Identification of a plastidial phenylalanine exporter that influences flux distribution through the phenylalanine biosynthetic network

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In addition to proteins, L-phenylalanine is a versatile precursor for thousands of plant metabolites. Production of phenylalanine-derived compounds is a complex multi-compartmental process using phenylalanine synthesized predominantly in plastids as precursor. The transporter(s) exporting phenylalanine from plastids, however, remains unknown. Here, a gene encoding a Petunia hybrida plastidial cationic amino-acid transporter (PhpCAT) functioning in plastidial phenylalanine export is identified based on homology to an Escherichia coli phenylalanine transporter and co-expression with phenylalanine metabolic genes. Radiolabel transport assays show that PhpCAT exports all three aromatic amino acids. PhpCAT downregulation and overexpression result in decreased and increased levels, respectively, of phenylalanine-derived volatiles, as well as phenylalanine, tyrosine and their biosynthetic intermediates. Metabolic flux analysis reveals that flux through the plastidial phenylalanine biosynthetic pathway is reduced in PhpCAT RNAi lines, suggesting that the rate of phenylalanine export from plastids contributes to regulating flux through the aromatic amino-acid network.
he aromatic amino acid L-phenylalanine is a vital constituent of proteins in all living organisms and serves as a versatile precursor for thousands of indispensable metabolites. In humans, phenylalanine is an essential amino acid obtained through the diet primarily from plant-based sources, either directly or indirectly. Humans can convert phenylalanine to L-tyrosine for incorporation into proteins, as well as to synthesize thyroid hormones and brain chemicals such as L-dopamine, epinephrine and norepinephrine. In plants, phenylalanine metabolism is even more prevalent and diverse. In fact, plants direct 20–30% of photosynthetically fixed carbon to the production of phenylalanine and phenylalanine-derived compounds, which constitute approximately 30–45% of plant organic matter and have profound impacts on growth and development (for example, lignin), reproduction (for example, phenylpropanoids and benzenoids) and defence (for example, salicylic acid, tannins and flavonoids).

Production of phenylalanine-derived compounds in plants is a complex multi-compartmental process relying on phenylalanine mainly synthesized in plastid starches, to first be exported to the cytosol across both the inner and outer plastid envelope membranes. Due to its low permeability coefficient (2.5 × 10⁻¹⁰ cm s⁻¹ for phenylalanine versus 2 × 10⁻⁴ cm s⁻¹ for water in egg phosphatidylcholine) and simple diffusion of phenylalanine through membranes cannot meet the cytoplasmic demand, and therefore its passage is predicted to be protein mediated. In general, the plastid outer envelope is believed to contain pores and channels that confer partial discriminatory transport of metabolites, while the inner membrane, in conjunction with over 100 different transporters, serves as a selective permeability barrier. Within the outer plastid envelope, an outer envelope protein, OEP16 (ref. 16), a member of the plant preprotein and amino-acid transporter superfamily, forms cation-selective high-conductance channels with remarkable permeability for amines and amino acids, including phenylalanine. However, there are still no reports on the characterization of inner envelope amino-acid transporters, despite the presence of several amino-acid biosynthetic pathways in plastids. Phenylalanine is utilized throughout the cell in plant primary and specialized metabolism; thus, its transport across membranes is not limited to plastids. While intercellular amino-acid transporters have been uncovered through recent studies on long-distance nitrogen transport in plants, to date very little is known about intracellular transport of amino acids.

Phenylalanine transport (uptake) has been well studied in microbes. In yeast (Saccharomyces cerevisiae) phenylalanine uptake occurs via the broad substrate transporters AGP1 and GAP1, the high-affinity tryptophan permease TAT2, and the branched-chain amino-acid permeases BAP2 and BAP3 (ref. 25). In Escherichia coli, phenylalanine uptake proceeds via four distinct systems: (i) PheP (T.C. 2.A.3.1.1), a specific transport system that prefers phenylalanine over tyrosine and does not accept L-tryptophan; (ii) AroP, a general amino-acid transport system actively transporting phenylalanine, tyrosine and tryptophan with high affinity; (iii) TyrP, a tyrosine-specific system transporting phenylalanine at mM, but not μM, concentrations; and (iv) LIV-I/LS, a branched-chain amino-acid transporter system with broad substrate specificity capable of transporting phenylalanine only at very high concentrations.

