each sample. Samples were vortexed and centrifuged to separate the aqueous phase. Chloroform and water

were added to the aqueous phase at a ratio of 1:1.5:5. Samples were mixed and centrifuged, and the upper aqueous phase was passed through a MILLEX GX 0.22µm filter (UFC30GV0S) followed by a MILLIPORE BIOMAX 5KMWL MEMBRANE filter (UFV5BCC00). The final filtrate was used in the HPLC analysis using a ZORBAX Rapid Resolution HT Eclipse Plus C18 1.8 µm 3.0x100 mm column. The HPLC analysis was carried out using a protocol described by Agilent Technologies (Woodward et al. 2007). An Agilent Technologies 1200 series automated liquid chromatography system was used to analyze the samples. Samples were derivatized pre-column with OPA (o-Phthalaldehyde) and FMOC (9-Fluorenylmethyl chloroformate). All chromatographs were processed in the Agilent Chemstation software suite. The area under the signal peaks was calibrated by the standard calibration curve and normalized by the internal standard norvaline and sarcosine concentration, then all the concentration data were back calculated to the concentration in the samples in nmol.gram⁻¹FW of shoot. Amino acid concentration in roots was expressed in nmol.2seedlings⁻¹ to reflect a relatively accurate concentration of amino acids identified in the root tissues and data for the two genotypes was compared accordingly.

Results

In-silico characterization of PAR1/LAT4 indicates its role in amino acid transport

In Arabidopsis, PAR1/LAT4 is a 495-aa protein with a molecular weight of 54.8 kDa. A secondary structure analysis predicted 10 transmembrane domains in the PAR1/LAT4 protein with both the N- and C-termini in the cytoplasmic side (Fig.

1a). An InterPro scan identified PAR1/LAT4 as an amino acid/polyamine transporter 1 (IPR002293) family protein containing PF13520 (amino acid permease) and PTHR11785:SF78 (glutamate/GABA antiporter) domains. InterPro analysis predicted its biological process to be amino acid transmembrane transport (GO: 0003333), and its molecular function to be amino acid transmembrane transport activity (GO: 0015171). Similar features were also identified in the other four members of the PAR1/LAT4-family (supplementary table S1). A domain search identified amino acid permease domain PF13520 conserved in all five members of the PAR1/LAT4-family proteins (Fig. 1b). A NCBI blast search against the PDB protein database using BLASTP2.5.1 (Altschul et al. 1997) identified *E. coli* L-arginine/Agmatine antiporter (AdiC, pdb/5J4/A) as the closest protein with 25% sequence identity. PAR1/LAT4 shares a number of conserved regions with its family members (supplementary figure S2). A motif search in the PAR1/LAT4 protein along with related proteins including LATs in Arabidopsis, *Oryza sativa*, *Homo sapiens*, and *Mus musculus* identified six common motifs in these proteins (Fig. 1c). Sequences of these motifs were searched again in the Arabidopsis, *Oryza sativa*, and *Homo sapiens* protein databases to see which known proteins carry these motifs, and if they can be associated with a common function. These motifs were identified in a number of other proteins including AtCAT1-9, AtBAT1, OsBAT1, OsPUT1, and HsSLC7A7 among the annotated/known proteins. These motifs searched in the *Saccharomyces cerevisiae* protein database identified proteins of diverse family and function, however, motif 1 was common in transporter proteins such as

ScMUP3, ScAGP2, ScLYP1, ScAGP1, ScDIP5, ScALP1, ScCAN1, ScBAP3, ScTAT1, ScMUP1, ScGNP1, ScHNM1, ScAGP3, ScSSY1, ScTPO1, ScTPO5, ScGAP1, ScHIP1 all of which are associated with amino acid and/or polyamine transport. When OsLATs were excluded in the motif search, motifs identified (supplementary figure S3) were different from what is shown in Fig. 1c. The new motif 1, when searched in the Arabidopsis protein database, was also found to be conserved in AtCAT1-9, AtBAT1, AtAAP2, 4, and 6.

