Synthesis and use of stable isotope labelled internal standards for quantification of phosphorylated metabolites by LC-MS/MS

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Supplementary Material and Methods

Growth of plants and cell cultures

Arabidopsis thaliana [L.] Heynh., accession Col-0 was grown for 5 weeks under 8/16 h day/night cycles at an average irradiance of 120 µmol photons m^{-2} s^{-1}, temperatures of 22/20 °C and relative humidities of 60/75 %, or for 3 weeks under 16/8 h day/night cycles at an average irradiance of 140 µmol photons m^{-2} s^{-1}, temperature of 20 °C and relative humidity of 75 %. Plants were grown in soil in individual 6-cm diameter pots, with water/gas permeable plastic membrane (Aquafol, Meyer, Germany) covering the soil surface. Maize (*Zea mays* L. cv. B73) seeds were germinated in the dark at 28 °C for 3 days in sealed petri dishes containing moistened filter paper. Seedlings were transferred to soil in 10-cm diameter pots and grown for 5 days under 16/8 h day/night cycles at an average irradiance of 105 µmol photons m^{-2} s^{-1}, temperatures of 22/18 °C and relative humidity of 70 %. Subsequently, maize plants were grown under 14/10 h day/night cycles at an average irradiance of 480 µmol photons m^{-2} s^{-1}, temperatures of 25/22 °C and relative humidities of 30/65 %. Wheat (*Triticum aestivum* cv. Anza) was grown in soil in 10-cm diameter pots under 16/8 h day/night cycles at an average irradiance of 105 µmol photons m^{-2} s^{-1}, temperatures of 22/18 °C and relative humidity of 70 %. Tobacco (*Nicotiana tabacum*) was grown in soil in 13-cm diameter pots under 16/8 h day/night cycles at an average irradiance of 175 µmol photons m^{-2} s^{-1}, temperatures of 25/20 °C and relative humidity of 55 %. Tomato (*Solanum lycopersicum)* cv. M82 and potato (*Solanum tuberosum* cv. Desirée) plants were grown as described in [1] and [2], respectively.

Chlamydomonas reinhardtii CC1690 cells were grown in a medium containing 5 mM HEPES, 17.5 mM acetate, 1 mM K-phosphates, Beijerinck salts (7 mM NH_4Cl, 0.34 mM $CaCl_2$, and 0.41 mM $MgSO_4$), and trace salt solution [184 µM H_3BO_3, 77 µM $ZnSO_4$, 26 µM $MnCl_2$, 18 µM $FeSO_4$, 7 µM $CoCl_2$, 6 µM $CuSO_4$, and 1 µM $(NH_4)_6Mo_7O_{24}$ in a 5-litre bioreactor flushed with 5 % CO_2, with a constant irradiance of 41 µmol photons $m^{-2}s^{-1}$ and temperature of 24°C. Cultures were maintained at a constant cell density (4.3×10^6 cells/mL) for five days as described in [3] before harvest.

Yeast (*Saccharomyces cerevisiae* pYX212) cells from a single colony grown on yeast extract- peptone-dextrose medium were used to inoculate 2 mL of minimal medium [-Ura] with 2 % (w/v) glucose, and incubated overnight at 28 °C with constant shaking at 200 rpm. The overnight culture was used to inoculate (1:500 dilution) 10 mL of the minimal medium in a 50 mL Erlenmeyer flask and incubated as before for 14.5 h before harvesting at an OD_{600} of around 0.5. Cell concentrations and corresponding cell volumes during growth of yeast cells were determined from four independent cultures with a Z2™ Coulter Counter® (Beckman Coulter Inc., CA, USA) in a size range from 3.083 µm to 12.3 µm, using a 100-fold dilution of the cultures, and averaging two counts. The resulting cell number mL^{-1} was proportional to the measured OD_{600} up to 0.7 (R^2 = 0.994). A standard curve obtained by linear regression of OD_{600} vs. cell number mL^{-1} allowed calculation of the cell number per mL culture at a given OD_{600}.

E.coli K-12 MG1655 cells grown on a Luria-Bertani medium (LB) agar plate were used to inoculate a pre-culture in MOPS minimal medium (TEKNOVA Inc., CA, USA) containing 0.2 % (w/v) glucose and grown overnight at 37 °C with 200 rpm constant shaking. This culture was used to inoculate a main culture at a 1:30 dilution in 30 mL of pre-warmed minimal medium in

a 100 mL Erlenmeyer flask and incubated as described above for 4 h before harvesting at an OD_{600} of around 0.5. Colony forming units (cfu) for *E. coli* MG 1655 was determined during growth of the bacteria. Aliquots of two to three independent cultures grown as described above were diluted $1:1\times10^5$ or $1:5\times10^5$ and 20 µL were plated on LB agar plates. After incubation of the plates at 37 °C overnight the cfu mL^{-1} of cell culture were calculated as the number of bacterial colonies per plate × volume plated (mL) × dilution factor. The cfu mL^{-1} culture were proportional to the measured OD_{600} up to 0.7 (R^2 = 0.992). A standard curve obtained by linear regression of OD_{600} vs. cfu mL^{-1} allowed calculation of the number of cells per mL culture at a given OD_{600}.

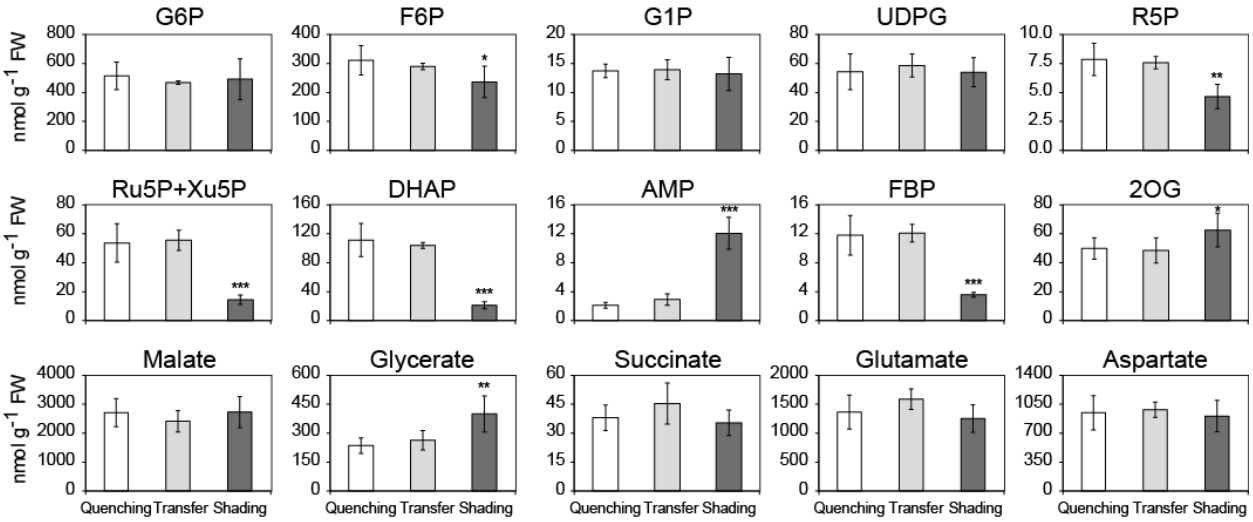
Optimization of sampling of photosynthetic tissues from plants

To be physiologically meaningful, metabolite measurements from any organism must not only be accurate, but also reliably reflect the *in vivo* concentrations in the living tissue or cells. Photosynthetic tissues present particular problems in this regard, because many photosynthetic intermediates combine high fluxes with small pool sizes, resulting in turnover times in the order of a second or less [4], [5], and so are potentially very sensitive to any change in light conditions during the harvesting procedure, before metabolism is quenched. Freeze-clamping of leaf tissue between two metal blocks pre-cooled in liquid nitrogen is an effective way of almost instantaneously quenching metabolism [6], [7], but is difficult to use with plants like Arabidopsis that have small leaves that grow as a rosette close to the soil. Pouring liquid nitrogen directly onto the plants was an alternative harvesting strategy employed by [5], but required the plants to be grown with a permeable plastic membrane over the soil to prevent the leaves being frozen to the soil, which might not always be desirable. This procedure is also potentially hazardous, and impractical for use within growth chambers because of the exposure of surrounding plants to cold nitrogen vapour and the likelihood of damage to the chamber from the inevitable spillage of liquid nitrogen. Therefore, we tested an alternative technique, which involves cutting the hypocotyl from underneath the rosette and then rapidly plunging the cut rosette into liquid nitrogen, taking care not to shade it from above at any time, thus keeping the leaves illuminated under the ambient light conditions throughout the harvesting procedure.

