

1 **Metabolic evidence for distinct pyruvate pools inside plant mitochondria**

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3 Xuyen H. Le^{1,2}, Chun-Pong Lee^{1,2}, Dario Monachello^{3,4}, A. Harvey Millar^{1,2*}

4 ¹ The University of Western Australia, School of Molecular Sciences, ² The ARC Centre of
5 Excellence in Plant Energy Biology, 35 Stirling Highway, Crawley, Perth 6009, Australia,

6 ³ Université Paris-Saclay, CNRS, INRAE, Univ Evry, Institute of Plant Sciences Paris-Saclay
7 (IPS2), 91405, Orsay, France & ⁴ Université de Paris, CNRS, INRAE, Institute of Plant
8 Sciences Paris-Saclay (IPS2), 91405, Orsay, France

9

10 **Corresponding author:** harvey.millar@uwa.edu.au, +61 8 6488 7245

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13 most of the experiments and data analysis, C.P.L. assisted with some of the mass spectrometry
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23 **Abstract**

24 The majority of the pyruvate inside plant mitochondria is either transported into the matrix
25 from the cytosol via the mitochondria pyruvate carrier (MPC) or synthesised in the matrix by
26 alanine aminotransferase (AlaAT) or NAD-malic enzyme (NAD-ME). Pyruvate from these
27 origins could mix into a single pool in the matrix and contribute indistinguishably to
28 respiration, or they could maintain a degree of independence in metabolic regulation. Here, we
29 demonstrated that feeding isolated mitochondria with U-¹³C-pyruvate and unlabelled malate
30 enables the assessment of pyruvate contribution from different sources to TCA cycle
31 intermediate production. Imported pyruvate is the preferred source for citrate production even
32 when the synthesis of NAD-ME-derived pyruvate was optimised. Genetic or pharmacological
33 elimination of MPC activity removed this preference and allowed an equivalent amount of
34 citrate to be generated from the pyruvate produced by NAD-ME. Increasing mitochondrial
35 pyruvate pool size by exogenous addition only affected metabolites from pyruvate transported
36 by MPC whereas depleting pyruvate pool size by transamination to alanine only affected
37 metabolic products derived from NAD-ME. Together, these data reveal respiratory substrate
38 supply in plants involves distinct pyruvate pools inside the matrix that can be flexibly mixed
39 based on the rate of pyruvate transport from the cytosol. These pools are independently
40 regulated and contribute differentially to organic acids export from plant mitochondria.

41

42 **Significance statement**

43 Pyruvate is the primary respiratory substrate for energy production to support plant growth and
44 development. However, it is also the starting material of many other pathways. Prioritisation
45 of respiratory use over other competing pathways would enable a level of control when
46 pyruvate is delivered to mitochondria via the mitochondrial pyruvate transporter. We
47 demonstrated the existence of two distinct pyruvate pools in plant mitochondria suggesting
48 inner mitochondrial organisation allows metabolic heterogeneity, hence metabolic
49 specialisation. This explains why NAD-ME flux into plant respiration is low and confirms the
50 prominent link between imported pyruvate and energy production. This compartmentation also
51 reveals how NAD-ME supplies substrate to the mitochondrial pyruvate exporter in plants,
52 especially during C4 metabolism.

53 Introduction

54 Pyruvate is the main product of cytosolic glycolysis and the fuel for aerobic respiration in most
55 organisms (1). It is oxidised by the mitochondrial pyruvate dehydrogenase complex (PDC),
56 producing acetyl-CoA which enters the tricarboxylic acid (TCA) cycle and generates reductant
57 equivalent for ATP production. In plants, the majority of pyruvate in the mitochondrial matrix
58 is supplied from three sources: (i) transport from the cytosol via the mitochondria pyruvate
59 carrier (MPC), (ii) alanine aminotransferase (AlaAT) that interconverts alanine and pyruvate,
60 and (iii) oxidative decarboxylation of malate via NAD-malic enzyme (NAD-ME). We have
61 recently shown that the combined action of MPC and the AlaAT is responsible for providing
62 the bulk of pyruvate to the TCA cycle in *Arabidopsis thaliana*, while NAD-ME only has a
63 minor role under both *in vivo* and *in vitro* conditions (2). This observation is in contrast to the
64 long-standing view that NAD-ME is a major contributor to pyruvate-dependent, TCA-cycle
65 linked respiration due to high malate availability and oxidation rate compared to other
66 respiratory substrates and the low level of pyruvate in plant cells (3-5). But is consistent with
67 *in vivo* labelling studies has revealed ME activity accounts for only 3% of the pyruvate
68 synthesized in respiring maize root tips (6, 7) and 1% in *Xanthium strumarium* leaves (8). It is
69 plausible that views about the centrality of the respiratory role of malic enzyme have been
70 significantly influenced by its activity at low pH in purified enzyme samples or isolated
71 mitochondria (7).

72 We found mitochondria isolated from an MPC1 loss-of-function mutant (*mpc1*) possess a
73 higher NAD-ME-dependent pyruvate production rate (2), indicating that a metabolic switch
74 may exist to control pyruvate supply and usage in order to meet specific metabolic and energy
75 demands under different conditions. One possible explanation for this phenomenon is that plant
76 mitochondria operate separate pyruvate pools: an imported pyruvate pool that sustains the TCA
77 cycle, and NAD-ME-derived pyruvate that serves as an emergency valve and is only switched
78 into TCA cycle metabolism when pyruvate import into the matrix is insufficient to satisfy
79 cellular energy demand.

80 One known mechanism in plant cells to separate metabolic pools is substrate channelling, either
81 (i) direct channel formed by interacting enzymes or (ii) increasing the local concentration of
82 enzymes by bringing enzymes together into clusters, rather than having them distributed
83 through the cell. It preferably facilitates the transfer of substrates from one active site of one
84 enzyme to the other sequential enzymes with minimal mixing substrates to the common pool

85 to minimize usage of substrates by competed pathways (9). The occurrence of substrate
86 channelling has been studied in the TCA cycle, making it one of the preceded examples of
87 metabolic direct sequential channelling (10, 11). The associative pairing of sequential enzymes,
88 called a metabolon has a kinetic advantage over free enzymes scouting for substrates from a
89 single metabolic pool (12), for example between malate dehydrogenase (MDH), citrate
90 synthase (CS) (12-16). Similarly, purinosome that is responsible for *de novo* purine synthesis
91 is an enzyme cluster in which reaction rate is enhanced by increased enzyme concentration is
92 probabilistic rather than direct (17). It was also suggested by the clustering model that flux
93 would increase by 6-fold for a 2-step pathway and over 100-fold for a 3-step pathway compared
94 to freely diffusing enzymes within a cell (18).

95 In this study, we explored the evidence for channelling-like phenomena in respiratory
96 metabolism before the TCA cycle, in the import of pyruvate via MPC and its delivery to the
97 TCA cycle. This necessitated the use of an intact mitochondrial system to allow pyruvate
98 transport and use by respiratory metabolism. We assessed the case for channelling by
99 monitoring how imported pyruvate interacts and competes with pyruvate generated by NAD-
100 ME in the matrix using co-feeding of mitochondria with multiple labelled and unlabelled
101 substrates. Our results revealed a preferential usage of transported pyruvate rather than NAD-
102 ME-derived pyruvate by pyruvate dehydrogenase complex (PDC) and TCA cycle enzymes
103 which could be reversed when MPC1 was absent or its activity chemically inhibited. This
104 flexibility indicates the presence of separate pyruvate pools in the matrix and suggests the
105 occurrence of a regulatory system that is more flexible than physical substrate channelling of
106 pyruvate between MPC and PDC, more akin to the proposed compartmentation of
107 mitochondrial metabolism and the apparent movement of metabolites between compartments
108 in models of mammalian cell metabolism (19-21).

109 **Results**

110 **Transported pyruvate is converted to citrate but when generated from NAD-ME it is** 111 **preferentially exported from isolated mitochondria**

112 Using selective reaction monitoring-mass spectrometry (SRM-MS) assays to trace the fate of
113 two substrates simultaneously (2, 22), we assessed the relative contribution of MPC1 and
114 NAD-ME to metabolites derived from the pyruvate pool in mitochondria from Col-0. Isolated
115 mitochondria were subjected to a series of $^{13}\text{C}_3$ -pyruvate concentrations ranging from 0 to 500
116 μM with a fixed concentration of 500 μM malate at pH 6.4 to determine if a high NAD-ME

117 activity competes with MPC for supplying pyruvate to the TCA cycle *in vitro*. This level of
118 acidity maximises NAD-ME activity (23). Under these conditions, imported malate can either
119 be oxidised to oxaloacetate by MDH or oxidised to pyruvate via NAD-ME. Citrate is then
120 synthesised by combining oxaloacetate with acetyl-CoA made either from exogenously
121 supplied pyruvate or from pyruvate formed in the matrix via NAD-ME (24). The pyruvate used
122 in this process can be distinguished by isotopic forms (Figure 1A, 1E). The relative amount of
123 labelled and unlabelled citrate exported to the extra-mitochondrial medium can be used to
124 assess the amount of respiratory pyruvate supplied by MPC and NAD-ME, respectively.

125 As expected, increasing exogenous $^{13}\text{C}_3$ -pyruvate concentration correlated with enhanced
126 production and export of $^{13}\text{C}_2$ -citrate and $^{13}\text{C}_2$ -succinate due to increased pyruvate import
127 (Figure 1B, 1C). Interestingly, unlabelled pyruvate (via NAD-ME) was released from the
128 mitochondria at high rates (at 14 nmol/min/mg protein with 500 μM provided pyruvate), at
129 least seven fold compared to other unlabelled metabolites (Figure 1G). When a smaller
130 concentration of $^{13}\text{C}_3$ -pyruvate (0 or 50 μM) was supplied, a lower amount of NAD-ME-
131 derived pyruvate from malate exported was detected (7-9 nmol/min/mg protein). However, the
132 amount of unlabelled citrate and succinate exported by mitochondria was independent of the
133 unlabelled pyruvate available (Figure 1E, 1F, Supplemental Figure S1A). From these data, we
134 concluded that the amount of exogenous $^{13}\text{C}_3$ -pyruvate had little impact on the entry of NAD-
135 ME-derived pyruvate into the TCA cycle, suggesting a mechanism to select the origin of
136 pyruvate for entry to the TCA cycle exists in plant mitochondria. Especially when providing
137 mitochondria with 500 μM pyruvate, MPC-derived citrate was more than 4 times higher in
138 concentration than NAD-ME-derived citrate.