A FWC signature motif in the PM_{asseq} domain at the C-termini, conserved in yeast amino acid permeases, that is responsible for plasma membrane association in amino acid transporters localized in the plasma membrane (Popov-Celeketic et al. 2016) was absent in all five LAT proteins.

Loss-of-function of PAR1/LAT4 causes increased sensitivity to exogenous leucine

In our growth assays, *par1-5* seedlings were visibly smaller than the corresponding WT seedlings in MS medium that contained nitrate, and in peat-based growth medium that contained both organic and inorganic forms of nitrogen (supplementary figure S1c-d). However, growth studies with branched-chain amino acids (BCAAs) such as leucine, isoleucine, or valine as sole source of nitrogen did not support sufficient growth, to allow a comparison between the two genotypes. Thus, we used gradually increasing concentration of leucine at 0mM, 1mM, and 2mM with 3mM nitrate in the MS-agar medium and observed growth of *par1-5* in comparison with WT. Mutant *par1-5* seedlings produced 55% biomass compared to WT on MS medium containing 3mM nitrate and 0mM leucine (Fig. 2a, b). In the presence of 1mM leucine in the growth medium, *par1-*

5 seedlings produced 48% of the biomass compared to the corresponding WT seedlings (Fig. 2b). In the presence of 2mM leucine, *par1-5* seeds had low germination rates compared to the corresponding WT seedlings. In the presence of 2mM leucine, *par1-5* seedlings showed a significant 98% reduction in biomass compared to WT on the same plate.

PAR1/LAT4 transcripts are present in all organs in maturing plants

Analyzing the *PAR1/LAT4* expression using the public database Arabidopsis eFP Browser (At-TAX) showed *PAR1/LAT4* mRNA in leaf, stem, flower, silique, whole seedling, and shoot apex. At a tissue-specific level, the Arabidopsis eFP Browser showed *PAR1/LAT4* expression in the leaf mesophyll and guard cells, stem epidermis, and fruit carpels (<http://bbc.botany.utoronto.ca/efp/>). Li et al. (2013) demonstrated ubiquitous expression of *PAR1/LAT4* throughout Arabidopsis development. We conducted an organ-specific quantitative RT-PCR to see the *PAR1/LAT4* transcript level in various organs. The *PAR1/LAT4* transcripts were detected in all organs with a higher abundance in the rosette leaves (Fig. 3).

Dark treatment causes leucine accumulation in par1-5

A radio labeled leucine uptake study showed no variation in leucine uptake between *par1-5* and WT (Fig. 4a, b). Studies were repeated with seedlings treated in the dark. When seedlings were grown in the dark for 27 hours prior to the isotopic feeding, *par1-5* seedlings accumulated significantly more leucine as measured by the ^{14}C - and ^3H - accumulation, compared to WT (Fig. 4c, d). Increased accumulation of leucine by *par1-5* was also observed in leaf protoplasts

of dark-treated plants. The average diameter of leaf protoplasts in WT was 0.51 μm , while it was 0.38 μm in *par1-5*, a 25% decrease (Fig. 4e). However, WT protoplasts accumulated 43.5 $\text{pmol} \cdot 10^5 \text{cells}^{-1} \cdot \text{hr}^{-1}$ ^{14}C -leucine, whereas *par1-5* protoplasts accumulated 52.7 $\text{pmol} \cdot 10^5 \text{cells}^{-1} \cdot \text{hr}^{-1}$, a 21% increase (Fig. 4f).

PARI/LAT4 shows a cumulative growth reduction in the absence of sucrose

To test whether the reduced availability of sucrose could affect *par1-5* growth, an assay was used with the level of nitrogen kept constant but sucrose supplement altered. In the presence of 1% sucrose in the growth medium, *par1-5* showed 32% and 12% reduction in total biomass and root length, respectively, compared to the corresponding WT (Fig. 5). This was consistent with previous results that showed reduced biomass of *par1-5* compared to WT in MS medium and in soil (supplementary figure. S1, Fig. 2). Without sucrose in the growth medium, reductions in biomass and root length in *par1-5* were 51% and 52%, respectively, compared to the corresponding WT (Fig. 5a-c). In the absence of sucrose, *par1-5* showed a significant 54% and 57% reduction in biomass and root length, respectively, compared to *par1-5* growth with sucrose while no significant growth reduction was observed in WT grown with or without sucrose (Fig. 5a-c).