To assess whether this harvesting technique was reliable, we compared metabolite levels in plants harvested by careful transfer into liquid nitrogen after excision ("Transfer") with plants quenched directly by pouring liquid nitrogen on to them ("Quench"). A third set of plants ("Shaded") was taken from under the lights for approx. 2 s before cutting the rosettes and plunging them into liquid nitrogen, to mimic the effects of shading during harvest. Metabolites were measured by LC-MS/MS [5], with the extracts being spiked with the SIL-ISs described above to ensure accurate measurements. There were no significant differences between the "Quench" and "Transfer" samples in the levels of any of the measured metabolites (Figure S3). In contrast, R5P, Ru5P+Xu5P, DHAP and FBP were significantly (P<0.001) lower in the "Shaded" samples, consistent with the very short turnover times estimated for these metabolites: 0.08, 0.33, 0.24 and 0.79 s, respectively [5]. AMP and glycerate were both significantly (P<0.001) higher in the "Shaded" samples than those harvested in the light (Figure S3), likely reflecting a rapid drop in the adenylate charge as photophosphorylation stalls, and restriction of glycerate kinase activity by low ATP levels. These results demonstrate the critical importance of the harvesting

technique when sampling illuminated leaf material. Direct quenching with liquid nitrogen might still be necessary for harvesting plants during very short pulse-labelling experiments to measure photosynthetic fluxes [8], [9], but our results show that the "Transfer" technique is just as effective as the less practical and more hazardous "Quench" technique for routine metabolite measurements in Arabidopsis rosettes.

Figure S3: Effect of harvest procedures on Arabidopsis metabolite content. Arabidopsis plants were harvested by pouring liquid nitrogen directly on the rosette leaves in the light field ("Quenching"), by cutting and transferring the rosette leaves into liquid nitrogen under the light field ("Transfer") or away from the light field ("Shading"). Values are means ± SD (*n* = 6, except for G1P where *n* = 5). Significant differences from values obtained in quenched material according to Student's *t* test are indicated by asterisks (*P < 0.05, **P < 0.01 and ***P < 0.001).



Sample collection

Five-week-old and 3-week-old Arabidopsis plants were harvested by pouring a large volume of liquid nitrogen over the rosette leaves in such a way as to avoid shading the rosette. All frozen plant material above the plastic foil was collected and stored at -80°C. Alternatively, rosette leaves from 3-week-old Arabidopsis plants were cut and transferred into liquid nitrogen while being kept constantly under the same irradiance without shading. In addition, 3-week-old Arabidopsis plants were removed from the light source, the rosette leaves cut and transferred into liquid nitrogen. Fully expended leaves 5 and 6 of 4-week-old maize plants, leaf 4 from the base of 5-week-old tobacco plants and all leaves of 3-week-old wheat plants were harvested by cutting and transferring them into liquid nitrogen while being kept constantly under the growth irradiance without shading. Pericarp from green and red tomato fruits was harvested at 35 DAP (days after pollination) and 55 DAP, respectively, according to [10]. Potato tubers from 8-week-old plants were harvested as described in [2]. *Chlamydomonas reinhardtii* cells were harvested as described in [3]. Yeast and *E. coli* cultures were grown until an OD_{600} of approximately 0.5. Two replicates of two mL from three individual cultures of yeast and six replicates of an *E. coli*

culture were harvested by vacuum filtration (PVDF membrane, 25 mm, 0.45 µm pore size, Sigma-Aldrich) at 28°C and 37°C, respectively, as described in [11]. Filters were immediately transferred into Eppendorfs tubes and frozen in liquid nitrogen. The liver from a female mouse (NMRI, six-month-old, obtained from JANVIER) was removed about 40 min after its death and immediately frozen in liquid nitrogen. All harvested materials were stored at -80°C until analysis. Frozen tissue was ground to a fine powder using either a Retsch ball-mill at liquid nitrogen temperature or a cryogenic grinding robot [12].

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Supplementary Tables

Table S1: Specific compound-dependent MS parameters for selected reaction monitoring (SRM) and SIL-IS amounts added to extracts. Abbreviations: G6P, glucose 6-phoshate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; UDPG, UDP glucose; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; Xu5P, xylulose 5-phophate; DHAP, dihydroxy-acetone-phosphate; AMP, adenosine monophosphate; FBP, fructose 1,6-bisphosphate; 2-OG, 2-oxoglutarate.

Compounds	Parent ion ([M-H]$^-$)	Mass	Collision energy	Preferred product ion		SIL-IS added
	formula		(ev)	Formula	Mass	(µM)
[6,6-$^2H_2$]G6P	$C_6H_{10}{}^2H_2O_9P^-$	261	22	$[H_2PO_4]^-$	97	1
[1,6-$^{13}C_2$]F6P	$^{12}C_2C_4H_{12}O_9P^-$	261	22	$[H_2PO_4]^-$	97	0.5
[U-^{13}C]G1P	$^{13}C_6H_{12}O_9P^-$	265	31	$[PO_3]^-$	79	0.5
[$^{13}C_{11}$,$^{15}N_2$]UDPG	$C_4{}^{13}C_{11}H_{23}{}^{15}N_2O_{17}P_2^-$	576	28	$^{13}C_9H_{12}{}^{15}N_2O_9P^-$	334.1	0.5
[2,3,4,5-$^{13}C_4$]R5P	$C_5H_8{}^2H_2O_8P^-$	233	10	$[H_2PO_4]^-$	97	0.2
[2,3,4,5-$^{13}C_4$]Ru5P/Xu5P	$C_5H_8{}^2H_2O_8P^-$	233	10	$[H_2PO_4]^-$	98	0.4
[U-^{13}C]DHAP	$^{13}C_3H_6O_6P^-$	172	10	$[H_2PO_4]^-$	97	0.5
[$^{13}C_{10,}{}^{15}N_5$]AMP	$^{13}C_{10}H_{13}{}^{15}N_5O_7P^-$	361.1	21	$[PO_3]^-$	79	0.5
[U-^{13}C]FBP	$^{13}C_6H_{13}O_{12}P_2^-$	345	27	$[H_2PO_4]^-$	97	2
[1,2,3,4-$^{13}C_4$]2-OG	$C_4{}^{13}CH_5O_5^-$	149	12	$-CO_2$	105	0.75
[2,3,3-$^2H_3$]malate	$C_4H_2{}^2H_3O_5^-$	136	13	$-H^2HO$	117	10
[2,3,3-$^2H_3$]glycerate	$C_3H_2{}^2H_3O_4^-$	108	11	$C_2H_2{}^2H_1O_3^-$	76	1
[U-^{13}C]succinate	$^{13}C_4H_5O_4^-$	121	10	$-{}^{13}CO_2$	76	1
[2,3,3-$^2H_3$]glutamate	$C_5H_5{}^2H_3NO_4^-$	149	14	$-H^2HO$	130	8
[2,3,3-$^2H_3$]aspartate	$C_4H_3{}^2H_3NO_4^-$	135	13	$-CO_2$	91	6

Table S2: Amounts of substrates and products in the reaction mixture pre- and post-HVPE purification. Compounds present in each reaction mixture are presented and if possible were quantified. n.d. stands for not determined.