139 In order to confirm the limited entry of NAD-ME-derived pyruvate into the TCA cycle, NAD-
140 ME double mutants *nad.me1/nad.me2* (*me1.me2*) and triple mutants of NAD-ME and the MPC
141 complex *nad.me1/nad.me2/mpc1* (*me1.me2.mpc1*) were fed with a mixture of 500 μM pyruvate
142 and 500 μM malate. Our previous study showed that mitochondria from wildtype plants (Col-
143 0) at pH 6.4 showed a rapid increase in unlabelled pyruvate generated from malate by NAD-
144 ME, while *me1.me2* and *me1.me2.mpc1* did not due to the absence of the NAD-ME enzyme
145 and wildtype mitochondria at pH 7.2 also failed to accumulate unlabelled pyruvate (NAD-ME
146 activity is minor) (2). Labelled citrate and downstream labelled TCA metabolites derived from
147 $^{13}\text{C}_3$ -pyruvate imported via MPC were steadily produced and exported, proving that malate
148 oxidation to OAA by MDH, pyruvate oxidation by PDC and other TCA cycle machineries
149 were not defective in these mitochondria (2). Despite this, the rate of unlabelled citrate exported

150 by wildtype at pH 6.4 was extremely low and did not show a significant difference to that by
151 *mel.me2* and *mel.me2.mpc1* and Col-0 at pH 7.2 (Figure 1H), suggesting that pyruvate
152 produced by NAD-ME at pH 6.4 in the matrix was mostly exported and not oxidised by PDC
153 for citrate synthesis (Figure 1E). Other downstream unlabelled metabolites, such as 2-
154 oxoglutarate and succinate, showed similar trends with no significant amounts produced
155 (Figure 1I, Supplemental Figure S1B). This confirms the hypothesis that there is a preference
156 of using imported pyruvate for TCA cycle metabolism and we cannot treat pyruvate from
157 different origins as single pool.

158 **No evidence for physical interaction of MPC and PDC**

159 To investigate the possibility of substrate channelling which could help explain the observed
160 metabolic compartmentation phenomena, we first looked at reports of protein-protein
161 interaction in the mitochondria. But we found no evidence for PDC subunit proteins binding to
162 MPC1 in plants (25, 26), yeast (27, 28) or mammalian cells (15, 29-32). To look instead for
163 evidence of MPC1 binding to PDC subunits, we conducted a high-throughput yeast two-hybrid
164 (Y2H) assay using MPC1 as bait to screen for interactions with about 12000 proteins in the
165 Arabidopsis library to probe the possible interaction between MPC and PDC (Supplemental
166 Table 1). The library includes the PDC components E1 α , E1 β , E2 and E3 (At1g59900,
167 At1g24180, At5g50850, At3g52200, At3g13930, At1g54220, At1g48030, At3g17240) and
168 other TCA cycle enzymes. High throughput screening failed to identify any physical
169 interactions of MPC1 with PDC subunits, suggesting the presence of distinct pyruvate pools is
170 not likely to result from a clear physical association that promotes substrate channelling.
171 GRXS17 (At4g04950) and GRX480 (At1g28480) were included as positive control baits in
172 the same screen to validate the experiment, and known interactors were confirmed by colony
173 sequencing (for GRXS17, known interactors BolA2-AT5G09830, BolA4-AT5G17560 and
174 Dre2-AT5G18400 (33); for GRX480, known interactors TGA3-AT1G22070 and TGA7-
175 AT1G77920 (34)).

176 **The use of mitochondrial NAD-ME-derived pyruvate by PDC is stimulated by 177 eliminating MPC1 activity either genetically or chemically**

178 We next examined if the preference of PDC for MPC-pyruvate over NAD-ME-pyruvate is
179 constitutive or if it changes depending on the pyruvate-supply in isolated mitochondria. We
180 have recently shown that MPC1 is required for pyruvate import from the cytosol into
181 mitochondrial matrix by monitoring the increase in labelled citrate and export of downstream

182 TCA cycle metabolites at pH 7.2 (2). Under conditions that optimise NAD-ME activity (pH
183 6.4), the loss of MPC1 resulted in the lack of mitochondrial ^{13}C -pyruvate import, leading to a
184 substantial reduction in the export of labelled citrate, succinate and malate to the
185 extramitochondrial medium compared to wildtype and a *mcp1* complemented line (Figure 2A-
186 D, Supplemental Figure S2A). While there was a lack of usage of NAD-ME-derived unlabelled
187 pyruvate inside wildtype mitochondria, we observed a substantial and progressive increase in
188 unlabelled citrate, 2-oxoglutarate and succinate concentrations in the extramitochondrial
189 medium of *mcp1* samples (Figure 2E-G, Supplemental Figure S2B). Less unlabelled pyruvate
190 was exported by *mcp1* mitochondria compared to wildtype, indicating that more NAD-ME-
191 generated pyruvate was consumed by *mcp1* mitochondria than that by wildtype and the *mcp1*
192 complemented line (Figure 2H). This suggests that MPC1 loss-of-function enhanced the rate
193 of NAD-ME-derived pyruvate being oxidised by PDC significantly more than in wildtype.

194 We also included UK-5099, a non-competitive MPC inhibitor (35-38) into our feeding
195 experiments to mimic the effect of knocking out MPC1. Figure 2 showed no significant
196 difference in the usage of pyruvate produced from either NAD-ME or MPC between Col-0,
197 *mcp1* and *mcp1/gMPC1* in the presence of this MPC inhibitor. All UK-5099 treated
198 mitochondria showed a similar pattern of unlabelled pyruvate and citrate export to *mcp1*
199 mitochondrial without UK-5099 treatment (Figure 2F, 2H, Supplemental Figure s2). UK-5099
200 treatment blocked transport and usage of exogenously-provided pyruvate while it enhanced the
201 usage of pyruvate from NAD-ME in Col-0 and *mcp1/gMPC1* in the same way as knockout of
202 MPC in *mcp1*. The flexibility in using NAD-ME-derived pyruvate to provide carbon for the
203 TCA cycle when the primary source of pyruvate is unavailable further confirms that physical
204 channelling is not the cause of the preferential use of imported pyruvate for supporting the
205 TCA cycle.

206 To independently confirm this effect, we conducted a separate label swap mitochondrial
207 feeding experiment with a combination of pyruvate and $^{13}\text{C}_4$ -malate with/without UK-5099.
208 The results consistently showed that *mcp1* mitochondria and UK-5099 treated mitochondria
209 were able to use pyruvate made internally to mitochondria by NAD-ME to drive the TCA cycle,
210 but this did not occur in wildtype or the *mcp1* complemented line (Supplemental Figure S3). It
211 was evidenced by the steady increase in NAD-ME-derived citrate and succinate concentration
212 in the form of $^{13}\text{C}_6$ -citrate and $^{13}\text{C}_4$ -succinate (Supplemental Figure S3B). Taken together,
213 pyruvate from NAD-ME can only be efficiently accessed by PDC when MPC1 is either not
214 present or chemically inactivated. This means there are effectively two mitochondrial pyruvate

215 pools in plant mitochondria that can be accessed by PDC, but it depends on the presence of
216 pyruvate import.

217 **Total amount of TCA cycle metabolites produced from NAD-ME-generated pyruvate** 218 **remained unchanged regardless of genotypic or treatment effect**

219 Although the maximal activity of NAD-ME was shown to be similar in wildtype, *mpc1* and
220 *mpc1/gMPC1* (2), we surmised the difference in unlabelled pyruvate production rate between
221 wildtype and *mpc1* might underlie the variation in metabolic regulation under *in vitro*
222 conditions. In order to assess NAD-ME activity of wildtype, *mpc1* and *mpc1/gMPC1* with and
223 without UK-5099, we calculated and compared the export rates of the downstream products
224 from NAD-ME, namely unlabelled pyruvate, citrate, succinate and 2-oxoglutarate when they
225 were fed with 500 μ M $^{13}\text{C}_3$ -pyruvate and 500 μ M malate at pH 6.4. Unlabelled fumarate and
226 malate derived from this pathway cannot be measured as malate had the same isotopically
227 labelled form as exogenously provided malate, and fumarate production was comparatively too
228 low to be quantifiable. Our results showed that *mpc1* and UK-5099-treated mitochondria
229 displayed no difference in the total concentration of metabolites derived from the NAD-ME
230 pathway (Figure 3B, Supplemental Figure S4A) while they displayed obvious deficiency in
231 total MPC-derived metabolite amount to that of Col-0 (Figure 3A). However, the export rates
232 of individual NAD-ME-derived metabolites were clearly different; wildtype exported the
233 majority as NAD-ME-derived pyruvate whereas *mpc1* and UK-5099 treated mitochondria
234 converted more than half of this pyruvate into TCA cycle metabolites (Figure 3). Swapping
235 from supplying $^{13}\text{C}_3$ -pyruvate and unlabelled malate to $^{13}\text{C}_4$ -malate and unlabelled pyruvate
236 showed consistent results, indicating that malic enzyme activity was similar amongst genotypes
237 and treatments but the pyruvate generated had significantly different fates when MPC1 was
238 absent or non-functional (Supplemental Figure S4B-C). Thus, the bias in pyruvate usage is not
239 due to different pyruvate production rate by NAD-ME.

240 **Alanine aminotransferase (AlaAT) can consume NAD-ME-derived pyruvate but not** 241 **MPC imported pyruvate**

242 Our results so far suggested PDC can prioritise imported pyruvate over NAD-ME-derived
243 pyruvate for generating TCA cycle intermediates. To explore this possibility, we introduced a
244 competitive co-substrate, glutamate, to drive the mitochondrial alanine aminotransferase
245 (AlaAT) in the direction of pyruvate consumption (i.e. pyruvate + glutamate \rightarrow alanine + 2-
246 oxoglutarate) to compete for pyruvate in isolated mitochondria (Figure 4A, 4E) (39). In an

247 equilibrium system, pyruvate imported by MPC or generated by NAD-ME would be converted
248 by AlaAT and reduce the export rate of the corresponding citrate product. If one source of
249 pyruvate is preferential, the export rate of citrate derived from it would be resistant to change.
250 We found glutamate addition to mitochondria significantly deteriorated the unlabelled citrate
251 export rate and pyruvate export rate consistent with competition for unlabelled pyruvate in Col-
252 0 and the complemented line (Figure 4F-G, Supplemental Figure S5B). However, the rate of
253 $^{13}\text{C}_2$ -citrate export, $^{13}\text{C}_2$ -succinate and $^{13}\text{C}_2$ -malate did not change upon the addition of
254 glutamate in all three genotypes, suggesting restricted access to imported pyruvate by AlaAT
255 (Figure 4B-D, Supplemental Figure S5A). These results show that AlaAT is more likely to
256 have access to NAD-ME-derived pyruvate than imported pyruvate in wildtype. In *mpc1*
257 mitochondria, the export rate of unlabelled citrate derived from the NAD-ME pathway was not
258 altered in the presence of glutamate. This suggests that, in the absence of MPC, PDC can access
259 pyruvate from NAD-ME just with a lower rate in the presence of glutamate. Therefore, the
260 preferential use of imported pyruvate for supporting the TCA cycle could be explained by
261 distinct pools of pyruvate accessible to PDC depending on the rate of pyruvate import.