In the absence of sucrose, par1-5 accumulates free amino acids in the shoots

Free amino acids were analyzed in the roots and shoots of WT and *par1-5* seedlings, grown with or without sucrose in the growth medium (Fig. 5a). On medium without sucrose, the total free amino acid content in *par1-5* seedlings was 176% higher in the shoots and 80% lower in the roots compared to WT plants (Table 1). With sucrose in the growth medium, *par1-5* showed no significant

difference in the total free amino acid content in the roots and shoots compared to that in the corresponding WT (Table 1). The addition of sucrose increased the amino acid content in WT shoots by 67% and decreased it in *par1-5* shoots by 41% compared to the same genotype grown without sucrose. In roots, addition of sucrose caused an increase in the free amino acid content in WT and *par1-5* by 135% and 809%, respectively, compared to that in the same genotype grown in the absence of sucrose (Table 1). In the absence of sucrose in the growth medium, where the loss of PAR1/LAT4 function was evident by reduced biomass (Fig. 5a-c), *par1-5* accumulated free amino acids in the shoots (Table 1). The accumulation and distribution of free amino acids in *par1-5* was significantly altered compared to WT when sucrose supplement was altered in the growth medium.

Discussion

PAR1/LAT4-family transporters interact with a broad range of substrates

Phylogenetically, PAR1/LAT4 belongs to the L-type Amino acid Transporter (LAT) family and/or Polyamine H-Symporter (PHS) family (Okumoto and Pilot 2011; Wipf et al. 2002). PAR1/LAT4-family proteins conserve domains and motifs that are common in known amino acid and/or polyamine transporters (Fig. 1b-c). The predicted biological process and molecular function for all five PAR1/LAT4-family members were amino acid transport and amino acid transport activity (supplementary table S1). While *in-silico* characterization provided a strong suggestion that PAR1/LAT4-family has a role in amino acid transport, experimental evidences suggest that these transporters interact with a broad range

of substrates. Loss of PAR1/LAT4 function showed increased paraquat tolerance compared to WT suggesting that PAR1/LAT4 mediates paraquat transport (Dong et al. 2016; Li et al. 2013). Another member of this family, RMV1/LAT1 mediated increased polyamine uptake when over-expressed and increased paraquat tolerance when knocked out (Fujita et al. 2012), showed increased cadaverine sensitivity when over-expressed (Sagor et al. 2016) and mediated thiamine transport (Martinis et al. 2016). In this report we have demonstrated that PAR1/LAT4 plays a role in leucine transport. We investigated the role of PAR1/LAT4 in leucine transport by growing *par1-5* on MS medium containing nitrate and leucine, as well as, by conducting radio-labelled leucine uptake studies. We were particularly interested to investigate if *par1-5* shows variation in leucine uptake because PAR1 was initially classified as LAT4 and LATs in animal have higher affinity for BCAAs (Mastroberardino et al. 1998; Verrey 2003). Moreover, BCAAs were previously shown to produce significant growth inhibition when used as a source of nitrogen, making them a good substrate to study mutants for amino acid transporters (Chen et al. 2010; Forsum et al. 2008; Miflin 1969; Miflin and Cave 1972). We conducted studies with gradually increasing concentration of leucine with 3mM nitrate supplement to investigate growth variation in *par1-5* compared to WT. Leucine was used as a representative of the BCAA group, and 3mM nitrate was used because this concentration was sufficient to support seedling growth on plates for a period of up to four weeks. Mutant *par1-5* seedlings were hypersensitive to the presence of leucine in the growth medium (Fig. 2). In radio-labelled leucine uptake studies, *par1-5* showed

increased accumulation of leucine in leaf mesophyll cell protoplasts and in seedlings upon dark treatment (Fig. 4c, d, f). Our studies along with published reports indicate that PAR1/LAT4-family transporters have a broad substrate specificity including amino acid leucine, polyamine, paraquat, cadaverine, and thiamine. Amino acid transporters in both plant and animal systems, even when specific to only amino acids, are broadly specific, although they have a higher or lower affinity for certain types of amino acids such as acidic, basic, or neutral.