Reaction mixtures for	Compounds	Amounts (nmol)		Synthesis efficiency (%)	Amounts recovered post-purification (%)
		pre-purification	post-purification		
[6,6-$^{2}H_2$]G6P	[6,6-$^{2}H_2$]G6P	9467	8244	95	87
	ATP	n.d.	n.d.		
	ADP	2756	-		
	AMP	n.d.	n.d.		
	glucose	630	-		
[1,6-$^{13}C_2$]F6P	[1,6-$^{13}C_2$]F6P	8447	8129	84	96
	ATP	n.d.	n.d.		
	ADP	n.d.	n.d.		
	AMP	n.d.	n.d.		
	fructose	461	-		
[U-^{13}C]G1P	[U-^{13}C]G1P	9837	9350	98	95
	sucrose	97	-		
	fructose	9950	-		
	Pi	50µmol	n.d.		
[$^{13}C_{11}$,$^{15}N_2$]UDPG	[$^{13}C_{11}$,$^{15}N_2$]UDPG	9290	8026	77	86
	G1P	1100	-		
	UTP	n.d.	nd		
	UDP	n.d.	nd		
	UMP	n.d.	nd		
[2,3,4,5-$^{13}C_4$]R5P	[2,3,4,5-$^{13}C_4$]R5P	7867	1882	79	24
	[2,3,4,5-$^{13}C_4$]Ru5P/Xu5P	2800	369		
	ATP	n.d.	n.d.		
	ADP	13	-		
	AMP	6700	-		
	Pi	n.d.	n.d.		
[2,3,4,5-$^{13}C_4$]Ru5P/Xu5P	[2,3,4,5-$^{13}C_4$]Ru5P/Xu5P	9200	4826	92	53
	[2,3,4,5-$^{13}C_4$]R5P	2300	320		
	ATP	n.d.	n.d.		
	ADP	9	-		
	AMP	6600	-		
	Pi	n.d.	n.d.		
[U-^{13}C]DHAP	[U-^{13}C]DHAP	3807	1400	38	37
	[U-^{13}C]FBP	7300	-		
[$^{13}C_{10,}$ $^{15}N_5$]AMP	[$^{13}C_{10,}$ $^{15}N_5$]AMP	7984	5408	67	68
	ATP	n.d.	-		
	ADP	5	-		

Table S3: Percentage of cross-signal contributions between analytes and corresponding SIL-IS.

Compounds	Contribution (%) of	
	Analyte to SIL-IS	SIL-IS to analyte
G6P	1.41	0.12
F6P	1.41	0.12
G1P	0	0.17
UDPG	0	0
R5P	0	0
Ru5P	0	0
Xu5P	0	0
DHAP	0	0
AMP	0	0
FBP	0.40	2.99
2-OG	0	0

Table S4: Analyte amounts quantified with or without SIL-IS in various biological materials. Measurements were performed at dilutions 0.01, 0.02, 0.05, 0.1 and 1 (undiluted) for extracts from leaves of Arabidopsis, maize, tobacco and wheat. Extracts from tomato green and red fruits, potato tuber were in addition measured at dilution 0.2. Extracts from mouse liver, *Chlamydomonas reinhardtii*, yeast and *E. coli* at dilutions 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 (concentrated 2 times). Amounts are expressed as nmol g^{-1} FW with the exception of *Chlamydomonas reinhardtii*, yeast and *E. coli* where amounts are expressed as pmol 10^{-6} cells. *E. coli* amounts marked with [a] are in fmol 10^{-6} cells. Values are means ± SD (n = 3, except for undiluted potato tuber extracts where n = 2). n.d. stands for not determined, * indicates that the peak was not detected or too small to quantify and and ** indicates multiple or poorly resolved peak.

Supplementary Figures

Figure S1: Hexose phosphates, ATP, ADP and AMP separation by HVPE at pH 2.0 and 3.5. Each compound was loaded at 50 µg per spot. Electrophoresis was conducted at 4.5 kV for 20 min (pH 2.0) or 30 min (pH 3.5), and the spots were stained with molybdate reagent. Faintly stained spots are indicated with grey dotted circles. The origin is indicated by a gray line. The colored marker Orange G (orange dots) was loaded between each compound.

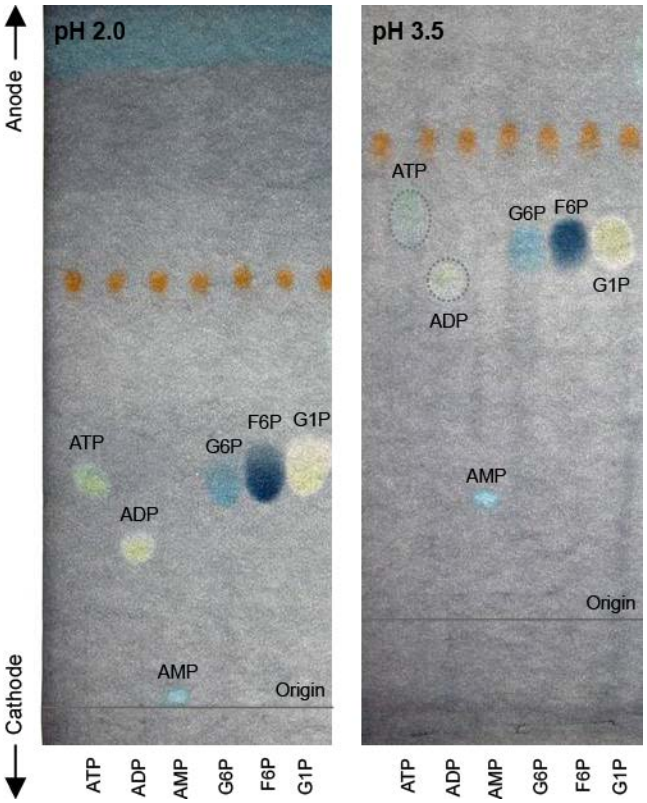


Figure S2: Electrophoretogram for the purification of [2,3,4,5-$^{13}C_4$]R5P. Markers run on the left side are Xu5P (1), ATP, ADP and AMP (2), Ru5P (3), Pi (4) and R5P (5). This part of the electrophoretogram was stained with molybdate reagent. Faintly stained spots are indicated with grey dotted circles. The main bands localized under 254-nm UV and originally marked with pencil are marked in grey. The origin is indicated by a gray line. The electrophoretogram was run at pH 3.5, 4.5 kV for 30 min. The colored marker Orange G (orange dots) was loaded between each compound.

