**Pyruvate uptakes into plastids of C₃ plants**

Plastids of primary endosymbiotic origin (i.e., plastids of the Archaeplastida) are bounded by two concentric envelope membranes that prevent the nonspecific diffusion of almost all polar molecules. Therefore, the controlled exchange of metabolites between plastids and the cytosol crucially depends on the activity of metabolite transport proteins located in the plastid envelope membrane. Pyruvate is an essential metabolite for several plastid metabolisms. In developing embryos of oilseed rape (*Brassica napus*), $^{13}$C-labeled pyruvate is imported into plastids via an unknown mechanism and contributes approximately one-third of the total metabolic flux to lipid biosynthesis, while phosphoenolpyruvate and glucose 6-phosphate import contribute the other two-thirds. In addition, branched-chain amino acid biosynthesis is strongly stimulated in isolated spinach chloroplasts by external provision of pyruvate, thereby providing further evidence for a crucial role of pyruvate transport across the chloroplast envelope.

**Proton-dependent type and sodium-dependent type C₄ plants**

Aoki et al. classified a wide range of C₄ plants, based on the two following uptake mechanisms: (i) C₄ plants belonging to the tribes of Andropogoneae and Arundinelleae, which show proton-dependent pyruvate uptake into chloroplasts, and (ii) other monocotyledonous and all dicotyledonous C₄ plants, which show sodium-dependent uptake into chloroplasts. The Andropogoneae and Arundinelleae plants belong to the NADP-malic enzyme (NADP-ME) type of C₄ photosynthesis, and the latter sodium-dependent pyruvate uptake species include all three subtypes of C₄ photosynthesis (i.e., NAD-malic enzyme (NAD-ME), NADP-ME, and
phosphoenolpyruvate carboxykinase subtypes)\textsuperscript{10}.

**The genera Flaveria and Cleome**

\(\text{C}_4\) photosynthesis has evolved independently multiple times within the angiosperms\textsuperscript{12}; for example, in the genera *Flaveria* and *Cleome*. The small genus *Flaveria* consists of 23 species, including both *bona fide* \(\text{C}_3\) and \(\text{C}_4\) species and intermediate species (\(\text{C}_3\)-\(\text{C}_4\), \(\text{C}_4\)-like) that all share a close genetic background\textsuperscript{12}. The genus *Flaveria* is therefore considered to be one of the best model systems for studying the evolution and mechanism of \(\text{C}_4\) photosynthesis\textsuperscript{33}. The genus *Cleome* recently emerged as a model system for the study of \(\text{C}_4\) photosynthesis due to its taxonomic proximity to the model plant *Arabidopsis thaliana*\textsuperscript{33}.

**Combination of different transcriptome datasets**

The importance of these candidate transporters in \(\text{C}_4\) photosynthetic metabolism is strongly supported by the fact that the orthologues of *BASS2* and *NHD1* genes have also been identified by comparative RNA-seq of the \(\text{C}_4\) species *Cleome gynandra* and \(\text{C}_3\) species *C. spinosa*\textsuperscript{16}. While *Flaveria* \(\text{C}_4\) species are classified as NADP-ME-type \(\text{C}_4\) plants, *Cleome* \(\text{C}_4\) species belong to the NAD-ME type. As noted above, the sodium-dependent pyruvate uptake mechanism is shared by both NADP-ME-type- and NAD-ME-type \(\text{C}_4\) plants. Since we found that *BASS2* and *NHD1*, but not *BASS4*, are commonly up-regulated in both comparative analyses (Supplementary Fig. 1), we assumed that *BASS4* has a specific function in NADP-ME type \(\text{C}_4\) plants, whereas *BASS2* and *NHD1* are associated with pyruvate-uptake activity in both plant genera.

**BASS2 amino acid sequences**

The *F. bidentis* *BASS2* and *F. pringlei* *BASS2* full-length cDNA sequences were
obtained from the RNA-seq contig data. Their amino acid sequences were 98.3% and 94.7% identical to that of *F. trinervia BASS2*, respectively.

**Evolutionary progression of BASS2 abundance in the genus *Flaveria***

The level of BASS2 gradually increased from *F. angustifolia* to *F. ramosissima* to *F. vaginata*, differing from the pattern observed for PEPC abundance, which gradually increased from *F. ramosissima* to *F. vaginata* to *F. brownii* (Supplementary Fig. 4). This result indicates that the increase of BASS2 protein level precedes the increase of PEPC abundance in the evolutionary sequence from C₃ to C₄ in the genus *Flaveria*.