262 Discussion

263 MPC plays an essential role in the mitochondrial pyruvate-supplying pathway by carrying more
264 supply to the TCA cycle *in vivo* than AlaAT or NAD-ME (2). MPC-dependent pyruvate import
265 alone can support normal growth and development of Arabidopsis seedlings without the
266 presence of the other two pathways, but not AlaAT or NAD-ME alone (2) which lead to
267 significant growth impairments. Here we show a regulatory mechanism exists to kinetically
268 prioritise MPC over other pyruvate-supplying pathways. Specifically, we provided genetic and
269 biochemical evidence for Arabidopsis mitochondria preferring imported pyruvate via MPC for
270 the TCA cycle operation over pyruvate synthesised inside the mitochondrial matrix by NAD-
271 ME. Our data indicated that imported pyruvate and matrix-derived pyruvate effectively operate
272 as two independent pools that do not homogeneously mix in the mitochondria. The rate of
273 contribution of different pathways to the mitochondrial pyruvate pool could not explain the
274 substantial bias of imported pyruvate usage over malate-derived pyruvate usage (Figure 1 and
275 4). The NAD-ME pathway provided about 90% of the total citrate in *mpc1* whereas MPC
276 accounted for more than 90% of the total citrate in *me1.me2* (Supplemental Figure S6). When
277 both pathways were available, we would expect their relative contribution to the TCA cycle to
278 be much more evenly distributed. However, we found that 80% of citrate was still derived from
279 MPC-transported pyruvate (Supplemental Figure S6).

280 **Do MPC and PDC form a metabolon?**

281 Transient metabolons regulating the metabolic flux through the association and dissociation of
282 components were observed for the glycolytic pathway in mammals, yeast, and plants (40-42),
283 polyamine metabolism in plants (43), and secondary metabolic pathways in plants (44-47). Our
284 results could be interpreted as preliminary evidence for a metabolon existing between enzymes
285 of the mitochondrial pyruvate-supplying pathways in response to metabolic regulation and
286 substrate availability. However, to date there is no protein-protein interaction data to support a
287 physical association between MPC and any of the subunits of PDC, despite extensive studies
288 being performed to define mitochondrial protein-protein interactions in plants, yeasts and
289 mammals. These have included the use of affinity purification-mass spectrometry (AP-MS),
290 cross-linking mass spectrometry (XL-MS), proximity-dependent biotinylation, biomolecular
291 fluorescent complementation assay (BiFC), split luciferase, and Y2H screening assays (15, 25-
292 32). Also, quantitative proteomics in Arabidopsis mitochondria has shown the abundance of
293 PDC subunits are all ~5 times greater than MPC1 (48), hence at least 80% of PDC could not
294 be associated with MPC1 at any one time. This is inconsistent with a hypothesis that MPC1
295 could physically associate with all PDC catalytic sites and prevent interaction with pyruvate
296 from other sources. Rather, it appears more likely that heterogeneous zones inside
297 mitochondria may be the reason for the observed separation of pyruvate pools. PDC has been
298 suggested to have close association with the inner membrane as PDC isolation is more effective
299 with detergents in animals (49, 50) and a higher portion of PDC remains associated with
300 mitochondrial membranes in plants than other TCA cycle enzymes (51) while NAD-ME is a
301 soluble enzyme that is free in the matrix in animals (52-54) and plants (7, 51). Additionally,
302 immunolabelling studies in human cell cultures show that PDC is heterogeneously distributed,
303 being found in clusters within the matrix (55).

304 Hypotheses that there are multiple pyruvate pools were evident in literature long before the
305 pyruvate transporter itself was identified. U-¹³C-glucose or U-¹³C-lactate labelling in
306 mammalian cells was used historically to develop models of compartmentation of
307 mitochondrial metabolism that also suggests separate pyruvate pools. Most notably one
308 originating from glucose and is used for releasable citrate, and another pool of pyruvate that
309 seemed to function as a substrate for the TCA cycle (21). In *Saccharomyces cerevisiae*, there
310 is also evidence that pyruvate from different origins is used for different purposes, i.e.
311 exogenous pyruvate goes to PDH in mitochondria while glycolytic channels provide pyruvate
312 to pyruvate decarboxylase in the cytosol (56). In *Tetrahymena*, pyruvate-derived acetyl-CoA,

313 the product of PDC activity, is independent from acetate-derived acetyl-CoA pool (57).
314 Pyruvate-derived CoA is oxidised for respiration, while acetate-derived CoA is used for fatty
315 acid synthesis via citrate and fatty-acid derived acetyl-CoA is used for gluconeogenesis via
316 malate (58). Our results show that NAD-ME-derived pyruvate and imported pyruvate do not
317 mix in the mitochondrial matrix using an *in organello* system.

318 **The potential impact of pyruvate pools on primary metabolism**

319 Metabolic pathways with separate pools of metabolic intermediates occur in all three processes
320 of respiration; glycolysis, TCA cycle and the electron transport chain. A set of respiration-
321 specialised metabolite pools insulated from other metabolic processes and enabling rapid and
322 efficient energy production, is therefore a key feature of living organisms. There is good
323 evidence that glycolytic enzymes interact with VDAC proteins of the outer mitochondrial
324 membrane by anchoring glycolytic enzymes to the mitochondrial surface (42, 59, 60). Fructose
325 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate are preferred
326 to be consumed in glycolysis rather than diluted into the bulk cytosol as shown by stable isotope
327 dilution experiments in Arabidopsis (60). Our results show that the metabolic preference for
328 respiratory role is also true for imported pyruvate in the mitochondria. Most of pyruvate made
329 by glycolysis readily enters the TCA cycle to immediately generate reducing power for ATP
330 production without being used by other competing pathways (61). Similarly, in glial cells the
331 pyruvate pool with glycolytic origin, is more closely related to mitochondrial pyruvate, which
332 is oxidized via TCA cycle activity (62). Based on the rate of citrate export, imported pyruvate
333 is directed with high efficiency as consistently over 80% of citrate was made from imported
334 pyruvate (Supplemental Figure S6) to ensure the respiration efficiency.

335 An important component of metabolic regulation is specialization. Our results show that in
336 plants, the imported pyruvate pool is designated to provide a carbon backbone to make citrate,
337 whereas NAD-ME-derived pyruvate is destined to be exported to the cytosol for other cellular
338 roles (Figure 5). The activity of NAD-ME and the mitochondrial pyruvate exporter ensures the
339 integrity of the photosynthetic metabolism in C4 plants by the recycling of carbon
340 intermediates. The export of NAD-ME-derived pyruvate from mitochondria is essential for
341 PEP synthesis in the chloroplasts to accept CO₂ in the mesophyll cells (63-66). NAD-ME
342 releases CO₂ from malate which allow carbon incorporation by Rubisco in the bundle sheath
343 cells. The whole process helps to minimize photorespiration and energy wastage, thereby
344 increasing plant yield. While the identity of the plant mitochondrial pyruvate exporter is

345 currently unknown, our results suggest that the metabolic arrangement of pyruvate pools
346 already operate in C3 plants to facilitate the non-mitochondrial usage of NAD-ME-derived
347 pyruvate (67-69), albeit at a much lower rate than in C4 plants (70). In C3 plants like rice and
348 Arabidopsis, pyruvate generated by NAD-ME from imported malate is exported to be recycled
349 into phosphoenolpyruvate in the cytosol and plastids to restore the pH balance in response to
350 water stress (71, 72). Moreover, pyruvate exported from mitochondria is potentially the main
351 source of acetyl CoA to synthesise fatty acids in plastids for generating essential cellular
352 components and signals (73-75). In summary, our data shows a process by which there is a
353 specialized role of NAD-ME-derived pyruvate, when MPC is operating, not to supply
354 substrates for respiration but to maintain photosynthesis, biosynthesis and potentially mediate
355 metabolic stress responses. This metabolic specialisation explains the contribution of NAD-
356 ME to reduction of the matrix NADH pool and thus some respiratory flux in Arabidopsis and
357 other plants (6-8) but argues that the assumption of NAD-ME being a main source of pyruvate
358 for the TCA-cycle is not generally applicable.

359 Our findings show that pyruvate pools are both separated but also metabolically flexible,
360 proven by genetics and inhibitors. This metabolic distinction allows individual turn over, flux
361 regulation, equilibrium and hence specialised response of each metabolic pool to cellular and
362 environmental stimuli. The presence of distinct mitochondrial pyruvate pools due to
363 mitochondrial anatomical and biochemical features suggests similar regulation of other
364 mitochondrial metabolites could exist in order to improve the efficiency and dynamic nature
365 of the respiratory pathway (76). It has been suggested previously that there could be two
366 different malate pools in plant mitochondria when castor bean mitochondria were incubated
367 with ¹⁴C-pyruvate and malate. TCA cycle-generated ¹⁴C-malate is exported as no appreciable
368 radiolabel can be found in CO₂ (77). The existence of a MDH-CS metabolon also indicates that
369 there could be at least two pools of malate, one generates OAA for the TCA cycle and the other
370 consumed by other pathways such as NAD-ME (12-15). There is also a prima-facie case for at
371 least two pools of citrate due to a citrate synthase–citrate exporter interaction (78). Metabolic
372 plasticity is not compromised by having two mitochondrial pyruvate pools. By having a
373 flexible secondary pyruvate pool, plant mitochondria have the ability to use NAD-ME-derived
374 pyruvate to generate TCA intermediates as a fine-tuning regulation mechanism of pyruvate
375 metabolism rather than being alternative substrates for respiration. Understanding the
376 conditions and mechanism that enable NAD-ME contribution to the TCA cycle metabolism *in*

377 *vivo* will be beneficial for the incorporation of the superior C4 characteristics into future C3
378 crops.