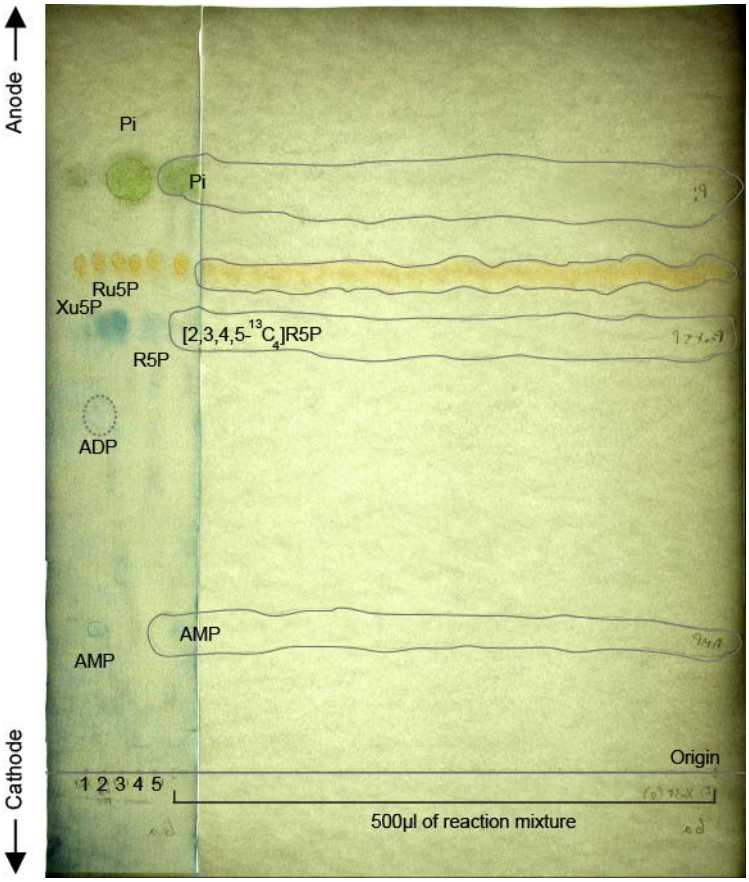
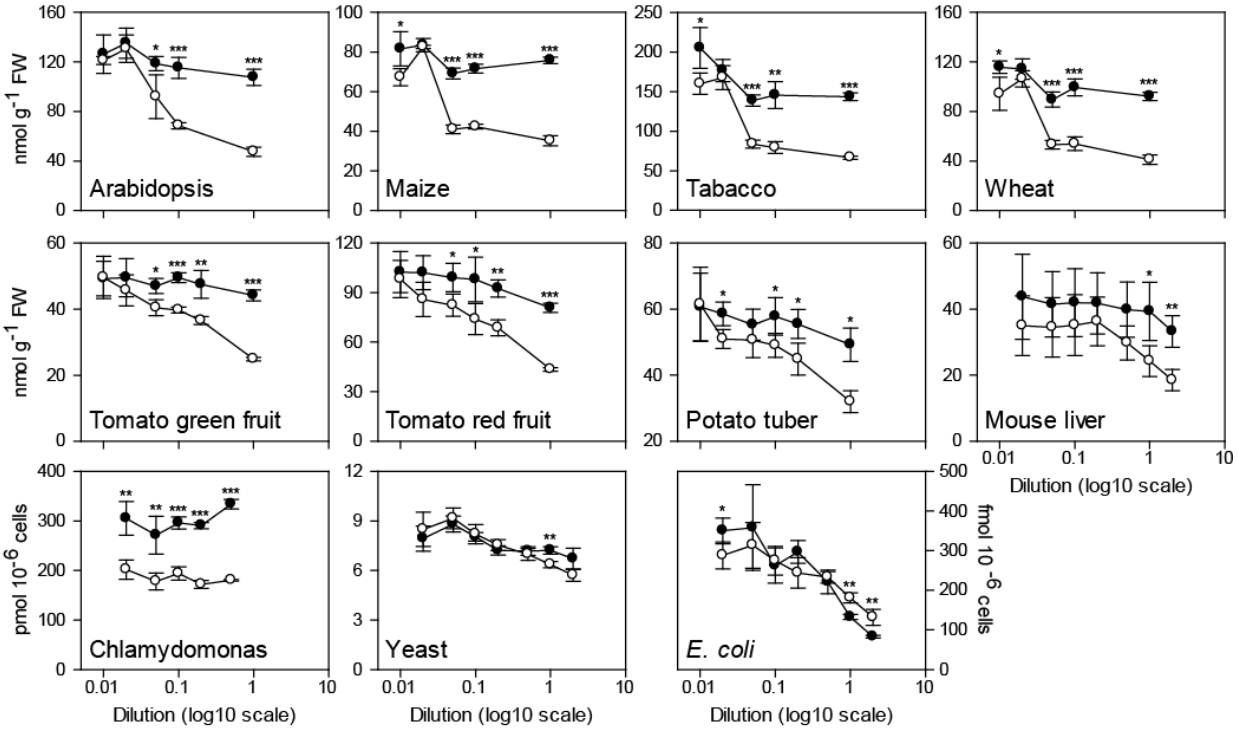
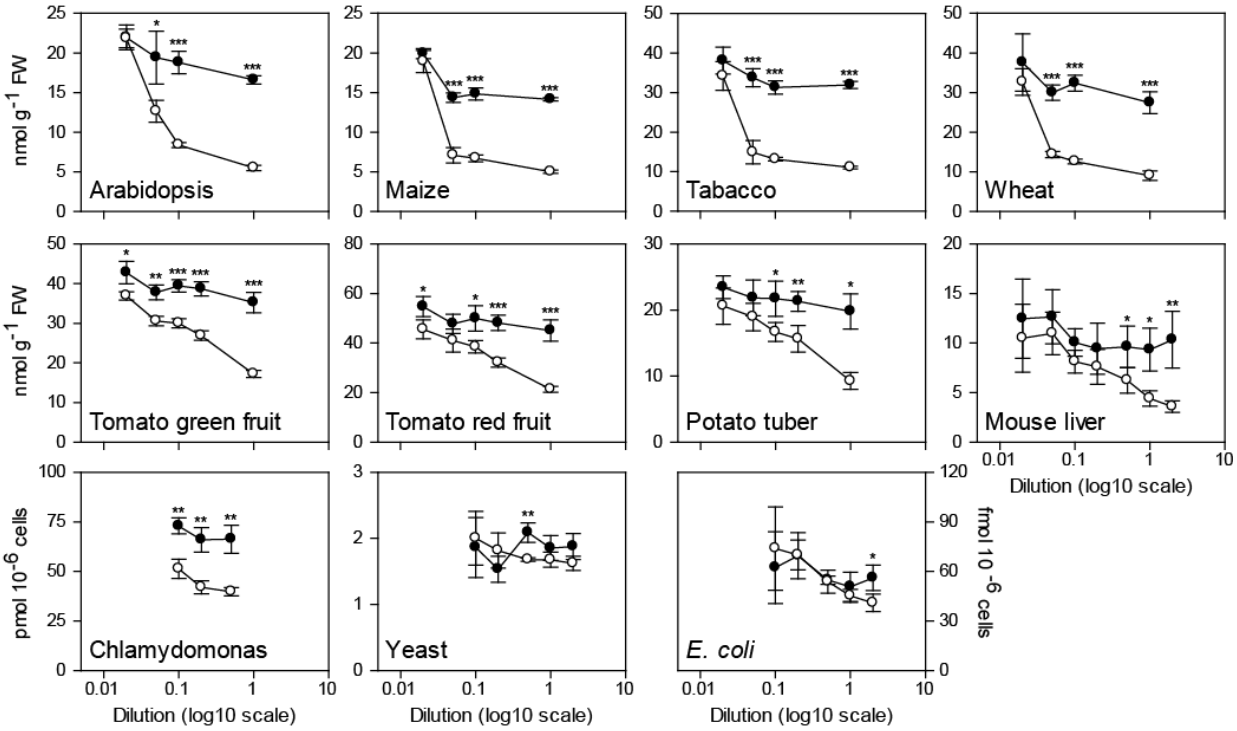


Figure S3: Quantification of analytes with (filled circles) or without (open circles) SIL-IS in various biological materials. F6P (**A**), G1P (**B**), UDPG (**C**), R5P (**D**), Ru5P+Xu5P (**E**), DHAP (**F**), AMP (**G**), FBP (**H**), 2-OG (**I**), malate (**J**), glycerate (**K**), succinate (**L**), glutamate (**M**) and aspartate (**N**). Measurements were performed in extracts from leaves of Arabidopsis, maize, tobacco and wheat diluted 100-, 50-, 20-, and 10-fold (indicated on the x-axis as 0.01, 0.02, 0.05 and 0.1, respectively) and undiluted (1). Green and red tomato fruits and potato tuber extracts were additionally diluted 5-fold (0.2). Extracts from mouse liver, *C. reinhardtii*, yeast and *E. coli* were measured after 50-, 20-, 10-, 5-, and 2-fold dilutions (indicated on the x-axis as 0.02, 0.05, 0.1, 0.2, and 0.5, respectively), undiluted (1) and in 2-fold (2) concentrated extracts. Values are means ± SD (n = 3, except for undiluted potato tuber extracts, where n = 2. Data are presented in Table S4 Supplementary Information. Significant differences from values obtained with signal corrected by SIL-IS according to Student's t test are indicated by asterisks (*P < 0.05, **P < 0.01, and ***P < 0.001).

A-F6P


nmol g-1 FW
Arabidopsis
Maize
Tabacco
Wheat
Tomato green fruit
Tomato red fruit
Potato tuber
Mouse liver
pmol 10-6 cells
Chlamydomonas
Yeast
E. coli
fmol 10 -6 cells
Dilution (log10 scale)