PAR1/LAT4 harbors a complex transport mechanism

LATs in animals exchange intra-cellular amino acids for extra-cellular amino acids at a 1:1 stoichiometry (Verrey 2003). Animal LATs require binding with a heavy-chain glycoprotein subunit to become functional (Mastroberardino et al. 1998; Pfeiffer et al. 1999; Rossier et al. 1999). Although Arabidopsis PAR1/LAT4-family proteins share low (~20%) sequence similarity with animal LATs (Fujita and Shinozaki 2014), the possibility that PAR1/LAT4 and members of its family interact with other subunits to localize and function properly remains to be tested. While RMV1/LAT1 was confirmed to be localized to the plasma membrane (Fujita et al. 2012), when GFP was fused to the c-terminus of PAR1/LAT4 and LAT3, they were localized to the Golgi apparatus and endoplasmic reticulum, respectively (Li et al. 2013). Knocking out and over-expressing *RMV1/LAT1* in Arabidopsis caused reduced, and increased paraquat uptake, respectively, compared to the control. However, in the polyamine uptake studies, *RMV1/LAT1* over-expression showed increased uptake while the mutant showed no difference compared to the control (Fujita et al. 2012). In studies with

PAR1/LAT4, the *par1* mutant showed reduced accumulation of paraquat in the chloroplast but no difference in paraquat uptake compared to the control (Li et al. 2013). In this report, the PAR1/LAT4 affected leucine transport in seedlings and leaf mesophyll cells when plants were treated in the dark (Fig. 4c, d, f). Dong et al (2016) demonstrated that a PAR1/LAT4 mutant's (*pqr2*) ability to provide paraquat tolerance is compromised when co-treated with paraquat and abscisic acid (Dong et al. 2016). While mutant *par1-5* showed reduced paraquat accumulation, it showed increased leucine accumulation, an opposite transport direction for these two substrates.

PAR1/LAT4 transports leucine upon dark induction

In our studies PAR1/LAT4 facilitated leucine transport *in vivo*. This ability was detected when plants were exposed to a prolonged dark period. Dark treatment was used to induce expression of the *PAR1/LAT4* gene in WT seedlings because microarray studies indicated that *PAR1/LAT4* transcript expression is highly induced under reduced light intensity or in continuous dark (Covington and Harmer 2007; Edwards and Millar 2007; Michael et al. 2008). Compared to WT, *par1-5* seedlings and leaf protoplasts showed increased accumulation of ¹⁴C-leucine and ³H-leucine (Fig. 4c, d, and f). Although, dark treatment was primarily used to induce *PAR1/LAT4* transcript level, and it caused significantly increased accumulation of leucine or its metabolic product in the *par1-5* seedlings and protoplast, subsequent expression analysis showed ubiquitous and constitutive expression of *PAR1/LAT4* in all organs throughout Arabidopsis development (Fig. 3) (Li et al. 2013). Increased accumulation of leucine in *par1-5* seedlings upon

dark treatment was more likely due to factors (triggered by dark treatment) that influenced the transport activity of PAR1/LAT4 rather than producing increased number of transcript. Thus, we considered testing other factors such as sucrose level that are altered during the dark cycle in plants. We have demonstrated that lack of sucrose in the growth medium causes a biomass reduction in *par1-5* seedlings (Fig. 5a-c), and significantly alters the accumulation and distribution of free amino acids in *par1-5* compared to WT (Table 1). These studies suggest that PAR1/LAT4 plays a role in transporting leucine, and this activity is influenced by dark cycle- derived physiological conditions such as low sucrose concentration.