379 **Materials and Methods**

380 **Plant material and growth conditions**

381 The MPC1 T-DNA insertion line SALK008465 was obtained from the Arabidopsis Biological
382 Resource Center (<https://abrc.osu.edu/>). *mpc1* and *mel.me2* seeds were previously
383 characterised and published (2, 7) and *mel.me2* seeds were obtained from Professor Verónica
384 G. Maurino (University of Bonn). *mel.me2.mpc1* and *mpc1/gMPC1* were generated and
385 confirmed as described previously (2).

386 Arabidopsis seeds were surface sterilized and dispensed into one-half strength Murashige and
387 Skoog liquid media (1.1g/L agar, 0.4 g/L MES, 10g/L sucrose) within enclosed, sterilized 100
388 mL polypropylene containers. The containers were rotated on the shaker in the long-day
389 conditions with 16 hours light, 8 hours dark and 60% humidity (110 $\mu\text{mol s}^{-1} \text{m}^{-2}$ light
390 intensity with tubular fluorescent lighting) and seedlings were harvested after two weeks.

391 **Isolation of mitochondria**

392 Mitochondria were isolated from 2-week-old Arabidopsis seedlings as described previously
393 (79).

394

395 **Substrate feeding of isolated mitochondria**

396 The detailed methods and materials for MS-based mitochondria feeding assays are described
397 previously (80). In short, 100 μg isolated mitochondria were mixed with substrates (a mixture
398 of pyruvate and malate), cofactors (2mM NAD^+ , 0.2 mM TPP and 0.012 mM CoA) and 1mM
399 ADP (for ATP synthesis) in a final volume of 200 μl . At specified time, this reaction mixture
400 was layered on top of silicon oil (AR200, 100 μl) which was layered above the stopping sucrose
401 solution (0.5 M sucrose, pH 1.0). Substrate transport was stopped by rapid centrifugation (12
402 000 g for 3 min) to harvest the mitochondria at the bottom of the tube. 5 μl of the extra-
403 mitochondrial medium (the top layer) was collected and extracted for quantitative analysis by
404 LC-SRM-MS.

405 **Analyses of metabolites by LC-SRM-MS**

406 Samples were analysed by an Agilent 1100 HPLC system coupled to an Agilent 6430 Triple
407 Quadrupole (QQQ) mass spectrometer equipped with an electrospray ion source as described
408 previously (2). Chromatographic separation was performed on a Kinetex C18 column, using
409 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) as the
410 mobile phase for binary gradient elution. The elution gradient was 18% B at 1 min, 90% B at
411 10 min, 100% B at 11 min, 100% B at 12 min, 18% B at 13 min, and 18% B at 20 min. The
412 column flow rate was 0.3 mL/min; the column temperature is 40 °C, and the autosampler was
413 kept at 10 °C. Selective reaction monitoring (SRM) transitions for targeted TCA cycle
414 metabolites and their isotopically labelled versions are shown previously (2). Data acquisition
415 was performed using Agilent MassHunter Workstation Data Acquisition software. Metabolite
416 quantitation of both unlabelled and labelled metabolites was carried out based on calibration
417 curves obtained with unlabelled authentic standards and normalized against internal standards.

418 **Interactome analyses**

419 The improved Y2H high-throughput binary interactome mapping liquid pipeline described (81)
420 is an adaptation of a previously developed interactome (82). The same low copy number yeast
421 expression vectors expressing DB-X and AD-Y hybrid proteins and the two yeast two hybrid
422 strains, *Saccharomyces cerevisiae* Y8930 and Y8800 were used. The reporter genes GAL2-
423 ADE2 and LYS2::GAL1-HIS3 are integrated into the yeast genome. Expression of the GAL1-
424 HIS3 reporter gene was tested with 1 mM 3AT (3-amino-1,2,4- triazole, a competitive inhibitor
425 of the HIS3 gene product). Y8800 MAT α and Y8930 MAT α yeast strains were transformed
426 with AD-Y and DB-X constructs, respectively and DB-X strains were tested for to be auto-
427 activation of the GAL1- HIS3 reporter gene in the absence of AD-Y plasmid. MPC1 was
428 cloned into DB-X construct acting as baits and screened against 12000 proteins in
429 Arabidopsis library cloned into AD-Y construct prior to Y2H screening.

430 Briefly, DB-X baits expressing yeasts were individually grown (30°C for 72 h) into 50-mL
431 polypropylene conical tubes containing 5 mL of fresh selective media (Sc-Leucine; Sc-Leu),
432 then pooled (max 50 individual bait yeast strains) and 50 μ L plated into 384-well low profile
433 microplates. Glycerol stocks of the (AD)-AtORFeome collection corresponding to 127 96-well
434 plates were thawed, replicated using the colony picker Qpix2 XT into 32 384-well plates filled
435 with 50 μ L of fresh selective media (Sc-Tryptophane; Sc-Trp) and incubated at 30 °C for 72 h.
436 Culture plates corresponding to the DB-baits pools and AD-collection were replicated into
437 mating plates filled with YEPD media and incubated at 30 °C for 24 h. Mating plates were

438 then replicated into screening plates filled with 50 μ L of fresh Sc-Leu-Trp-Histidine + 1 mM
439 3AT media and incubated at 30 °C for 5 days. Only diploid yeast with interacting couples can
440 growth in this media. In order to identify primary positives, the OD600 of the 384-well
441 screening plates was measured using a microplate-reader Tecan Infinite M200 PRO. Yeast
442 cultures identified as positive interactions were picked from selective media and protein pairs
443 were identified by de-pooling of DB-baits in a targeted matricial liquid assay in which all the
444 DB-baits were individually tested against all the positive AD-proteins. Identified pairs were
445 cherry-picked and checked by DNA sequencing.

446 **Statistical analysis**

447 All statistical analyses were performed using the two-sided *t* test function built in Excel 2010.
448 Statistical tests and the number of biological replicates are indicated in figure legends.
449 Biological replicates indicate samples that were collected from different batches of plants
450 grown under the same conditions except biological replicates for transcript analysis and
451 metabolite analysis were samples collected from different plants grown at the same time.

452

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458

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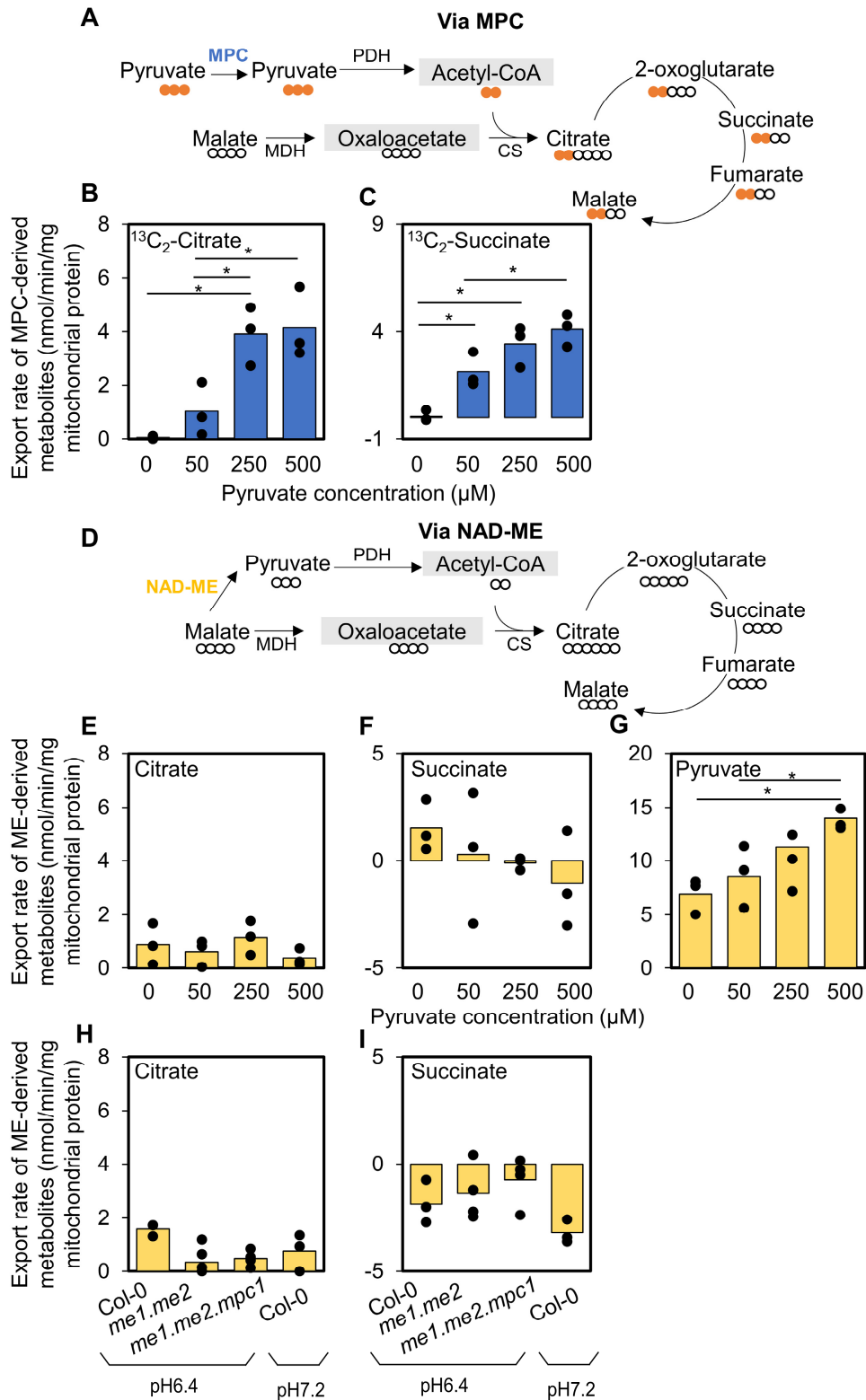
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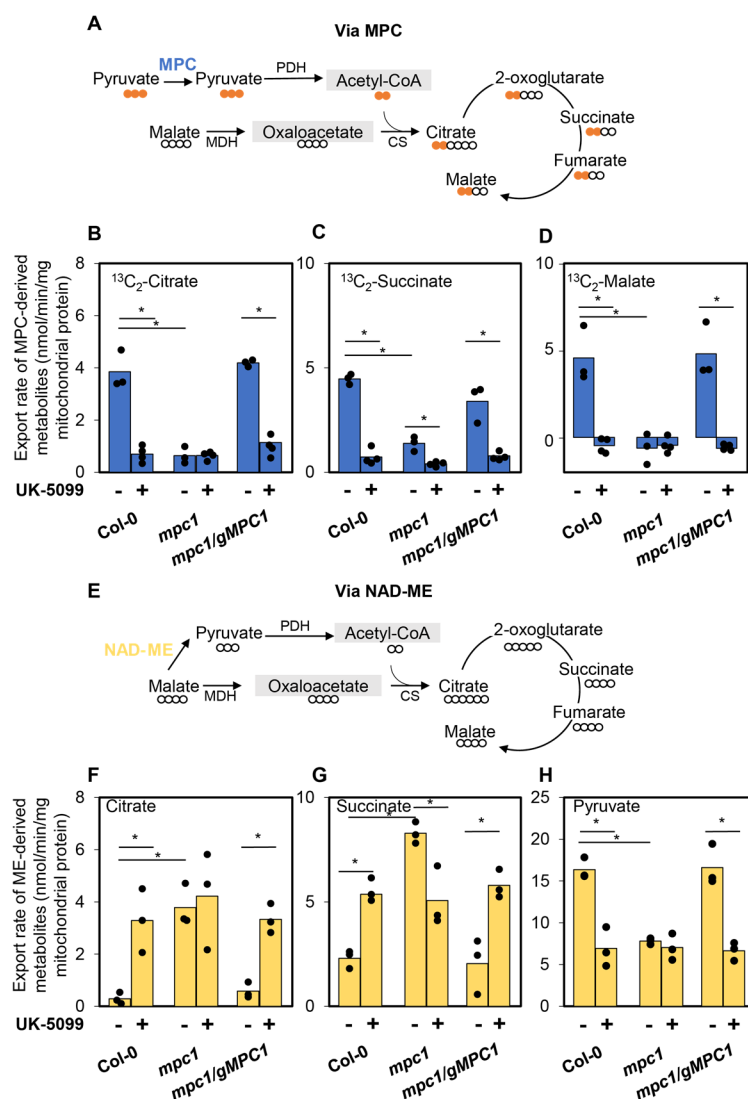
669 **Figures**