B-G1P


nmol g-1 FW
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Tabacco
Wheat
Tomato green fruit
Tomato red fruit
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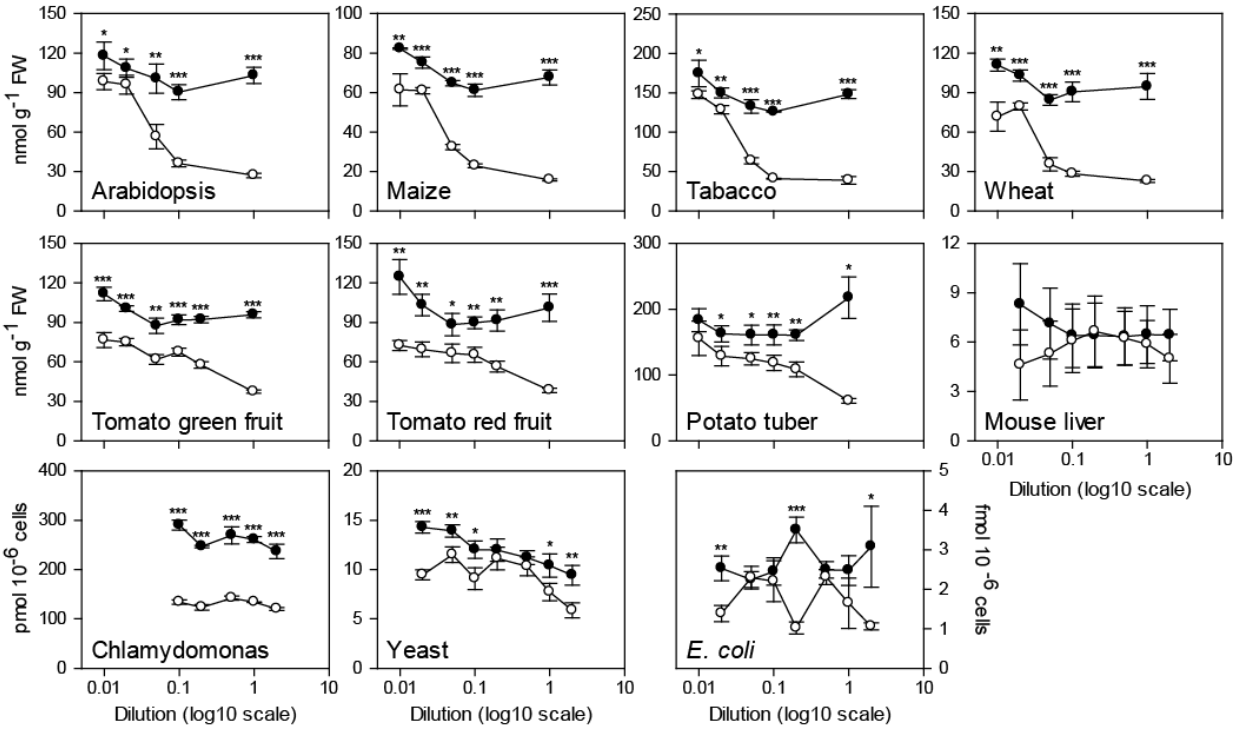
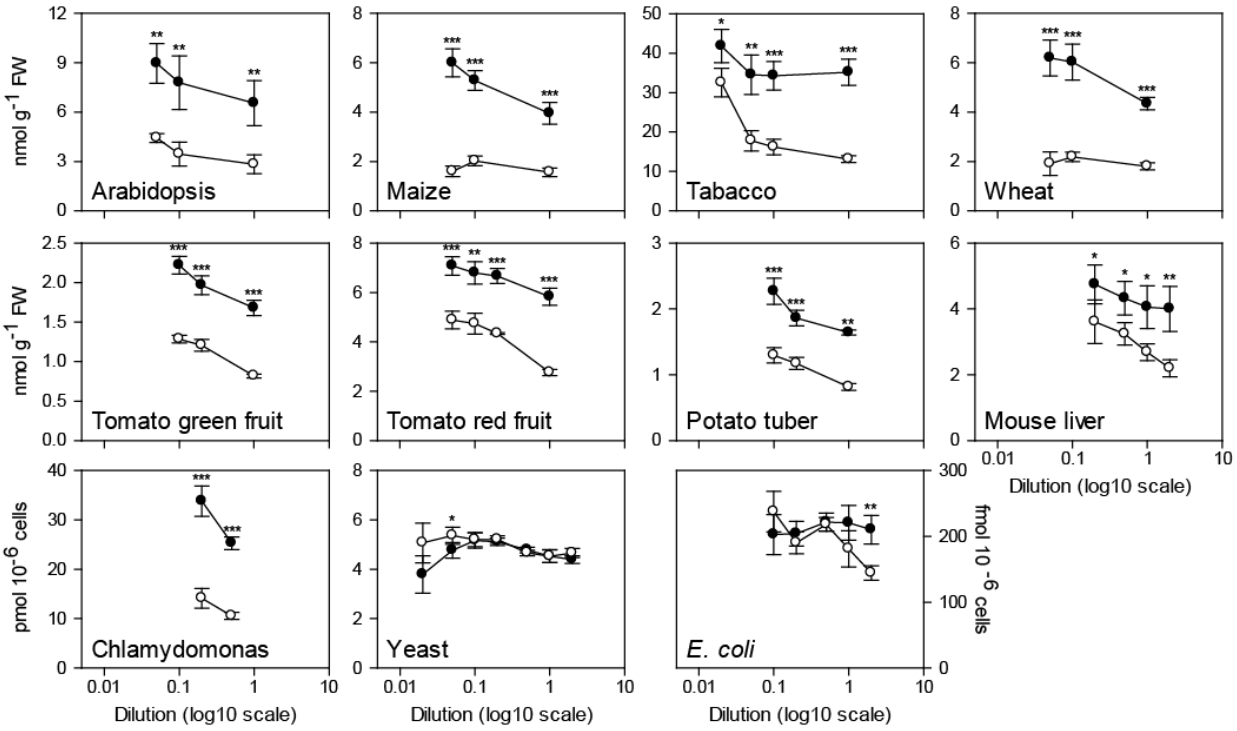
Yeast
E. coli
fmol 10 -6 cells
Dilution (log10 scale)

C-UDPG


nmol g-1 FW
Arabidopsis
Maize
Tabacco
Wheat
Tomato green fruit
Tomato red fruit
Potato tuber
Mouse liver
pmol 10-6 cells
fmol 10 -6 cells
Chlamydomonas
Yeast
E. coli
Dilution (log10 scale)