The flowers of Petunia hybrida cv Mitchell have emerged as an ideal model system for studying plant phenylalanine metabolism. The primary fate of phenylalanine in mature petunia flowers is towards production of cytosolically synthesized volatiles and emitted volatiles, as the flower opens. We hypothesized that a phenylalanine transporter may be also transcriptionally upregulated at this time. Since RNA-Seq is a quantitative approach, gene expression on day 2 after flower opening relative to buds was investigated based on the number of...
counts corresponding to each contig in the generated data sets. The counts are based on the number of reads that map to a given contig normalized for the total number of reads and length of the contig (fragments per kilobase of transcript per million mapped (FPKM) values), which were used by Cufflinks for differential gene expression. First, higher expression of genes encoding plastidial phenylalanine biosynthetic enzymes (chorismate mutase 1, CM1; prephenate aminotransferase, PPA-AT; arogenate dehydratase 1, ADT1) and cytosolic phenylalanine-utilizing enzymes involved in scent formation (phenylalanine ammonia-lyase 1, PAL1; phenylacetaldehyde synthase, PAAS) on day 2 relative to buds (Fig. 1 and Supplementary Table 1) was confirmed to validate the RNA-Seq data sets. Next, expression of the three petunia pheP homologues was examined. This analysis revealed that contig Ph19221 showed an average 1.8-fold decrease in counts on day 2 relative to buds, while contigs Ph21511 and Ph18042 both showed a 2-fold average increase (Fig. 1 and Supplementary Table 1). Therefore, only Ph21511 and Ph18042 were further examined as plastidial phenylalanine transporter candidate genes.

Recent analysis of the 5,800 transmembrane proteins in Arabidopsis thaliana revealed that 660 contained putative amino-terminal plastid transit peptides. Therefore, it was hypothesized that if contigs Ph21511 and Ph18042 encode plastidial transporters their cognate proteins should bear detectable N-terminal transit peptides. Analysis of the encoded Ph18042 protein by multiple subcellular prediction programs (WoLF PSORT: http://www.genscript.com/psort/wolf_psort.html; Predotar: https://urgi.versailles.inra.fr/predotar/predotar.html; TargetP: http://www.cbs.dtu.dk/services/TargetP/) revealed that it does not contain a transit peptide. In contrast, the first 43 amino acids of the encoded Ph21511 protein were predicted with a score of 0.837 by TargetP to serve as a plastid-targeting signal. To experimentally test for plastidial localization, the first 52 amino acids of Ph21511, which include all the residues prior to the first predicted transmembrane domain, were fused to the N-terminus of the GFP reporter protein and transiently expressed in tobacco leaves (Fig. 2a–f). Whereas the GFP control displayed fluorescence confined to the cytosol (Fig. 2a–c), the green fluorescence of the Ph21511-52-GFP construct resulted in co-localization with the red autofluorescence of chlorophyll in plastids (Fig. 2d–f). To independently verify the confocal microscopy result, total crude extracts and isolated plastids were prepared from 2-day-old petunia flowers and examined by immunoblotting using purified anti-Ph21511 antibodies. The apparent molecular mass of the detected protein, which migrated above the 50-kDa marker, from both crude extract and the plastid fraction was consistent with the calculated size (57.9 kDa) for mature (that is, without transit peptide) Ph21511 (Fig. 2g). Moreover, the detected signal from equal amounts

![Figure 1](image1.png) **Figure 1 | Identification of plastidial phenylalanine transporter candidates.** Grey boxes depict average fold change in gene expression in petunia flowers on day 2 postanthesis relative to the bud stage. Changes in expression are shown for petunia homologues of E. coli pheP genes encoding plastidial phenylalanine biosynthetic enzymes chorismate mutase 1 (CM1), prephenate aminotransferase (PPA-AT) and arogenate dehydratase 1 (ADT1), as well as genes for the cytosolic phenylalanine-utilizing enzymes phenylalanine ammonia lyase 1 (PAL1) and phenylacetaldehyde synthase (PAAS). Measurement of gene expression is based on the average number of sequenced fragments ± s.e.m. (n = 3 biological replicates) found for a given contig (gene) per kilobase of sequence per million reads (FPKM) from RNA-Seq analysis of petunia flowers collected at 20:00 h at the bud stage (day – 1) and on day 2 postanthesis.

![Figure 2](image2.png) **Figure 2 | Ph21511 is localized to plastids.** (a–c) Transient expression of GFP alone in leaves of Nicotiana benthamiana. (d–f) Transient expression of the first 52 amino acids of Ph21511 fused to the N-terminal end of GFP in leaves of Nicotiana benthamiana. Red pseudocolour of chlorophyll autofluorescence is shown in panels a and d. Green pseudocolour of GFP fluorescence is shown in panels b and e. Merged images of red and green pseudocolour from a and b and from d and e are shown in c and f, respectively. Scale bar, 5 μm. (g) Immunoblot using anti-Ph21511 antibodies against 60 μg protein from total crude extract (CE) and plastids (P) prepared from day 2 petunia flowers.
PhcAT transports all three aromatic amino acids. The use of whole-cell uptake assays in a heterologous E. coli expression system has been an informative approach for elucidating the biochemical function of plastid transporters. To determine if PhcAT is capable of transporting phenylalanine and/or the other aromatic amino acids, uptake assays with 14C-phenylalanine, 14C-tyrosine and 14C-tryptophan were performed with intact E. coli cells expressing recombinant PhcAT53-583 (N-terminal 6XHis-tagged PhcAT) without its predicted transit peptide or carrying an empty vector as a control. An immunoblot using purified anti-PhcAT (anti-Ph21511) antibodies against E. coli crude extracts verified that recombinant PhcAT53-583 was expressed in isopropyl-1-thio-β-D-galactopyranoside (IPTG)-induced cells carrying the empty vector.