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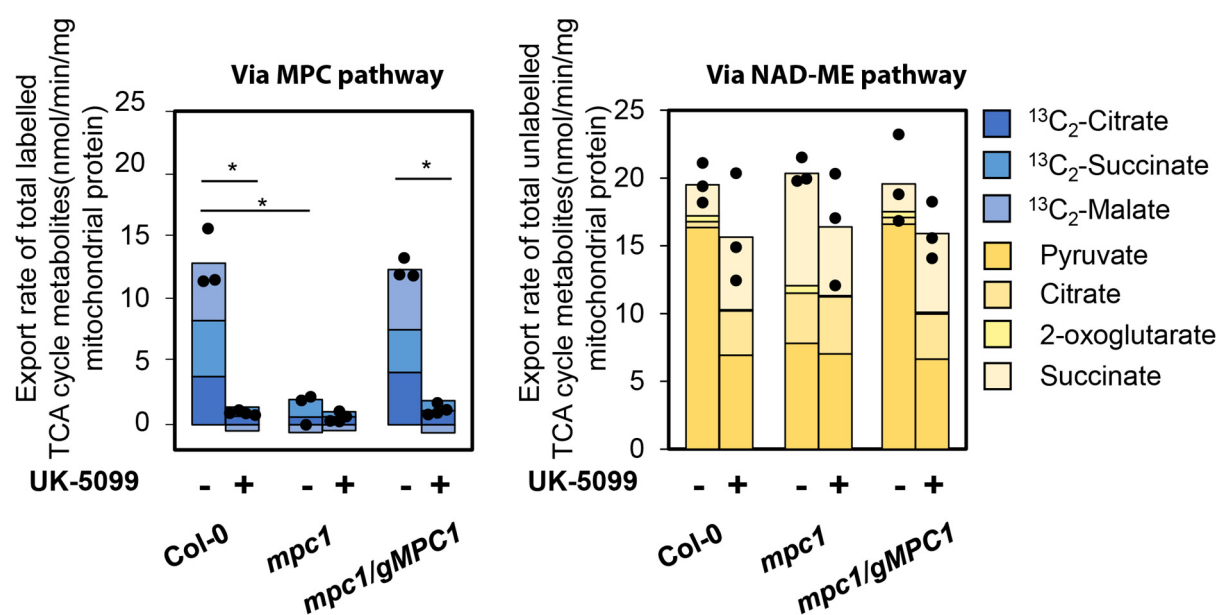
672 **Figure 1. The usage of imported pyruvate for TCA cycle is preferred to that of NAD-ME-derived**
673 **pyruvate by Col-0.** (A-G) Mitochondria were incubated and 0, 50, 250 and 500 μM of $^{13}\text{C}_3$ -pyruvate
674 in the presence of 500 μM malate and ADP at pH 6.4 to increase ME activity. The isotopic incorporation
675 patterns of labelled pyruvate and unlabelled malate into citrate via MPC (A) and via NAD-ME (D) are
676 shown. Bar graphs show export rates of (B) $^{13}\text{C}_2$ -citrate (via MPC), (C) $^{13}\text{C}_2$ -succinate (via MPC); (E)
677 citrate (via NAD-ME), (F) succinate (via NAD-ME), (G) pyruvate (via NAD-ME) of Col-0
678 mitochondria. (H-I) Col-0, *mel.me2* and *mel.me2.mpc1* mitochondria were incubated in a mixture of
679 500 μM malate and 500 μM $^{13}\text{C}_3$ -pyruvate at pH6.4 and pH7.2. Bar graphs compare the export rates of
680 (H) unlabeled citrate (via NAD-ME), (I) unlabeled succinate (via NAD-ME) of Col-0 versus mutant
681 mitochondria. Quantification was carried out using SRM-MS to directly assess substrate consumption
682 and product generation of substrate-fed mitochondria after separating mitochondria from the extra-
683 mitochondrial space by centrifugation through a single silicon oil layer. The rates were calculated from
684 time course values of metabolite concentration recorded in the extra-mitochondrial space after varying
685 incubation periods. Each bar represents averaged value from three or more replicates represented by
686 data points. Significant differences between different pyruvate concentrations and between wildtype
687 and mutants are denoted by asterisks based on Student's t-tests (*, $p < 0.05$). Abbreviations: PDH -
688 Pyruvate dehydrogenase, MDH – Malate dehydrogenase, CS – Citrate synthase.



689

690 **Figure 2: The loss of MPC1 changed the pyruvate usage pattern for generating TCA cycle**
 691 **intermediates.** Col-0, *mpc1* and *mpc1/gMPC1* mitochondria were incubated in a mixture of 500 μM
 692 malate and 500 μM $^{13}\text{C}_3$ -pyruvate at pH6.4. The isotopic incorporation patterns of labelled pyruvate and
 693 unlabelled malate into citrate via MPC (A) and via NAD-ME (E) are shown. Bar graphs show export
 694 rates of (B) $^{13}\text{C}_2$ -citrate (via MPC), (C) $^{13}\text{C}_2$ -succinate (via MPC), (D) $^{13}\text{C}_2$ -Malate (via MPC); (F) citrate
 695 (via NAD-ME), (G) succinate (via NAD-ME), (H) pyruvate (via NAD-ME). Quantification was carried
 696 out using SRM-MS to directly assess substrate consumption and product generation of substrate-fed
 697 mitochondria after separating mitochondria from the extra-mitochondrial space by centrifugation
 698 through a single silicon oil layer. The rates were calculated from time course values of metabolite
 699 concentration recorded in the extra-mitochondrial space after varying incubation periods. Each bar
 700 represents averaged value from three or more replicates represented by data points. Significant
 701 differences between controls and treatments are denoted by asterisks based on Student's t-tests (*, $p <$
 702 0.05).