D-R5P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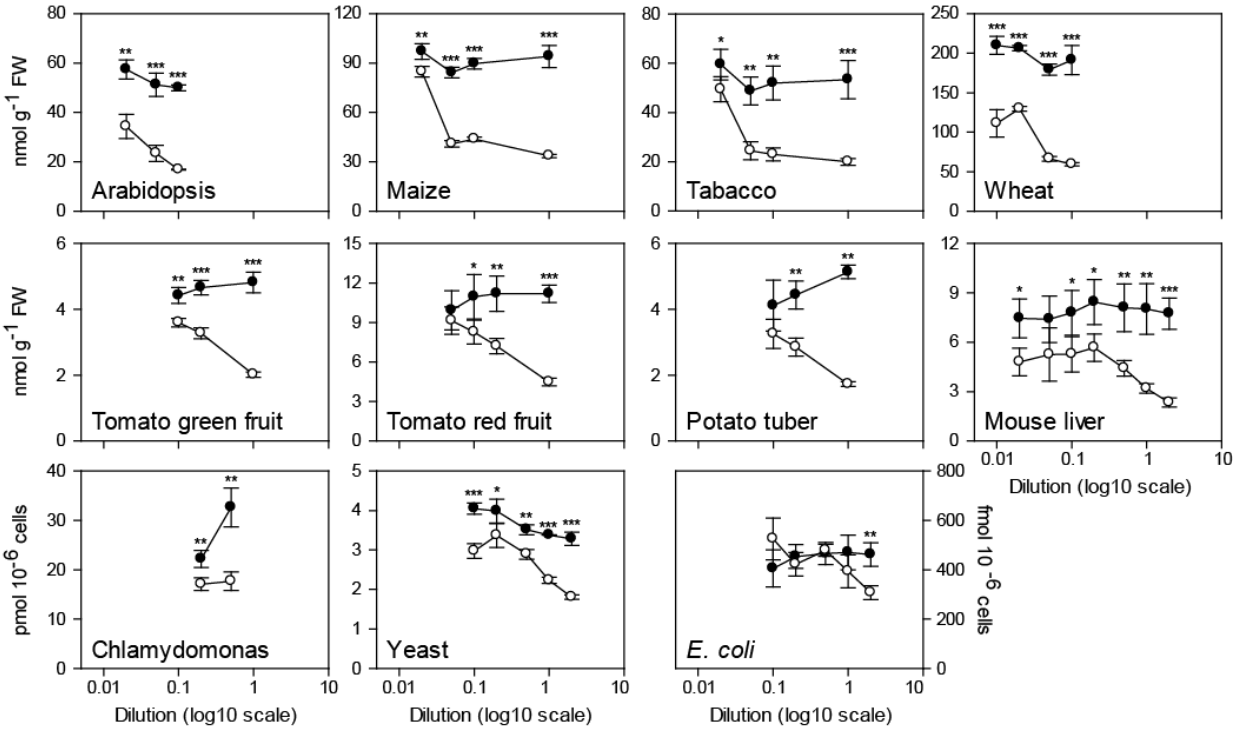
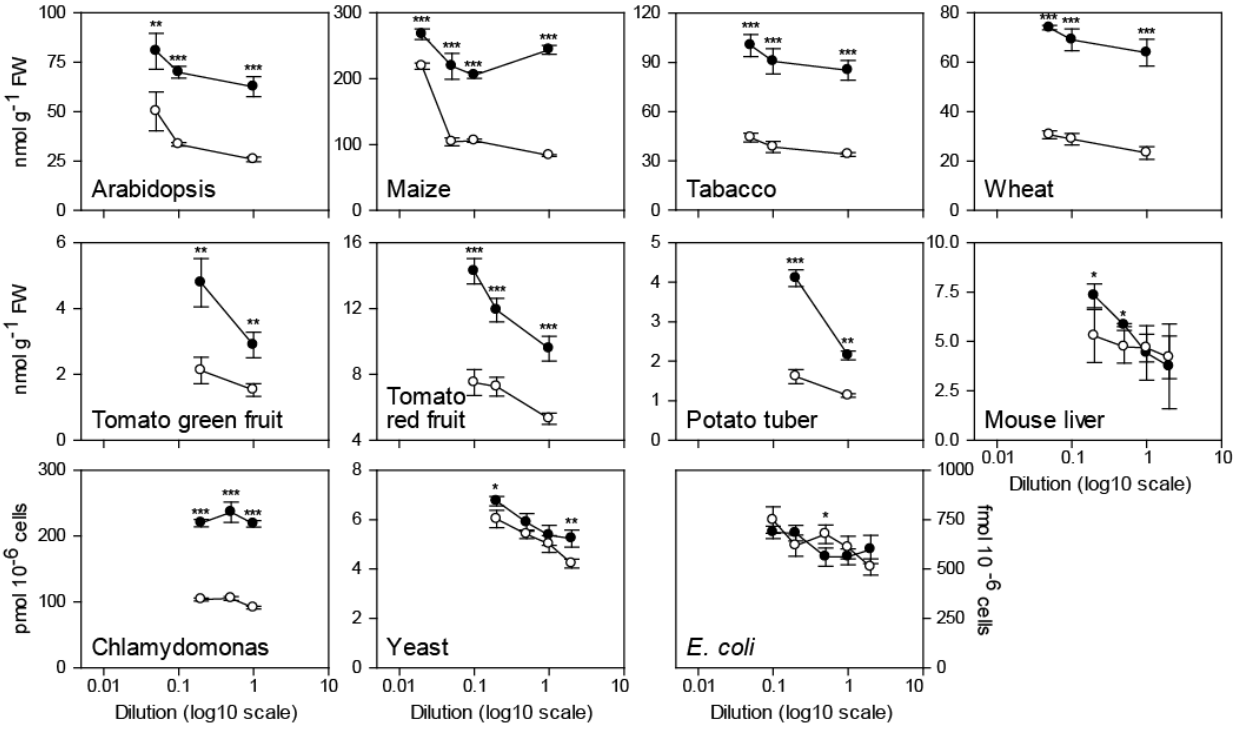
Arabidopsis
Maize
Tabacco
Wheat
Tomato green fruit
Tomato red fruit
Potato tuber
Mouse liver
pmol 10-6 cells
fmol 10 -6 cells
Chlamydomonas
Yeast
E. coli
Dilution (log10 scale)