Conclusion

This report showed PAR1/LAT4's ability to facilitate leucine transport and previous reports demonstrated its ability to import paraquat into chloroplast (Li et al. 2013). Depending on the substrate type and physiological conditions, PAR1/LAT4 showed transport characteristics which are similar to an antiporter.

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Table 1. Free amino acid analysis in the roots and shoots of *parl-5* seedlings in comparison with WT grown in the presence or absence of 1% sucrose in the growth medium.

	1% sucrose						0% sucrose					
	Shoot (nmol.g ⁻¹ FW)			Root (nmol.2seedling ⁻¹)			Shoot(nmol.g ⁻¹ FW)			Root (nmol.2seedling ⁻¹)		
Free AA	WT	<i>parl-5</i>	% Change	WT	<i>parl-5</i>	% Change	WT	<i>parl-5</i>	% Change	WT	<i>parl-5</i>	% Change
Asp	1635.98	2369.75	44.85	9.67	8.72	-9.90	1230.56	934.05	-24.10	4.13	0.22	-94.72
Glu	4086.46	3753.03	-8.16	32.20	26.89	-16.47	2252.21	3107.20	+37.96	11.78	0.45	-96.16
Asn	247.24	243.41	-1.55	7.40	5.42	-26.71	138.43	322.83	+133.20	2.60	0.28	-89.18
Ser	1842.05	1379.72	-25.10	18.98	12.85	-32.32	581.71	3187.72	+447.99*	5.26	0.96	-81.67
Gln	2126.76	1739.75	-18.20	45.52	32.53	-28.55	722.59	1268.02	+75.48	15.65	1.46	-90.66
His	39.59	37.63	-4.96	0.90	0.72	-20.13	32.67	68.49	+109.65	0.39	0.04	-90.46*
Gly	242.60	124.99	-48.48	2.74	1.98	-27.64	55.70	900.14	+1516.10	1.61	0.80	-50.07
Thr	583.26	466.22	-20.07	8.63	6.06	-29.84	192.27	825.91	+329.56*	2.81	0.35	-87.68
Arg	42.42	46.74	10.18	1.01	0.77	-23.49	41.87	134.07	+220.18*	0.53	0.13	-75.85
Ala	497.98	442.68	-11.10	5.13	3.82	-25.54	256.64	902.32	+251.58*	2.45	0.49	-79.84
Tyr	28.40	34.98	23.15	0.71	0.59	-16.07	43.37	225.40	+419.69*	0.45	0.24	-46.84*
Cys	4.81	10.45	117.23	0.19	0.15	-21.39	17.43	160.11	+818.34**	0.21	0.15	-25.69
Val	1785.28	2201.67	23.32	42.28	33.96	-19.69	2119.88	8235.10	+288.47*	25.66	8.11	-68.40
Met	22.81	28.10	23.21	0.43	0.34	-22.37	34.82	131.32	+277.13*	0.27	0.12	-56.36*
Trp	15.80	19.46	23.13	0.24	0.21	-10.52	20.89	97.51	+366.83*	0.20	0.08	-57.50*
Phe	39.98	36.13	-9.63	0.58	0.53	-8.44	42.17	247.73	+487.52**	0.34	0.19	-44.75
Ile	50.29	54.91	9.19	1.55	1.21	-22.08	56.07	301.07	+436.94*	0.84	0.29	-65.79*
Leu	48.85	57.11	16.92	2.10	1.77	-15.87	52.71	294.73	+459.19*	1.09	0.25	-77.36
Lys	26.16	39.96	52.77	0.81	0.67	-16.58	121.68	773.71	+535.84	0.85	0.71	-16.49
Total	13366.72	13087	-2.09	181.08	139.19	-23.13	8013.68	22117.43	176.00*	77.11	15.32	-80.13*

*, Pvalue <0.05; **, Pvalue <0.01;***, Pvalue <0.001. Each data is an average of three biological replications with two seedlings per replication.

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Figure legends

Fig. 1. *In-silico* characterization of the Arabidopsis PAR1/LAT4-family proteins.