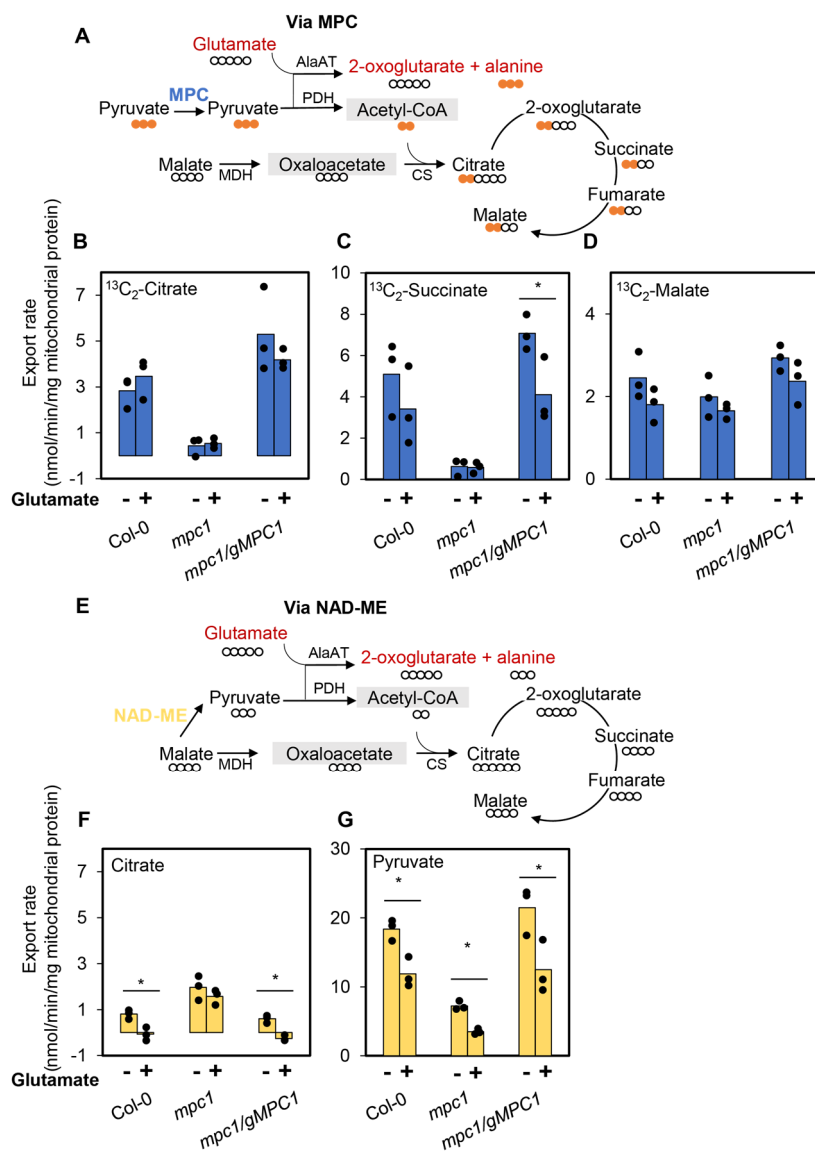
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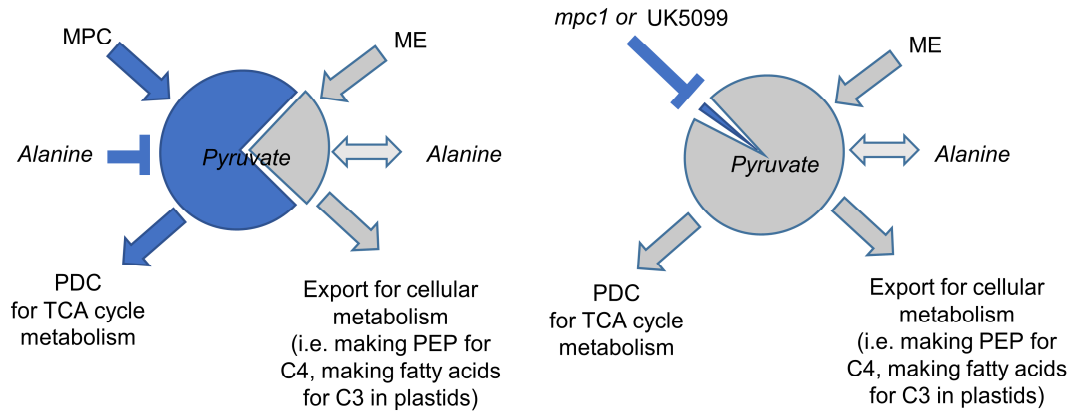
705 **Figure 3. The rate of accumulation of total extra-mitochondrial NAD-ME-derived metabolites in**
 706 **mitochondrial feeding experiments.** Bar graphs show the export rates of total extra-mitochondrial
 707 amount of MPC-derived metabolites (A) and NAD-ME-derived metabolites (B) when Col-0, *mpc1* and
 708 *mpc1/gMPC1* mitochondria were fed with 500 μM $^{13}\text{C}_3$ -pyruvate and 500 μM malate at pH 6.4 with and
 709 without UK-5099. Quantification was carried out using SRM-MS to directly assess substrate
 710 consumption and product generation of substrate-fed mitochondria after separating mitochondria from
 711 the extra-mitochondrial space by centrifugation through a single silicon oil layer. The total amount of
 712 MPC-derived metabolites were the sum of $^{13}\text{C}_2$ -citrate, $^{13}\text{C}_2$ -succinate, $^{13}\text{C}_2$ -malate. The total amount of
 713 NAD-ME metabolites were the sum of unlabelled pyruvate, citrate, 2-oxoglutarate and succinate. The
 714 rates were calculated from time course values of metabolite concentration recorded in the extra-
 715 mitochondrial space after varying incubation periods. Each stacked bar represents averaged value from
 716 three or more replicates. Data points represented the total amount of metabolites exported of
 717 independent replicates. Significant differences between controls and treatments are denoted by asterisks
 718 based on Student's t-tests (*, $p < 0.05$).

719



720

721 **Figure 4. The impact of removal of pyruvate by AlaAT on citrate production in Col-0, *mpc1* and**
 722 ***mpc1/gMPC1* mitochondria.** Mitochondria incubated with 500 μM malate and 500 μM ¹³C₃-pyruvate
 723 with and without 500 μM glutamate at pH 6.4. The isotopic incorporation patterns of labelled pyruvate
 724 and unlabelled malate into citrate via MPC (A) and via NAD-ME (E) are shown. Bar graphs show
 725 export rates of (B) ¹³C₂-citrate (via MPC), (C) ¹³C₂-succinate (via MPC), (D) ¹³C₂-malate (via MPC);
 726 (F) citrate (via NAD-ME), (G) pyruvate (via NAD-ME). Quantification was carried out using SRM-
 727 MS to directly assess substrate consumption and product generation of substrate-fed mitochondria after
 728 separating mitochondria from the extra-mitochondrial space by centrifugation through a single silicon
 729 oil layer. The rates were calculated from time course values of metabolite concentration recorded in the
 730 extra-mitochondrial space after varying incubation periods. Each bar represents averaged value from
 731 three or more replicates represented by data points. Significant differences between controls and
 732 treatments are denoted by asterisks based on Student's t-tests (*, p < 0.05).



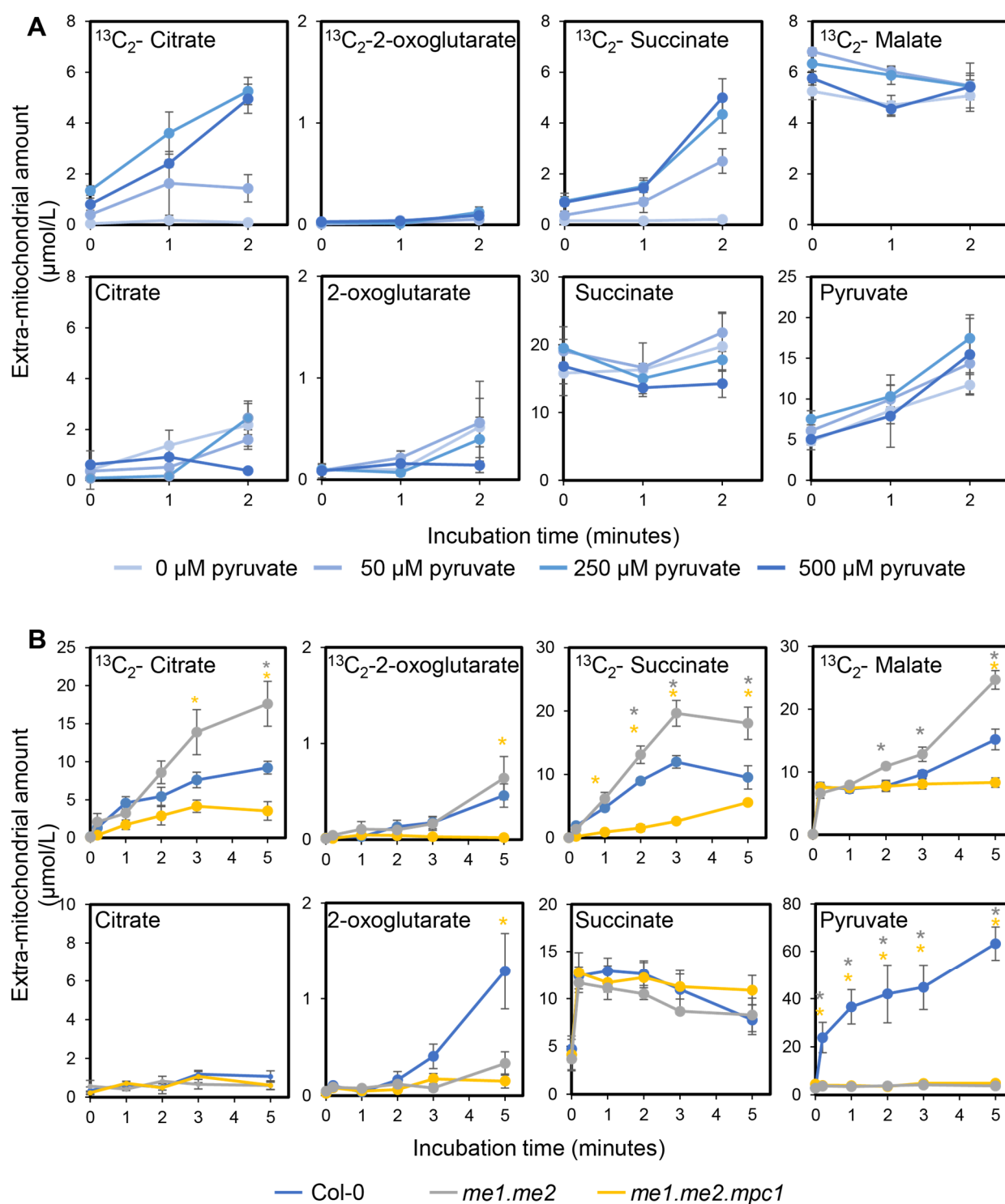
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734 **Figure 5. Schematic presentation of the source and sink of mitochondria pyruvate pools via MPC**
735 **(Blue) and NAD-ME (Grey).** In wildtype plants (left), the imported pyruvate pool is prioritised to be
736 accessed by PDC to make acetyl-CoA which is then condensed with OAA to enter the TCA cycle while
737 most of the NAD-ME-derived pyruvate pool is exported from the mitochondria for other cellular
738 processes. In *mpc1* (or UK5099 treated wildtype plants) the imported pool is no longer available and
739 the NAD-ME-derived pyruvate pool is used for both TCA cycle metabolism and export for other
740 purposes. AlaAT can readily use the NAD-ME-derived pyruvate pool to make alanine in both wildtype
741 and MPC-deficient mitochondria, but cannot access pyruvate imported by MPC.