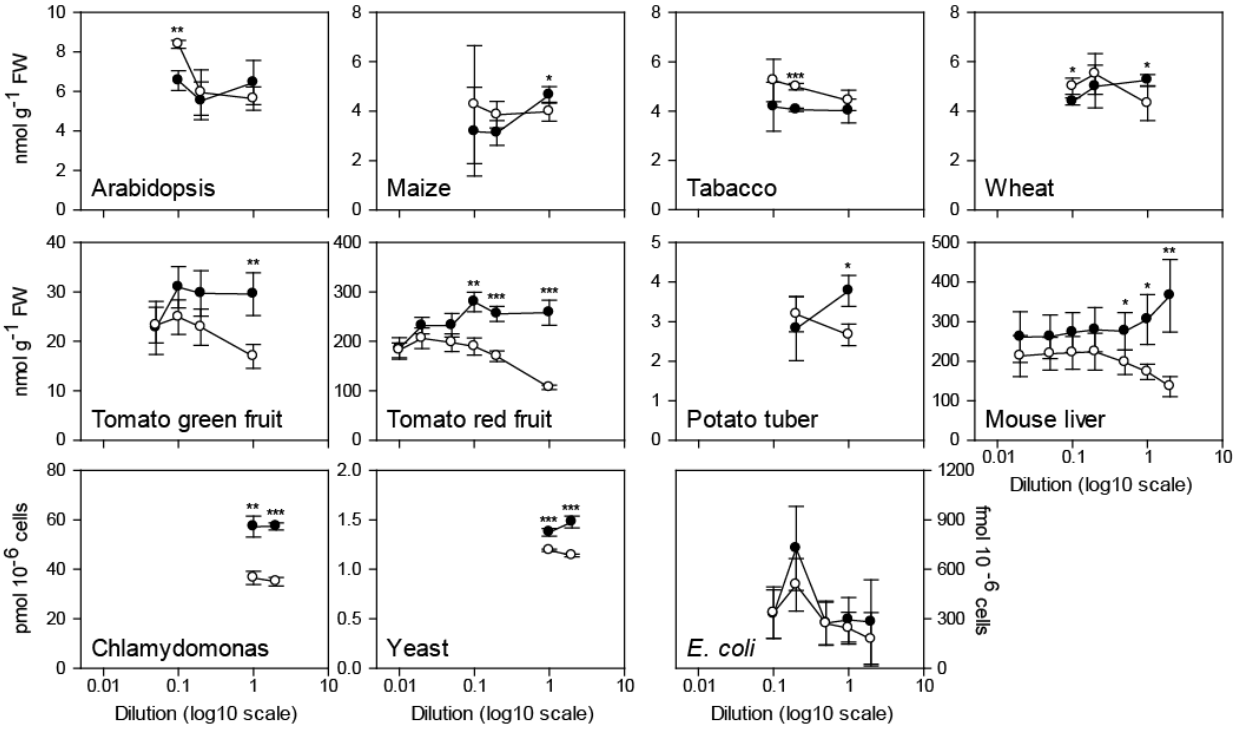
E-Ru5P+Xu5P



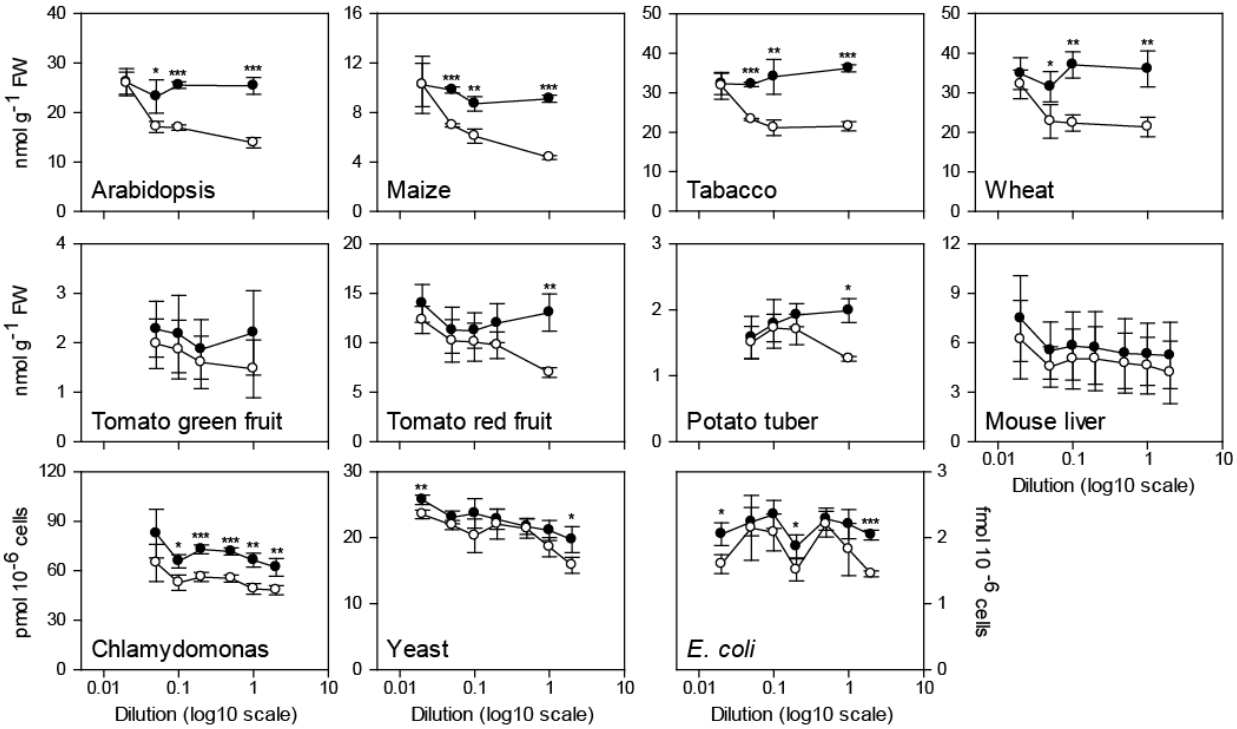
F-DHAP



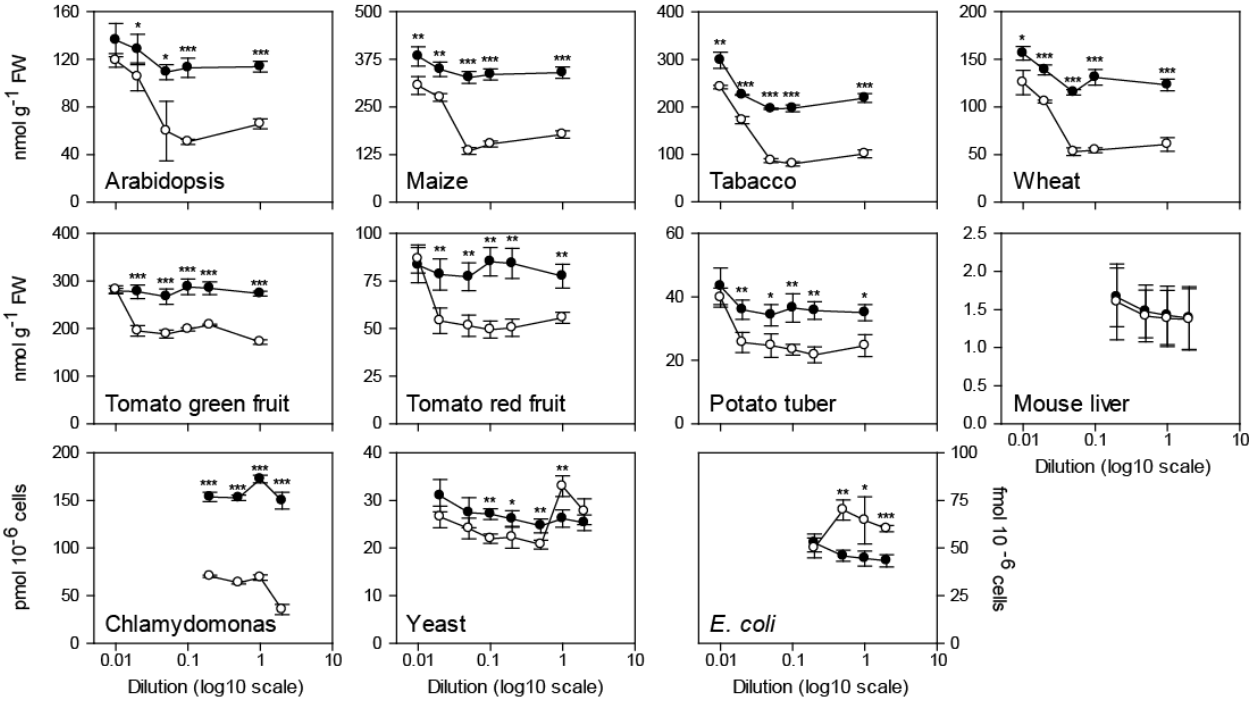
G-AMP


nmol g-1 FW
Arabidopsis
Maize
Tabacco
Wheat
Tomato green fruit
Tomato red fruit
Potato tuber
Mouse liver
Dilution (log10 scale)
pmol 10-6 cells
Chlamydomonas
Yeast
E. coli
fmol 10 -6 cells

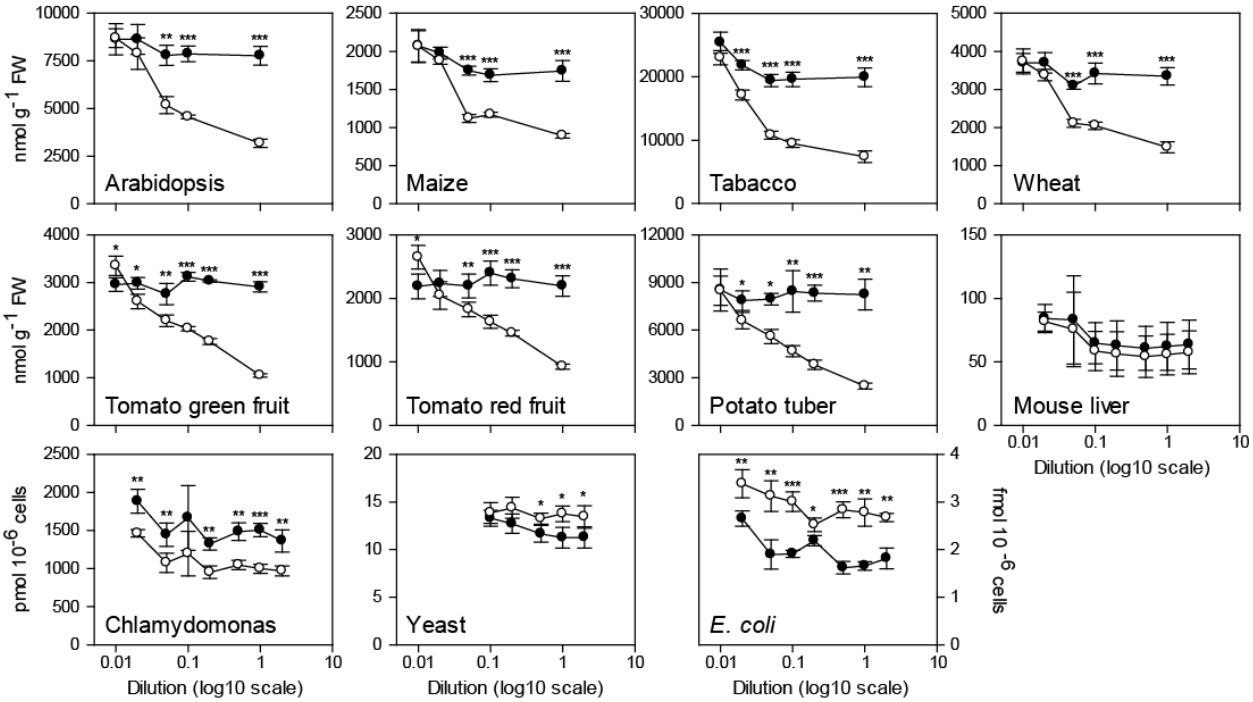
H-FBP


nmol g-1 FW
Arabidopsis
Maize
Tabacco
Wheat
Tomato green fruit
Tomato red fruit
Potato tuber
Mouse liver
Dilution (log10 scale)
pmol 10-6 cells
Chlamydomonas
Yeast
E. coli
fmol 10 -6 cells