Figure 4 | PhcAT aromatic amino-acid transport activity. (a) Immunoblots on 2.5 µg of whole-cell crude extracts prepared from E. coli carrying an empty pET28a vector or codon-optimized pET28a: PhcAT53-583 and 2.5 µg of purified PhcAT53-583. (b–e) Transport assays on whole E. coli cells carrying empty pET28a (white circles) or pET28a:PhcAT53-583 (black circles). An equal number of cells were incubated with 100 µM 14C-glucose as a negative control (b), 14C-phenylalanine (c), 14C-tyrosine (d) or 14C-tryptophan (e) for the indicated time periods until termination of the assays by rapid filtration. Data are presented as a percentage relative to the corresponding empty-vector control value at 2 min set to 100%. Data are means ± s.e.m. (n≥3 biological replicates).
pET28a::PhpCAT157-1749 construct, but not empty pET28a vector (Fig. 4a). As a negative control, 14C-glucose was used to test if overexpression of PhpCAT affected endogenous E. coli uptake activities. First, glucose was selected because it was not expected to be a substrate of PhpCAT. In addition, one of the native glucose transport systems is proton driven similar to the E. coli AroP uptake system, which is responsible for 80–90% of aromatic amino-acid transport. Since no difference in 14C-glucose uptake was detected between E. coli harbouring the empty vector and cells expressing PhpCAT53-583, this indicates that proton-driven metabolite transport, including the endogenous aromatic amino-acid uptake system, across the membrane was not perturbed (Fig. 4b). On the other hand, E. coli cells expressing PhpCAT53-583 accumulated significantly lower levels of 14C-phenylalanine (Fig. 4c), 14C-tyrosine (Fig. 4d) and, to a lesser extent, 14C-tryptophan (Fig. 4e), compared to the empty-vector control. The detected levels of radiolabelled amino acids result from the net difference between uptake into and efflux out of the cell. Since no decrease in uptake via the endogenous aromatic amino acid E. coli transport systems is presumably occurring, the reduced accumulation of aromatic amino acids in cells expressing PhpCAT53-583 is due to efflux via PhpCAT. These results demonstrate that all three aromatic amino acids are substrates of PhpCAT.

**Figure 5 | Metabolic profiling of petunia flowers from PhpCAT-RNAi and PhpCAT-overexpression lines.** (a) Effects of PhpCAT RNAi suppression. PhpCAT mRNA levels (n = 3 biological replicates), internal pools of shikimate, prephenate, arogenate, phenylalanine, tyrosine and tryptophan (n = 4), and total emission of phenylalanine-derived volatiles (n ≥ 3) were measured in three independent PhpCAT-RNAi lines and control PhpCAT-overexpression lines (white bars) and compared to control values (black bars), which were set at 100%. Data are means ± s.e.m. *P < 0.05, **P < 0.01 by two-tailed Student’s t-tests. Dotted lines indicate trafficking steps. White boxes with question marks indicate unknown transporters/transport steps. In (a) the ‘x’ over PhpCAT depicts downregulation. In (b) the arrow with ‘OX’ depicts overexpression. ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; CHA, chorismate; CM, chorismate mutase; E4P, erythrose 4-phosphate; PPA-AT, prephenate aminotransferase; PDET, prephenate dehydratase; PEP, phosphoenolpyruvate; PYY-AT, phenylpyruvate aminotransferase.

To further investigate the role of PhpCAT in vivo, PhpCAT was overexpressed under the control of a petal-specific promoter44. Three different lines showing 4.5–100-fold increase in PhpCAT transcript levels (Fig. 5b) were chosen for further metabolite analysis. Total emission of phenylalanine-derived volatiles in detached flowers increased by up to 18% in PhpCAT overexpressors compared to control (Fig. 5b), although the degree of increase differed for individual compounds (Supplementary Fig. 3). In addition, the levels of phenylalanine reduced and shikimate (up to 9% reduced) in PhpCAT-RNAi lines compared to control (Fig. 5a).