**Pyruvate transporters in proton-dependent type C₄ plants**

We did not detect a protein cross-reacting with an antiserum directed against the BASS protein in the C₄ species maize, sorghum, and *Miscanthus sinensis*, all of which were previously shown to possess the proton-dependent type of pyruvate transporter, or in the C₃ plant species rice and *C. spinosa* (Fig. 2e). These results are consistent with previous analyses of the maize chloroplast envelope proteome⁵, ²⁰, in which BASS2 was not detected, and indicates the presence of another gene encoding a proton-dependent-type pyruvate transporter in these plants. *Mep2* and *Mep4* have been postulated as good candidates for such a proton-dependent-type pyruvate transporter on the basis of comparative proteomics experiments⁵, ²⁰.

**Sodium-requirement of C₄ plants**

Interestingly, the sodium-dependent pyruvate uptake explains the requirement for sodium as a micronutrient in many C₄ species³⁷. Our findings also contribute to identification of the molecular target of the sodium nutrition.
Maintenance of the proton-gradient across the chloroplast envelope membrane

How is the proton gradient driving sodium export from the chloroplast maintained? We propose that the protons entering the chloroplast in exchange with sodium cations are required for the conversion of PEP, which is synthesized from pyruvate, ATP, and Pi, from its triple-negatively charged form \( \text{PEP}^{3-} \) to its double-negatively charged form \( \text{PEP}^{2-} \), which is the form required for the export of PEP from the chloroplast by the PEP/phosphate antiporter PPT in the C₄ cycle (Supplementary Fig. 12a). In summary, the proton entering the chloroplast in counter-exchange with sodium leaves the chloroplast bound to PEP, thereby stoichiometrically coupling pyruvate import to the export of PEP.

Pyruvate transporters in mitochondria

Extensive efforts have been made to identify organelle-localized pyruvate carriers, both in mitochondria and in plastids\(^{21}\). In mitochondria, pyruvate uptake across the mitochondrial membrane connects cytosolic glycolysis with the mitochondrial tricarboxylic acid cycle. A candidate for the yeast mitochondrial pyruvate carrier \( \text{YIL006w} \) was identified in 2003, using an inhibitor-based strategy\(^{38}\). However, that study did not provide positive (“gain-of-function”) evidence for a role of \( \text{YIL006w} \) in pyruvate transport. It was later shown that \( \text{YIL006w} \) actually functions as a mitochondrial \( \text{NAD}^+ \) transporter, not a pyruvate transporter\(^{39}\). The mitochondrial pyruvate importer has thus remained elusive.

References on supplementary information


Supplementary Figure 1. BASS2, BASS4 and NHD1 expressions in C₄ plants of the genus Cleome. BASS2 and NHD1 are abundantly expressed in C₄ species, while BASS4 is not abundant. C₃ plant Cleome spinosa; Cs, and a C₄ plant C. gynandra; Cg. Each read count in the RNA-seq dataset is indicated. The abbreviations of gene names are the same as in Fig. 1.
Supplementary Figure 2. BASS2 protein structure. a, Alignment of amino acid sequences of arbitrary selected plant BASS2s. Dots indicate residues identical to those above. Dashes denote gaps introduced to facilitate the alignment. Reversed (white-on-black) characters indicate residues identical in more than four of the six BASS2 sequences. Epitopes used as a peptide antigen are indicated by a black line. *F. trinervia* BASS2; FtBASS2, *F. bidentis* BASS2; FbBASS2, *F. pringlei* BASS2; FpBASS2, *Arabidopsis thaliana* BASS2; AtBASS2, *Solanum lycopersicum* BASS2; SlBASS2, *Oryza sativa* BASS2; OsBASS2. b, Hydropathy analysis of *F. trinervia* BASS2.
Supplementary Figure 3. Phylogenetic relationships of BASS. BASS protein sequences were retrieved from GenBank and aligned using MUSCLE 3.6. A neighbour-joining tree was created using MEGA4 with standard parameters. Bootstrap analysis was carried out with 1000 replicates and bootstrap values are indicated in the figure. A maximum likelihood tree was calculated with Treefinder, version October 2008 applying a WAG +G amino acid substitution model. Bootstrap analysis was carried out with 1000 replicates and bootstrap values are indicated in the figure. Accession numbers are indicated with species names. The abbreviations of *Flaveria* BASS names are the same as in Supplementary Fig. 2.
Supplementary Figure 4. Variation of BASS2 abundance among the *Flaveria* species.