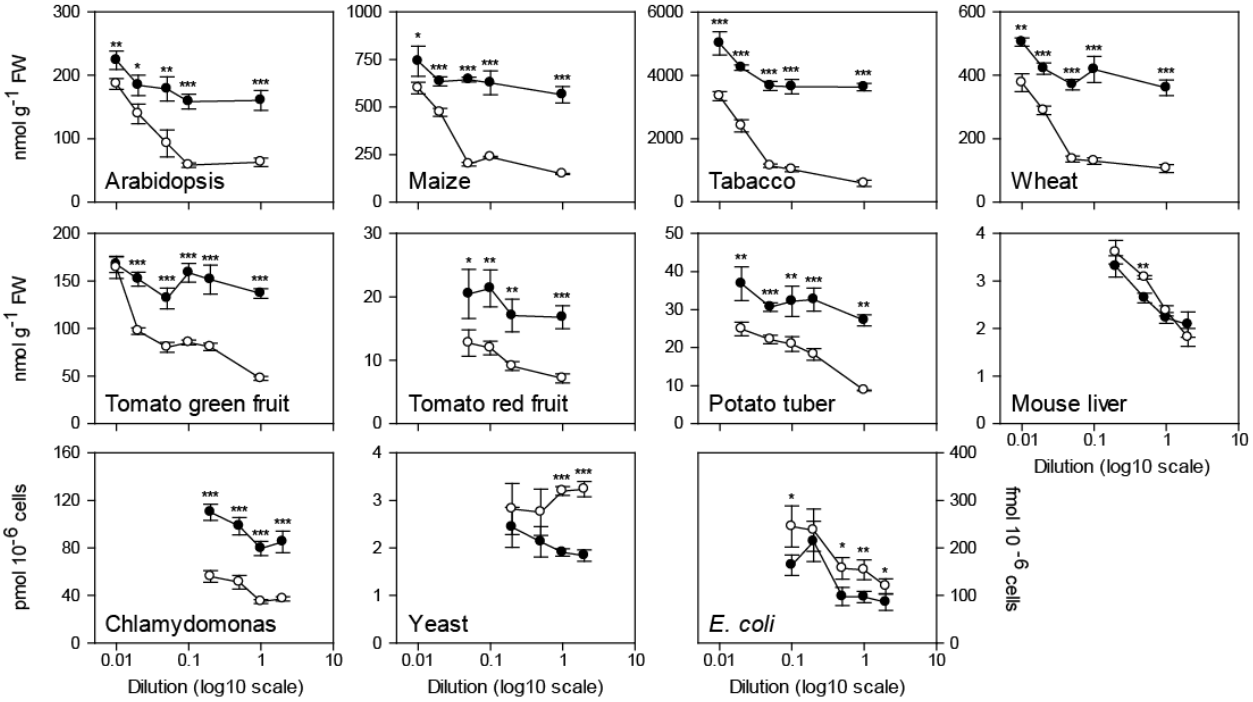
I-2OG



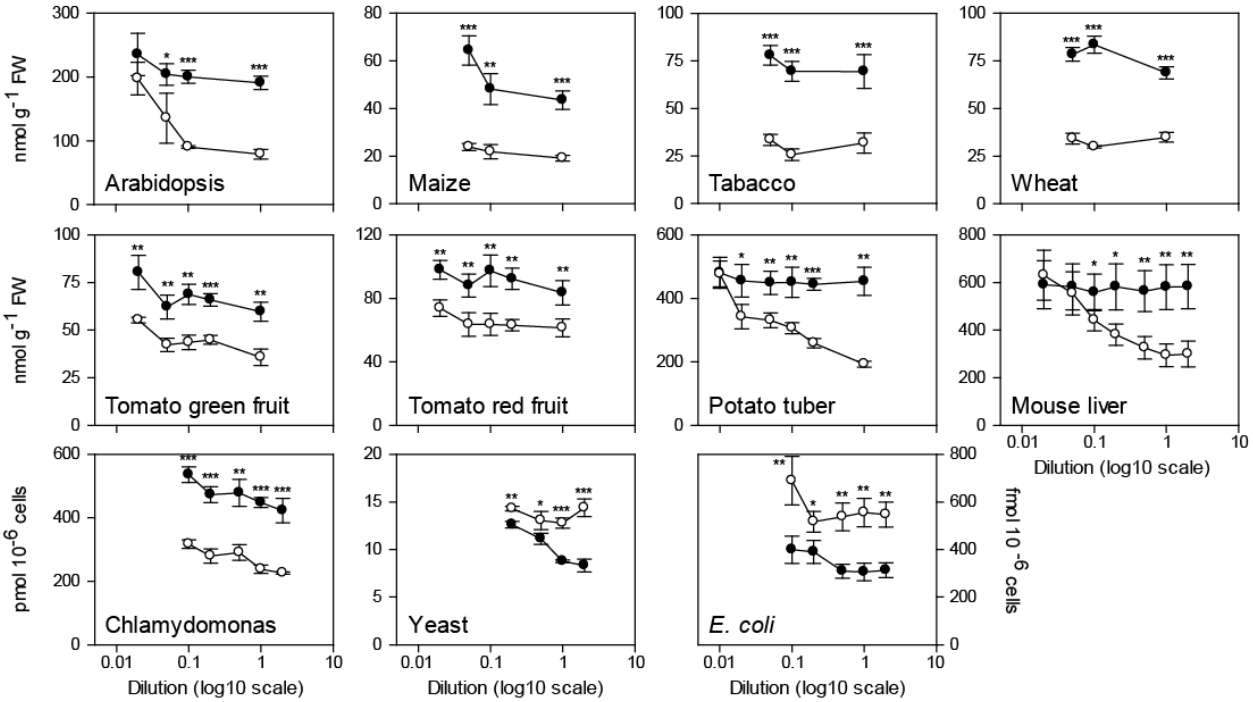
J-Malate



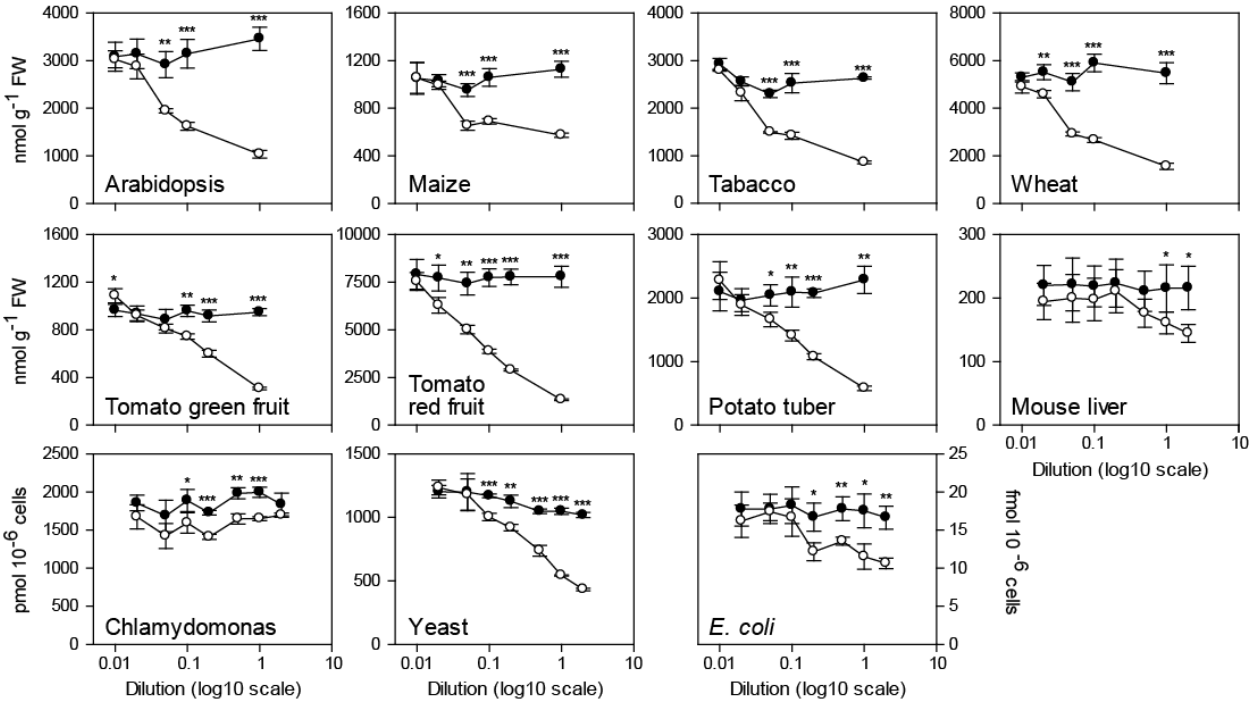
K-Glycerate



L-Succinate



M-Glutamate



N-Aspartate