**PhpCAT links plastidial–cytosolic phenylalanine metabolism.** To examine if PhpCAT functions in vivo as an exporter of phenylalanine from plastids, PhpCAT expression was downregulated using an RNAi strategy under control of a petal-specific promoter44. Three independent lines with 75–80% downregulated PhpCAT transcript levels (Fig. 5a) were selected for subsequent detailed metabolic profiling. Moreover, immunoblot analysis of crude extracts and purified plastid fractions from RNAi line 17 with purified anti-PhpCAT (anti-Ph21511) antibodies indicated that PhpCAT was downregulated at the protein level (Supplementary Fig. 1). Consistent with an in planta role in plastidial phenylalanine export, downregulation of PhpCAT led to 20–42% reduction in total emission of phenylalanine-derived volatiles relative to control (Fig. 5a), but the extent of decrease varied for each compound (Supplementary Fig. 2). Phenylalanine and tyrosine levels were also decreased in PhpCAT-RNAi lines by up to 42% compared to control (Fig. 5a), which was likely due to reduced amounts of their biosynthetic intermediates prephenate (25–52% decreased in PhpCAT-RNAi lines versus control) and arogenate (54–61% decreased in PhpCAT-RNAi lines versus control) (Fig. 5a). At the same time, only minimal decreases were observed in the pool sizes of tryptophan (5–18%
and tyrosine increased from 23 to 53% in PhpCAT overexpressors compared to control (Fig. 5b), likely as a result of elevated pools of phenylalanine biosynthetic intermediates prephenate and arogenate (11–16% and 22–48% increase in PhpCAT overexpressors versus control, respectively) (Fig. 5b). Moreover, the tryptophan pool displayed an increasing trend and no changes were detected in shikimate levels in PhpCAT overexpressors compared to control (Fig. 5b). Taken together, these data indicate that PhpCAT is involved in plastidial phenylalanine export in planta. Moreover, profiling of plastidial phenylalanine biosynthetic intermediates suggests that PhpCAT is involved in regulating flux through the aromatic amino-acid biosynthetic network.

PhiPCAT controls phenylalanine biosynthetic flux. Since downregulation of PhpCAT led to a decrease in the levels of phenylalanine, tyrosine, and their shared precursors prephenate and arogenate (Fig. 5a), we hypothesized that phenylalanine and tyrosine accumulate inside plastids of PhpCAT-RNAi lines and feedback inhibit the arogenate pathway (Fig. 6a). Recently we showed that plants also contain an alternative pathway that proceeds via phenylpyruvate to produce phenylalanine in the cytosol, and flux through this route increases when the plastidial biosynthetic pathway (via arogenate) is impaired12. In the alternative pathway, a cytosolic phenylpyruvate aminotransferase preferentially converts phenylpyruvate to phenylalanine using tyrosine as an amino donor12 (Fig. 6a). Interestingly, tyrosine cannot serve as an amino donor for PPA-AT in the plastidal arogenate pathway11. Taking advantage of this characteristic to distinguish between the two pathways, we employed metabolic flux analysis with stable isotopic labelling using 15N-tyrosine to determine the effect of reduced plastidial phenylalanine export on carbon flux through the parallel phenylalanine biosynthetic pathways.

Excised 2-day-old petunia flowers from control and PhpCAT-RNAi lines 9 and 17 were fed with 10 mM 15N-tyrosine starting at 18:00 h, harvested after 2, 4 and 6 h, and analysed by liquid chromatography-mass spectrometry (LC-MS) to determine phenylalanine and tyrosine pool sizes and isotope abundances. Similar to what was observed previously12, the labelling percentage and concentration of phenylalanine increased linearly over 6 h, as did the level of emitted volatiles (Supplementary Fig. 4). To assess the control PhpCAT exerts on metabolic fluxes through the phenylalanine biosynthetic network, a metabolic flux model was developed (see Methods). The simulation revealed that \( v_i \), the rate of synthesis through the plastidial arogenate pathway, was 32 and 44% lower \((P<0.05, \text{Student's} t\text{-test}, n \geq 3)\) at \( t_0 \) and \( t_6 \), respectively, in PhpCAT-RNAi lines compared to control (Fig. 6b and Supplementary Table 2). This finding is consistent with our hypothesis that reduced export of phenylalanine and tyrosine from plastids in PhpCAT-RNAi lines leads to feedback inhibition of the arogenate pathway. At the same time, the flux analysis showed that in control and the PhpCAT-RNAi lines, \( v_2 \), the flux through the cytosolic phenylpyruvate pathway, was minor at \( t_0 \) but significantly increased over the 6-h period (Fig. 6b and Supplementary Table 2). In addition, increase in the rate of \( v_2 \) in PhpCAT knockdowns was more rapid than in control \((P<0.01, \text{Student's} t\text{-test}, n \geq 3)\) (Fig. 6b and Supplementary Table 2), suggesting that more carbon flux is directed through the cytosolic pathway. Taking this into account with the decrease in flux through the arogenate pathway, the relative decrease in cytosolic phenylalanine production is considerably higher in PhpCAT-RNAi lines compared to control \((v_2/v_1 \text{ flux ratio of 0.44 versus 0.18, respectively, at } t_6)\) (Fig. 6b and Supplementary Table 2).