742

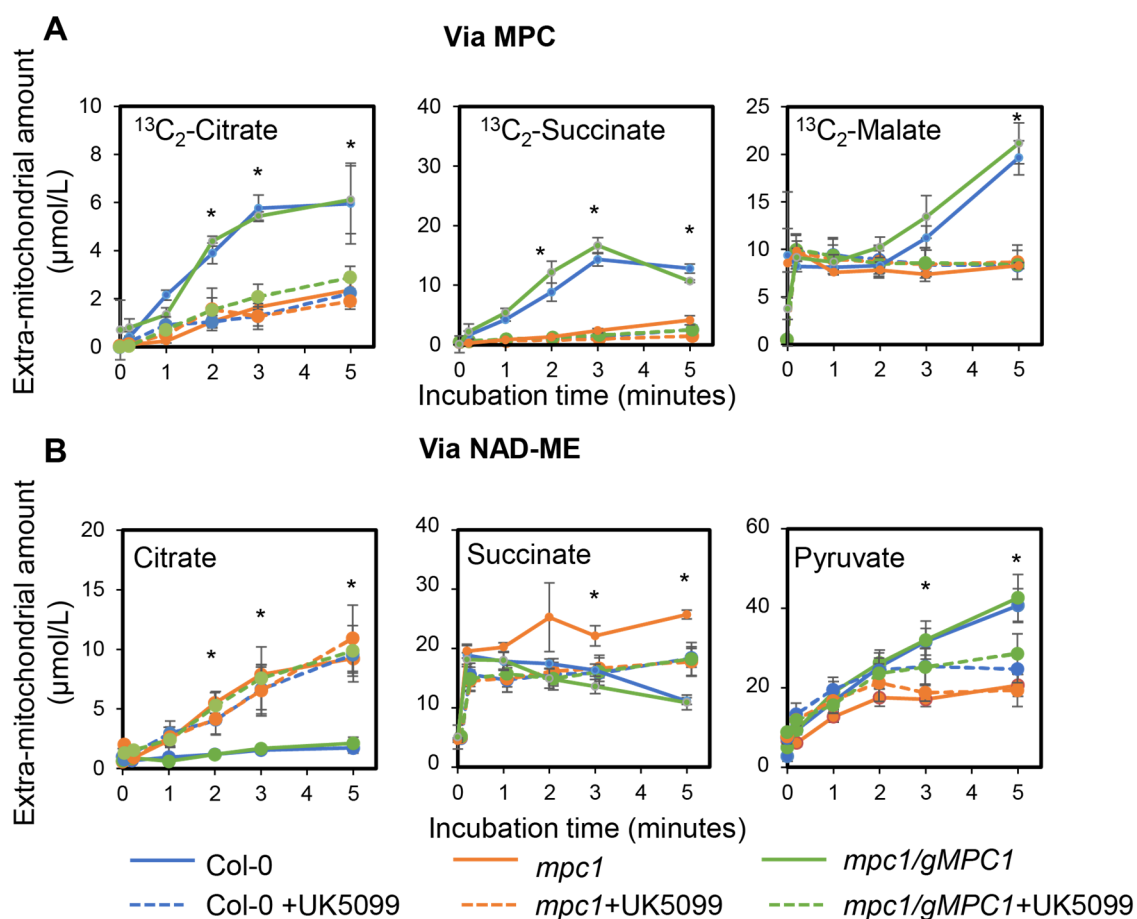
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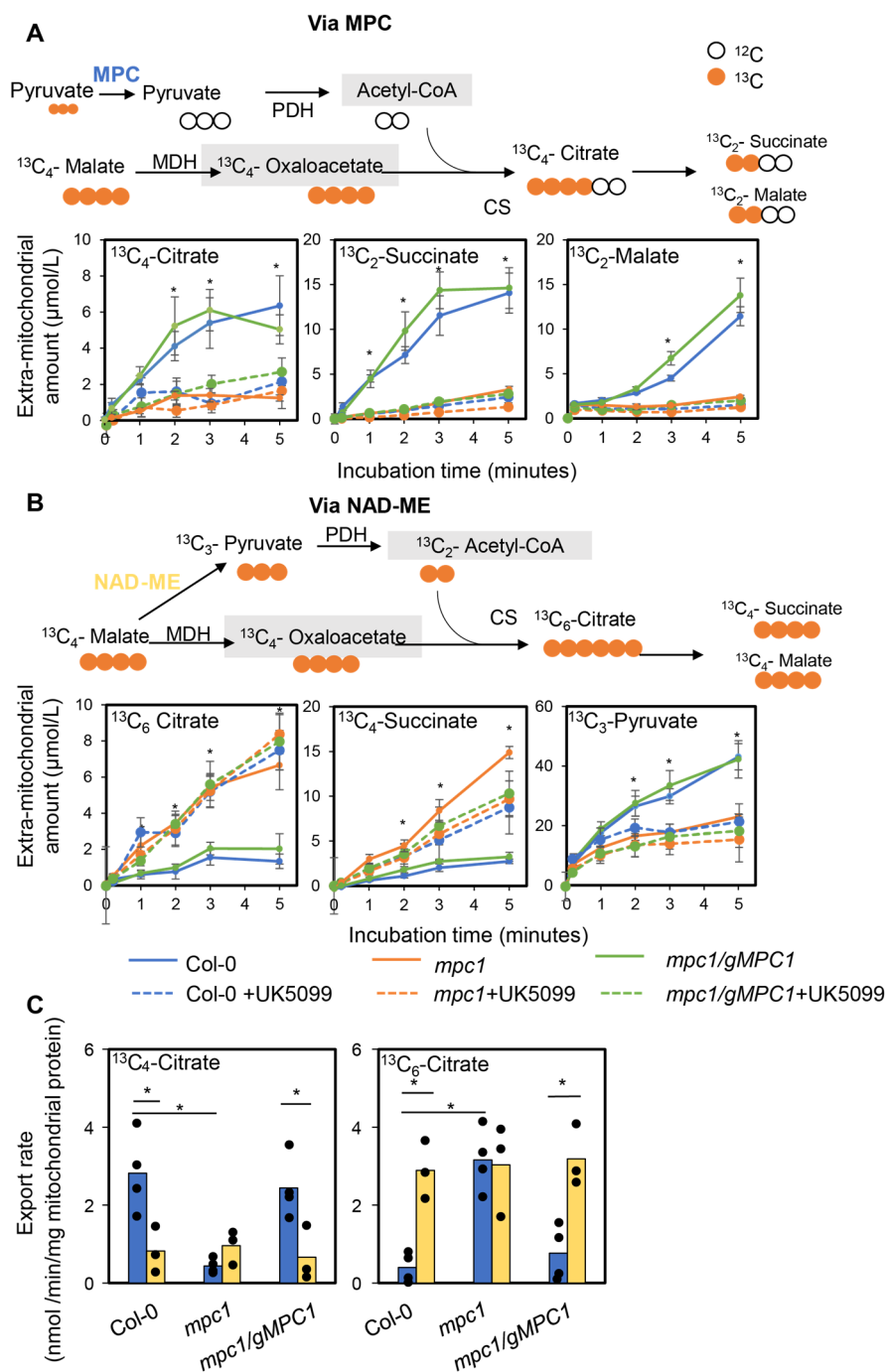
Supplemental Figure S1. ¹³C₃-Pyruvate and malate feeding to isolated mitochondria of Col-0, *me1.me2* and *mpc1.me1.me2*. Time courses of metabolite concentrations in the extra-mitochondrial space of isolated mitochondria incubated with 500 μM ¹³C₃-pyruvate and 500 μM malate via the MPC pathway (A) and via the NAD-ME pathway (B). All experiments were conducted in the presence of ADP at pH 6.4 to initiate substrate uptake and consumption by both pathways. Metabolic reaction was stopped by centrifugation through a single silicon oil layer by which the mitochondrial pellet was separated from the extra-mitochondrial medium. Unused substrate and exported products in the extra-mitochondrial medium were quantified using LC-SRM-MS. Each data point represents averaged value from three or more biological replicates with error bars indicate standard error (n≥3). Significant differences between *mpc1*, Col-0 and *mpc1/gMPC1* are denoted by asterisks based on Student's t-tests (*, p < 0.05). (Supports Figure 1).

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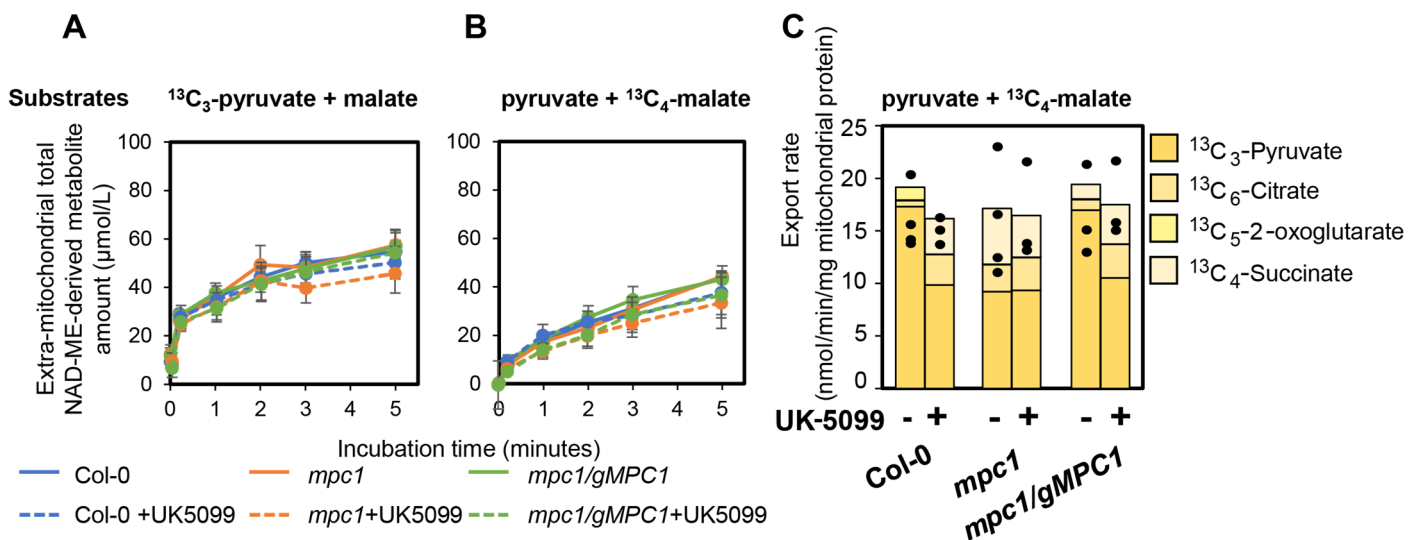
Supplemental Figure S2. $^{13}\text{C}_3$ -Pyruvate and malate feeding to isolated mitochondria of Col-0, *mpc1* and *mpc1/gMPC1*. Time courses of metabolite concentrations in the extra-mitochondrial space of isolated mitochondria incubated with 500 μM $^{13}\text{C}_3$ -pyruvate and 500 μM malate via MPC pathway (A) and via NAD-ME pathway (B). All experiments were conducted in the presence of ADP at pH 6.4 to initiate substrate uptake and consumption by both pathways. Metabolic reaction was stopped by centrifugation through a single silicon oil layer in which the mitochondrial pellet was separated from the extra-mitochondrial medium. Unused substrate and exported products in the extra-mitochondrial medium were quantified using LC-SRM-MS. Each data point represents averaged value from three or more biological replicates with error bars indicate standard error ($n \geq 3$). Significant differences between *mpc1*, Col-0 and *mpc1/gMPC1* are denoted by asterisks based on Student's t-tests (*, $p < 0.05$) (Supports Figure 2).