Crude protein was prepared from the leaves of indicated *Flaveria* species at noon, and then subjected to the immunoblot analysis with anti-BASS2 antibody (upper panel). Crude protein was loaded on lane in accordance with the proposed evolutional order from C\textsubscript{3} to C\textsubscript{4} *Flaveria* species\textsuperscript{12}. The same membrane was reprobed with an anti-PEPC antibody (middle panel). The lower panel shows CBB-staining as a loading control.
**Supplementary Figure 5. BASS2 protein levels in different tissues.** Crude protein extract was prepared from total leaf (T), bundle sheath strands (B), mesophyll cells (M), and mesophyll cell chloroplasts (Mc). PEPC and RubisCO large subunit (RbcL) were detected immunologically as a mesophyll-cell cytosolic marker and as a bundle-sheath-cell chloroplast marker, respectively.
Supplementary Figure 6. Plastid-localization of the BASS2::GFP. GFP fluorescence of root epidermal cells of transgenic tobacco was observed. The left panel, GFP fluorescence; the right panel, bright field. Bar = 10 µm.
Supplementary Figure 7. Recombinant protein expressed in *E. coli*. a, The recombinant BASS2 protein (the center panel) and NHD1 protein (right panel) were immunologically detected. The CBB-stained gel was shown as a loading control (the left panel). Arrowheads indicate the BASS2 and NHD1 signals, and an asterisk indicates a non-specific signal. b, Plasma membrane preferable accumulation of the recombinant BASS2. The crude protein extract of recombinant *E. coli* was prepared with the extraction buffer without detergent by sonication. After slow speed centrifugation (12,000 × g for 15 min), the resulting supernatant fraction was subjected to ultracentrifugation (100,000 × g for 40 min). Both pellet fractions (low centrifugation pellet, LC ppt; ultracentrifugation pellet, UC ppt) were dissolved with extraction buffer containing detergent and an equal volume of the solution was subjected to immunoblot analysis. The left panels indicate BASS2 single expression, and the right panels indicate the dual expression system. BASS2 proteins were immunologically detected (the top panel) and the NHD1 protein (the middle panel) was detected with its artificial tag (S-tag). The bottom panels are CBB stained gels as loading controls.
Supplementary Figure 8. Age-dependent BASS2 transcript abundance from publicly available microarrays. To compare the expression data on leaves, microarray data on plants grown on soil under continuous light were selected.
Supplementary Figure 9. BASS2 expression in *A. thaliana*. **a**, The 2-kb-upstream from the first ATG site was introduced into the site just before the β-glucuronidase (GUS) gene in pBI101 vector. Localizations of the promoter-driven GUS activities in transgenic lines are shown. Age of the plant is indicated in each panel and the leaf-order is also indicated in the 19-day-old plant. **b**, Variation of BASS2 protein levels in 19-day-old plants are immunologically detected. Leaves were collected in accordance with the leaf order as indicated, crude proteins were extracted, and subjected to the immuno-detection (upper panel). The lower panel is a CBB-stained gel as a loading control.
Supplementary Figure 10. *bass2* mutant. **a,** T-DNA insertion sites on the *BASS2* gene are indicated. **b,** RNA gel-blot analysis of *BASS2* in 10-day-old plants. Ten micrograms of total RNA was loaded on each lane and the lower panel indicates EtBr-staining gel as an RNA loading control. **c,** Protein gel-blot analysis of *BASS2* in 7-day-old plants. Fifty micrograms of crude protein was subjected to an immunoblot analysis. Five micrograms of crude protein from *F. trinervia* was also loaded as a positive control. The lower panel shows CBB-staining as a loading control.
Supplementary Figure 11. Isopentenyl diphosphate synthesis pathways in plants and pyruvate as an initial substrate for MEP pathway. Cytosolic pathway is inhibited by mevastatin treatment. Synthesized IPP can be exchanged between cytosol and plastids. Glyceraldehyde 3-phosphate, G3P. Hydroxymethylglutaryl-CoA, HMG-CoA. HMG-CoA reductase, HMGR. Isopentenyl diphosphate, IPP. Mevalonic acid, MVA.
Supplementary Figure 12. BASS2 function in plants. a, The coupling reaction model of BASS2 and NHD1 in sodium-dependent pyruvate uptake. The model also displays the stoichiometric coupling of the pyruvate import to PEP export. b, BASS2 functions as a sodium-dependent pyruvate transporter at mesophyll cell plastids in the C₄ carbon cycle (right) and at plastids in developing leaves of A. thaliana in MEP pathway (left).