Discussion

Eukaryotic metabolic networks are often spread throughout multiple subcellular compartments separated by organelar membrane barriers, through which precursors, intermediates and end products must pass. While some metabolites may passively diffuse across membranes, others require protein-mediated transport, as is the case for phenylalanine synthesized in plastids. The current study took advantage of the distinctive transcriptional developmental regulation of phenylalanine metabolism in petunia flowers to identify a plastidial amino-acid transporter, PhpCAT, which is capable of transporting all three aromatic amino acids (Fig. 4). Increases and decreases in the levels of phenylalanine-derived volatiles, as well as in phenylalanine, tyrosine, and their biosynthetic intermediates prephenate and arogenate in PhpCAT overexpression and RNAi lines, respectively (Fig. 5), support an in vivo role for PhpCAT as a plastidial aromatic amino-acid exporter. Moreover, flux through the phenylpyruvate pathway, which produces phenylalanine in the cytosol, was increased when PhpCAT was downregulated (Fig. 6b).

It is likely that while the total cellular pools of phenylalanine and tyrosine decrease in PhpCAT-RNAi lines compared to control (Fig. 5a), these amino acids accumulate inside the plastid and feedback inhibit the shikimate and/or aromatic amino-acid biosynthetic pathways. Disproportional effects on the concentrations of phenylalanine, tyrosine, and their precursors prephenate and arogenate compared to shikimate and tryptophan (Fig. 5a) may point to feedback inhibition of CM1 (Fig. 6a) in PhpCAT-RNAi lines. Previously it was shown that petunia CM1 activity is not inhibited at the same concentration (50 μM) of phenylalanine and tyrosine56 known to regulate Arabidopsis CM1 (ref. 47). However, in light of the high demand for phenylalanine in petunia flowers, and the fact that inhibition constants \((K_i)\) of other plant CMs are in the range of 0.3–1.1 mM3, it is possible that petunia CM1 is inhibited at higher concentrations of phenylalanine. To test this, we assayed preparations of petunia plastids for CM activity in the presence of a range of phenylalanine concentrations (Supplementary Figs 5 and 6). Indeed, petunia plastidial CM activity was found to be inhibited by phenylalanine with an IC50 of 748 μM (Supplementary Fig. 6). Thus, the metabolic phenotype on the arogenate pathway observed in PhpCAT-RNAi lines (Fig. 5a) could be attributed to feedback inhibition of petunia CM1. Conversely, in PhpCAT- overexpression lines, levels of phenylalanine, tyrosine, prephenate and arogenate were increased compared to control (Fig. 5b), which may indicate reduced levels of phenylalanine and tyrosine in plastids leading to the relaxation of naturally occurring feedback regulation of their biosynthetic enzymes, including CM1. The fact that tryptophan also trended to increase in PhpCAT overexpression lines compared to control (Fig. 5b) might further imply reduced feedback restrictions on tryptophan biosynthesis and/or the shikimate pathway.