- a. A cartoon representation showing the 10 transmembrane domains in PAR1/LAT4 protein with both C- and N-termini in the cytoplasmic side.
- b. Amino acid permease domain PF13520 is conserved in all five members (LAT1-5) of the Arabidopsis PAR1/LAT4-family. Grey bar shows the length of amino acid sequence and red bar shows the position and extent of the domain in each protein.
- c. A block diagram of non-overlapping tiling of motifs found common in the Arabidopsis PAR1/LAT4- family and related proteins in *Oryza sativa*, *Homo sapiens*, and *Mus musculus* identified using MEME motif search tool. Each colored box represents a specific motif in the protein. The order of the motifs indicates their position within each protein sequence.

Fig. 2. Mutant *par1-5* shows increased sensitivity to exogenous leucine

- d. Mutant *par1-5* seedlings are more sensitive to exogenous leucine compared to WT. Variable concentrations of L-leucine were added to 0.5xMS medium with 3mM nitrate as the source of nitrogen.
- e. A quantitative representation shows significantly increased sensitivity of the *par1-5* mutant seedlings compared to WT to the presence of variable concentrations of leucine measured by the reduction in fresh-weight of biomass. The relative percentage of growth was calculated assuming the biomass obtained by the corresponding WT as 100%. Error bar represents standard deviation of

three to five biological replications with two seedlings per replication. Photos shown in panel A were taken when the seedlings were two-week old. Each growth condition had three or more plate replications. Each growth experiment was repeated more than twice with similar results. *, Pvalue <0.05; **, Pvalue <0.01; ***, Pvalue <0.001)

Fig. 3. Organ specific expression analyses in mature plants through quantitative RT-PCR detects *PAR1/LAT4* transcripts in all organs.

The relative quantitation was calculated using an average expression of *PAR1/LAT4* in whole plants as a calibrator and *EF1 α* as an endogenous control. The error bar represents the average difference between the RQ_{min} and RQ_{max} of three biological replications each with three technical replications.

Fig. 4. Radio-labeled leucine uptake for two hours shows increased accumulation of L-leucine measured by ¹⁴C- and ³H- signal in *par1-5* compared to WT when seedlings were treated in dark for 27 hours prior to isotopic feeding.

- a. Seedlings exposed to light for six hours post night-time show no variation in ¹⁴C- accumulation between WT and *par1-5*.
- b. Study as in 'a' shows no variation in ³H- accumulation between WT and *par1-5*.
- c. Seedlings, kept in dark for 27 hours prior to isotopic feeding, show significantly increased accumulation of ¹⁴C- in *par1-5* compared to WT.
- d. Study as in 'c' shows significantly increased accumulation of ³H- in *par1-5* compared to WT.

e. Diameter measurement shows significantly smaller size of leaf protoplasts in *par1-5* compared to WT. N=100.

f. Leaf protoplasts of *par1-5* show significantly increased accumulation of ^{14}C - compared to WT. N= 3 to 5 vials with 4×10^5 cells in each vial from 10 independent plants.

In studies a-d Error bar represents standard deviation of three biological replications. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001.

Fig. 5. In the absence of sucrose in the growth medium, *par1-5* shows cumulative growth reduction compared to WT

a. Mutant *par1-5* shows reduced biomass and root length compared to WT in the presence of 1% sucrose in the growth medium. In the absence of sucrose, *par1-5* seedlings show further reduction in both biomass and root length compared to those in the presence of sucrose.

b. A quantitative representation of the reduction in biomass in *par1-5* compared to WT in the presence or absence of sucrose in the growth medium. N = 4, with two seedlings per replication.

c. A quantitative representation of the reduction in root-length in *par1-5* compared to WT in the presence or absence of sucrose in the growth medium. N= 10.

The percentage of root-length or biomass was calculated by assuming the average growth obtained by the corresponding WT as 100%. The root length measurement and plate photos were taken when the seedlings were nine-day old.

For the dry weight of biomass, two-week old seedlings were dried at 70°C for four hours before taking weight. All statistical significance tests were carried out on average value of the biological replications derived from raw data. Error bar represents standard deviation. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001.

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Fig. 1

Fig. 2

Fig. 3

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