Downregulation of PhpCAT expression in petunia flowers by 75–80% led to 20–42% reduction in the emission of phenylalanine-derived volatiles (Fig. 5a). The smaller reduction in volatiles relative to PhpCAT expression is likely the result of one or a combination of several factors. (i) It is possible that the remaining amount of PhpCAT transporter activity was sufficient to sustain the observed volatile emission rate. (ii) In addition or alternatively, there could be other specific and/or non-specific amino-acid transporters, which are not homologous to E. coli PhEP, involved in exporting phenylalanine from plastids. (iii) Another possibility is the contribution of a phenylalanine storage pool in the vacuole to the production of phenylalanine-derived products. Vacuoles,
Figure 6 | Metabolic modelling of the phenylalanine biosynthetic network in control and PhpCAT-RNAi lines. (a) Scheme depicting cytosolic formation of phenylalanine and potential feedback inhibition mechanisms involved in plastidial phenylalanine biosynthesis. In PhpCAT-RNAi lines, phenylalanine and tyrosine may accumulate in plastids and feedback inhibit arogenate dehydratase (ADT) and arogenate dehydrogenase (ADH), respectively, as well as chorismate mutase 1 (CM1). If plastidial ADTs also function as prephenate dehydratases (PDTs), then the enzymatic conversion of prephenate to phenylpyruvate may also be subject to feedback inhibition by phenylalanine. It is unclear if phenylpyruvate utilized in the alternative phenylalanine biosynthetic pathway originates from the plastid, or from cytosolic conversion of chorismate exported from the plastid. White boxes with question marks indicate unknown transporters/transport steps. AS, anthranilate synthase. For remaining abbreviations, see Fig. 5. (b) Flux models representing the phenylalanine biosynthetic network in 2-day-old petunia flowers from control and PhpCAT-RNAi lines. Computer-assisted metabolic modelling was performed using pool sizes and isotopic abundances of phenylalanine and tyrosine, and measurements of phenylalanine-derived volatile emission from petunia petal tissue supplied with 10 mM 15N-tyrosine for up to 6 h. The rate of plastidial flux ($v_1$) in PhpCAT-RNAi lines was found to be significantly lower than control based on comparisons of absolute fluxes and two-tailed Student’s $t$-tests corrected by Bonferroni method ($P < 0.05$). The rate of change in cytosolic flux ($v_2$) in PhpCAT-RNAi lines was found to be significantly higher than control ($P < 0.01$) based on paired-sample two-tailed Student’s $t$-tests of slopes (see Methods for additional details). $n = 3$ for control and $n = 6$ for PhpCAT-RNAi lines.
which are separated from cytosol by a single, semi-permeable membrane called the tonoplast, are involved in temporary and long-term storage of numerous metabolites, including free phenylalanine. In recent years, tonoplast-localized transporters have been discovered in plants and shown to be an integral part of a complex cellular network controlling plant metabolism. Two Arabidopsis amino-acid transporters, AtCAT2 and AtCAT4, have been shown to localize to the vacuolar membrane. Though the physiological function of AtCAT4 is still unknown, AtCAT2 has been shown to regulate free amino-acid levels in Arabidopsis leaves. Interestingly, AtCAT2 is the most similar Arabidopsis CAT homologue to Ph18042 (Supplementary Fig. 7), the other E. coli PhcP homologue with increased gene expression in petunia flowers on day 2 postanthesis (Fig. 1 and Supplementary Table 1). Similar to AtCAT2, Ph18042 is also predicted to localize to the vacuole and it seems conceivable that it may contribute to modulating cytosolic phenylalanine levels in petunia flowers.

(iv) Finally, in PhpCAT-RNAi lines the phenylpyruvate pathway could partially compensate for the shortage of phenylalanine being exported from the plastid. Recently it was shown that flux through the cytosolic phenylpyruvate pathway (Fig. 6a) increases when entry into the arogenate pathway is genetically blocked. Similarly, the present study shows that the phenylpyruvate pathway has higher relative contribution when plastidial phenylalanine export is impeded in PhpCAT-RNAi lines (Fig. 6b). It still remains an open question at which upstream step flux is redirected towards the phenylpyruvate pathway. It is possible that CM1 is feedback inhibited by phenylalanine and tyrosine accumulated in plastids of PhpCAT-RNAi lines; thus, flux may be redirected at the level of chorismate (Fig. 6a). Plants contain a cytosolic isofrom of CM, CM2, with higher affinity for chorismate than their plastidial CM1 counterparts, which may produce prephenate in the cytosol for the alternative phenylalanine biosynthetic pathway (Fig. 6a). This scenario implicates the involvement of a plastidial chorismate transporter and cytosolic prephenate dehydratase (PTD), neither of which has been discovered. Alternatively, phenylpyruvate may be generated in the plastid via dehydratation of prephenate by moonlighting ADT (or an unidentified PTD) and then exported through an unknown transporter to the cytosol (Fig. 6a). However, PhpCAT-RNAi lines have reduced prephenate levels and the catalytic efficiency of PPA-AT11 is much higher than those of the known ADTs with moonlighting function in sink tissues as a transporter of amino acids, including phenylalanine. Fusion of GFP to the N-terminus of AtCAT6 protein to the plastid was confirmed by the presence of a transit peptide predicted to serve as a plastid-targeting signal. On the other hand, Arabidopsis chloroplast targeted CAT7 is known to function, it possesses a putative transit peptide predicted to serve as a plastid-targeting signal. On the other hand, AtCAT6 has previously been shown to function in sink tissues as a transporter of amino acids, including phenylalanine. Fusion of GFP to the N-terminus of AtCAT6 revealed that it is localized to the plasma membrane. However, it should be further investigated if AtCAT6 is dual localized, because similar to Arabidopsis CAT7 and PhcP, it contains a predicted transit peptide (with a TargetP score of 0.949).

At the cellular level, transporters are integral parts of metabolic networks as they mediate interactions between multiple pathways across different subcellular compartments. Metabolic flux analysis performed in the current study strongly supports the idea that transporters, such as PhcP, further exert control over fluxes through metabolic networks by influencing organellar metabolite concentrations, which in turn biochemically regulate enzymes. Thus, development of organellar metabolic-targeting profiles, coupled with stable isotope labelling, will be needed to further dissect the role PhcP plays in feedback control of the aromatic amino-acid network.

Methods

Genetic transformation. 

Generation of RNA-Seq data sets. RNA was isolated from corollas of at least eight wild-type P. hybrida cv. Mitchell diploid plants (W-115; Ball Seed Co., West Chicago, IL) grown under standard greenhouse conditions with a light period from 18:00 until 21:00 h. For the PhpCAT-RNAi construct, DNA containing two spliced prephenate synthase fragments of the coding region corresponding to nucleotides 30–577 and 30–368, the latter in an antisense orientation to create a hairpin structure, was synthesized (Genscript, Piscataway, NJ). 5′-EcoRI and 3′-BamHI sites were added for directional subcloning into pRNA69 containing the Clarkia brevior linuslinus synthase (LIS) petal-specific promoter44. The resulting cassette containing the LIS promoter and the synthetic PhcP hairpin fragment was cut out with SacI/NotI and ligated into the binary vector pART27C (strain GV3101)-mediated transformation of P. hybrida cv. Mitchell diploid using the standard leaf disk transformation method.

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and assigning weight to each transformed value. Linear model coefficients were then calculated using limma’s design matrix, contrast matrix and log-transformed values. Linear model was fitted using empirical Bayes method and differences between counts for two experimental conditions were calculated, which were then adjusted for multiple hypothesis testing. Additionally, mapping of each sample to the transcripts using TopHat resulted in 8,0278, which were then used by the Cufflinks (v 2.0.2) suite of programs38. In all, 96.98% of the contigs found to be differentially expressed by Cufflinks (v 2.0.2) were also differentially expressed by both edgeR and voom.

qRT-PCR. Sample collection, RNA isolation and qRT-PCR were performed as previously described35. Briefly, samples were collected from the tissues indicated in (DE3) and amino acids preceding the first predicted transmembrane domain of pK7FWG259 via GatewayTM technology, resulting in an in-frame fusion with the specific primers 5'-AGCGACCTTACAAGGACCAATGATCCTCCCTTCTACAATAAAA-3' and reverse primer 5'-CCGTTTCATAATGCACCAAGAGCCGCGTTTCTTCGA-3'. PhcAT expression was analysed relative to the reference gene elongation factor 1-2 which was amplified using primers 5'-CCTGGTCAAATGTCACACATTGAGACCAGACCT-3' and forward primer 5'-CATATGAGCTCGGTTGCATATAATGTCGAC-3'. qRT-PCR data. Then every 6-min estimates of C0 and f0 were calculated along the experimental period every 6 min using equations (3) and (4). The whole simulation was performed in Matlab R2013a environment (The MathWorks, Inc., Natick, MA). Variances in the estimated slopes were derived with a standard linear regression procedure as described48, while setting the intercepts as constants being the basal flux at 0 time. Since flux values were derived from the estimated trend slopes and other experimental measurements, flux variances can be derived by considering the propagation of errors based on the following equation:

\[ \sigma_f^2 = \sum_i \sigma_y^2 (\frac{df}{dy})_i \]

where \( y = f(x_1, \ldots, x_n) \).

Preparation of plastids. Plastids were isolated from approximately 25 g of 1–3-day-old petals of control, PhcAT-RNAi line 9 and PhcAT overexpression line 3 petunia plants. Flowers were kept in the dark for 2 h prior to plastid preparations to deplete starch. Petals were placed in a chilled blender containing 200 ml ice-cold medium A (0.5 M sorbitol, 10 mM MES/NaOH pH 6.3, 4 mM Na-ascorbate, 4 mM cysteine, 2 mM MgCl2, 1.5 mM KH2PO4, 1 mM MnCl2, 1 mM EDTA-Na2) and homogenized with three 2-s pulses. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA). An aliquot of filtrate (crude extract) was taken from each preparation and set aside, and the remainder was centrifuged at 6,000 g, 4 °C for 5 min. The pellet was washed twice in medium B (0.33 M sorbitol, 10 mM MES/NaOH pH 7.6), resuspended in 2 ml of medium B, and layered over a discontinuous PercollTM gradient consisting of 80% (2 ml) and 25% (6 ml) PercollTM prepared in medium B. After centrifugation (9,200 g, 4 °C for 20 min in a swinging bucket rotor with no brake applied), the intact plastid fractions (approximately 1.5 ml) were collected from the interface of the PercollTM layers, washed twice in medium B, and finally resuspended in 50 mM NaH2PO4 (pH 8.0) with 5% (v/v) glycerol, flash frozen and stored at −80 °C.

Immunoblots. Immunoblot analysis was performed essentially as described previously49 using petunia crude extracts and plastids, E. coli crude extracts, and the purified PhcAT-R.T38. Protein sample concentration was determined by the Bio-Rad Bradford protein assay. To solubilize membrane proteins, protein-loading dye containing 10% SDS was added to protein samples, which were then incubated at room temperature for 3 h, and centrifuged at 5,000g for 5 min to pellet debris. Immunodetection was performed on 2.5 μg protein using purified rabbit anti-PhcAT polyclonal antibodies (1:13,000) generated against a synthetic peptide CMIDDPAPGSAFGM (Genscript, Piscataway, NJ; http://www.genscript.com/ custom-polynucleotide-antibody-production-services.html). A goat anti-rabbit IgG horseradish peroxidase conjugate (1:3,000) was used as secondary antibody (Bio-Rad). Films were exposed at −70 °C, and images were visualized using an enhanced chemiluminescence reagent (PerkinElmer, Waltham, MA) according to the manufacturer’s protocol, and exposing the gels on Eastman Kodak X-OART.
AR film. An uncrropped immunoblot corresponding to Fig. 4a is shown in Supplementary Fig. 9.

Chlorisomat mutase assays. CM assays were performed using protein prepared from petunia plastids according to a previously published method. Briefly, corellia tissue was collected from day 2 flowers at 21:00 h, immediately frozen in liquid nitrogen, ground with mortar and pestle, and lyophilized at −80 °C. A 0.1 mbar for a minimum of 72 h. Approximately 0.3 g of dried tissue was resuspended in 2.5 ml heptane/tetrachloroethylene (density = 1.32 g ml−1) and homogenized in a ball mill. The resulting suspension was layered atop a freshly prepared heptane/tetrachloroethylene density gradient, then centrifuged for 90 min at 13,000g, 4 °C. The resolved gradient was divided into six fractions; each fraction was divided into half and all solvent evaporated under nitrogen flow. For enzyme assays, the resulting residue was resuspended in 1 ml of protein resuspension buffer (50 mM Tricine-NaOH, 10% glycerol, pH 8.4). Insoluble debris was pelleted by centrifugation, and the supernatant desalted into protein resuspension buffer before use in assays. Marker assays were completed for plastids (NADP+/-glyceraldehyde-3-phosphate dehydrogenase) and cytosol (alcohol dehydrogenase) as described previously. Plastid content peaked in the second heaviest fraction (7–12 ml as measured from the bottom of the gradient, approximate density 1.49–1.45 g ml−1), as 46% of total recovered GAPDH activity was found in that fraction, compared to 18% of ADH activity. This fraction was used for subsequent analysis.

CM assays were performed as described previously, except that they were adapted to high-performance liquid chromatography detection as follows. Following reaction incubation and subsequent conversion of prephenate to phenylpyruvate and chorismate, enzymatic reactions were targeted neutralized using 50 ml of 1 M NaOH, and 10 ml of the reaction mixture was analysed by high-performance liquid chromatography using the Waters Atlantis C18 column (3 mm, 2.1 × 150 mm) held at 35 °C, with an 8-min linear gradient of 5–70% acetonitrile in 1% formic acid at a flow rate of 0.4 ml min−1. Phenylpyruvate and chorisomat were detected by absorbance at 288 nm and quantified by comparison to authentic standards. For phenylpyruvate product confirmation, MS/MS was completed using an Agilent 6410 Triple Quadrupole LC-MS. Data were collected for product ion scans on a precursor m/z of 163.1 in negative mode using a collision energy of 20 V.

Targeted metabolite profiling. Petunia volatiles (benzaldehyde, benzyl alcohol, phenylacetaldehyde, methylbenzoate, phenylethanol, benzylacetate, eugenol, isoeugenol, vanillin, benzylbenzoate and phenethylbenzoate) were collected from detached flowers of control, PhpCAT-RNAi and PhpCAT overexpression lines (minimum of three flowers per biological replicate) from 18:00 to 22:00 h on day 2 postanthesis by a closed-loop stripping method and analysed by gas chromatography-MS as previously described. Internal pools of the aromatic amino acids were determined by capillary gas chromatography, which encodes the phenylalanine-specific transport system of Escherichia coli. J. Bacteriol. 173, 3622–3629 (1991).

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Author contributions
J.R.W., M.G. and N.D. conceived the project and designed research; J.R.W., M.G., L.G., J.A.M., H.Y., R.J., J.H.L. and R.M.M. performed research; J.R.W., M.G. and N.D. analysed the data; J.T.S. and J.T. analysed RNA-Seq data; J.R.W. and N.D. wrote the paper. All authors read and edited the manuscript.

Additional information
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