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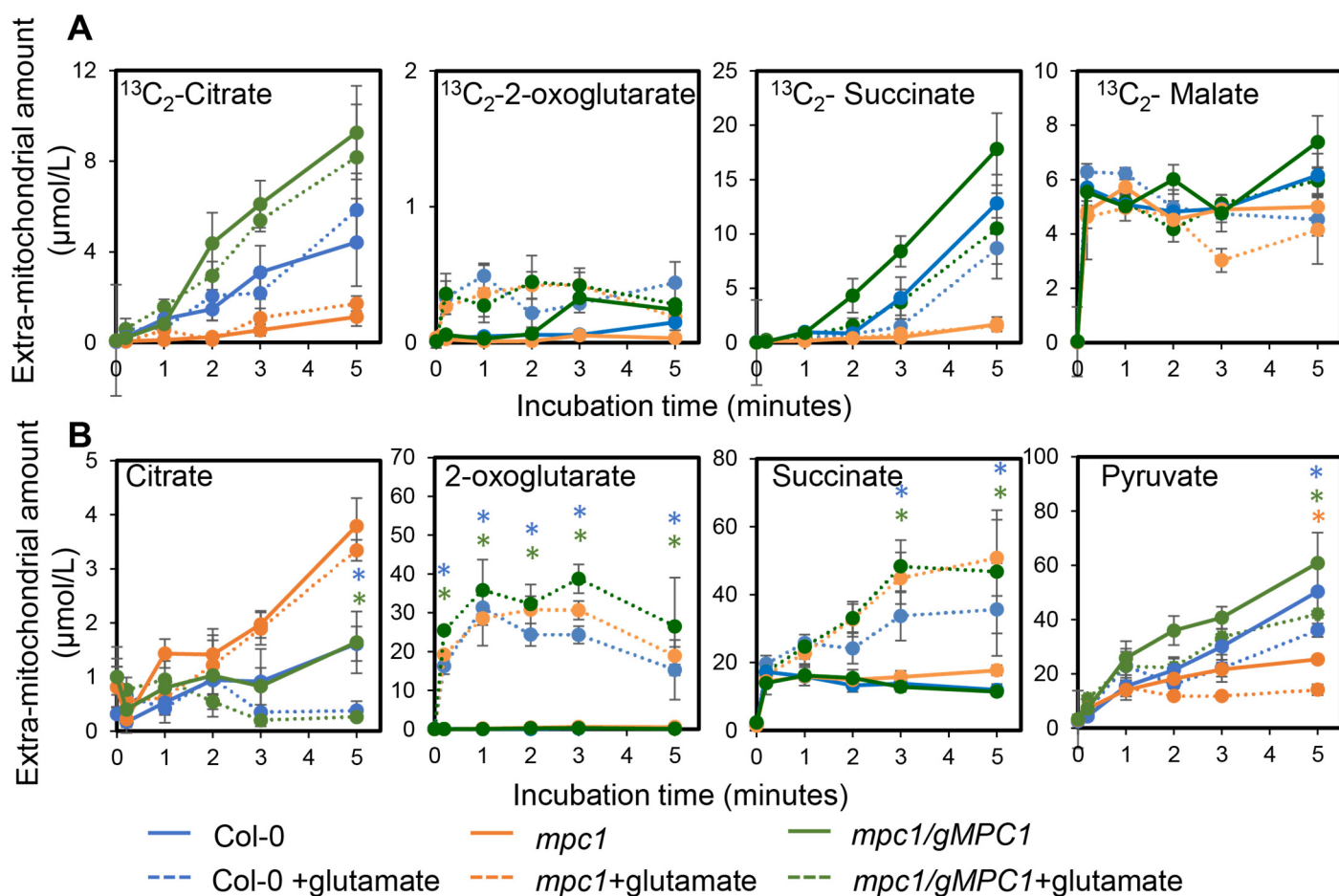
Supplemental Figure S3. Pyruvate and $^{13}\text{C}_4$ -malate feeding to isolated mitochondria of Col-0, *mpc1* and *mpc1/gMPC1*. Time courses of metabolite concentrations in the extra-mitochondrial space of isolated mitochondria incubated with 500 μM pyruvate and 500 μM $^{13}\text{C}_4$ - via MPC pathway (A) and via NAD-ME pathway (B). All experiments were conducted in the presence of ADP at pH 6.4 to initiate substrate uptake and consumption by both pathways. Metabolic reaction was stopped by centrifugation through a single silicon oil layer in which the mitochondrial pellet was separated from the extra-mitochondrial medium. Unused substrates and exported products in the extra-mitochondrial medium were quantified using LC-SRM-MS. Each data point represents averaged value from three or more biological replicates with error bars indicate standard error ($n \geq 3$). Significant differences between *mpc1*, Col-0 and *mpc1/gMPC1* are denoted by asterisks based on Student's t-tests (*, $p < 0.05$) (C) Bar graphs show the rates calculated from time course values of metabolite concentration recorded in the extra-mitochondrial space after varying incubation periods. Each bar represents averaged value from three or more replicates represented by data points. Significant differences between controls and treatments are denoted by asterisks based on Student's t-tests (*, $p < 0.05$). (Supports Figure 2).

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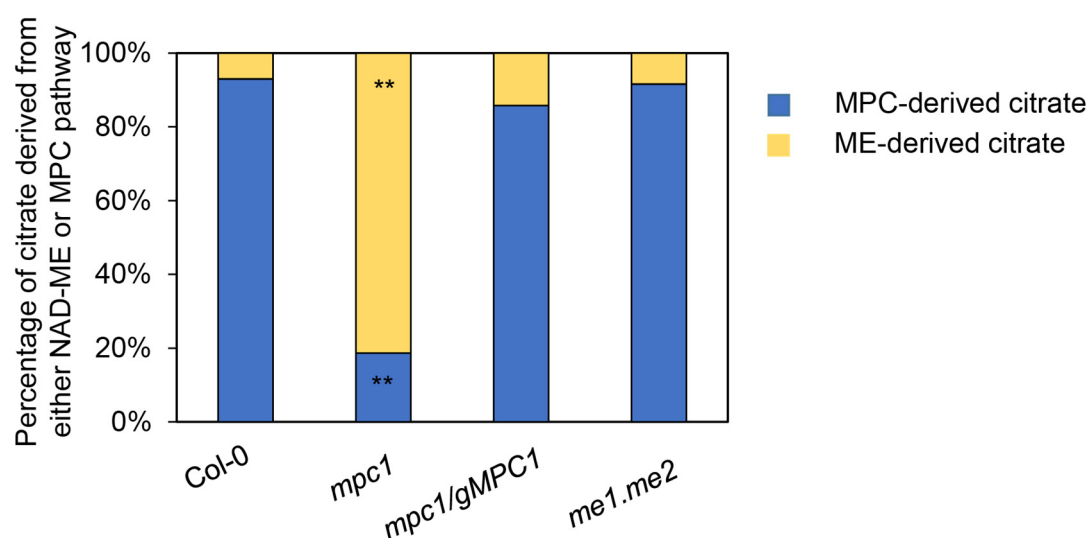
Supplemental Figure S4. The total amount and the rate of metabolites exported from mitochondria that were made via the NAD-ME pathway. The total amount of NAD-ME derived metabolites was calculated from time course experiments of either pyruvate and $^{13}\text{C}_4$ -malate feeding (A, including unlabeled citrate, 2-oxoglutarate, succinate, pyruvate) or pyruvate and $^{13}\text{C}_4$ -malate feeding (B, including $^{13}\text{C}_6$ -citrate, $^{13}\text{C}_5$ -2-oxoglutarate, $^{13}\text{C}_4$ -succinate, $^{13}\text{C}_3$ -pyruvate) to isolated mitochondria of Col-0, *mpc1* and *mpc1/gMPC1*. All experiments were performed in the presence of ADP at pH 6.4 to initiate substrate uptake and consumption by both pathways. Metabolic reaction was stopped by centrifugation through a single silicon oil layer in which the mitochondrial pellet was separated from the extra-mitochondrial medium. Unused substrates and exported products in the extra-mitochondrial medium were quantified using LC-SRM-MS. Each data point represents averaged value from three or more biological replicates with error bars indicate standard error ($n \geq 3$). Significant differences between *mpc1*, Col-0 and *mpc1/gMPC1* are denoted by asterisks based on Student's t-tests (*, $p < 0.05$). (C) Bar graphs show the calculated export rate of all metabolites combined which were made from ME-derived pyruvate after 5 minutes feeding the mitochondria with pyruvate and $^{13}\text{C}_4$ -malate. Each stacked bar represents averaged value of the indicated metabolite from three or more replicates. Data points represented the total amount of ME-derived metabolites exported in independent replicates. Significant differences between controls and treatments are denoted by asterisks based on Student's t-tests (*, $p < 0.05$). (Supports Figure 3).

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Supplemental Figure S5. $^{13}\text{C}_3$ -Pyruvate and malate feeding to isolated mitochondria of Col-0, *mpc1* and *mpc1/gMPC1* with or without the addition of glutamate. Time courses of metabolite concentrations in the extra-mitochondrial space of isolated mitochondria incubated with 500 μM $^{13}\text{C}_3$ -pyruvate and 500 μM malate with or without the addition of glutamate. All experiments were conducted in the presence of ADP at pH 6.4 to initiate substrate uptake and consumption via both MPC and NAD-ME pathways. Metabolic reaction was stopped by centrifugation through a single silicon oil layer in which the mitochondrial pellet was separated from the extra-mitochondrial medium. Unused substrate and exported products in the extra-mitochondrial medium were quantified using LC-SRM-MS. Line graphs show the amount of $^{13}\text{C}_2$ -citrate (A), citrate (B) and pyruvate (C) during 5-minute incubation. Each data point represents averaged value from three or more biological replicates with error bars indicate standard error ($n \geq 3$). Significant differences between controls (straight lines) and treatments (dotted lines) are denoted by asterisks based on Student's t-tests (*, $p < 0.05$) (Supports Figure 4).

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Supplemental Figure S6. The proportion of citrate derived from difference sources of pyruvate. Isolated mitochondria incubated with 500 μM $^{13}\text{C}_3$ -pyruvate and 500 μM malate with or without the addition of glutamate in the presence of ADP at pH 6.4 to initiate substrate uptake and consumption to initiate substrate uptake and consumption via MPC and NAD-ME pathways. Metabolic reaction was stopped by centrifugation through a single silicon oil layer by which the mitochondrial pellet was separated from the extra-mitochondrial medium. The amount of citrate (labelled and unlabelled) in the extra-mitochondrial medium were quantified using LC-SRM-MS. Bar graphs show the percentage of $^{13}\text{C}_2$ -citrate (blue) and citrate (yellow) derived from the MPC and NAD-ME pathways, respectively. Each bar is the averaged value from three or more biological replicates ($n \geq 3$). Significant differences between wildtype and mutants are denoted by asterisks based on Student's t-tests (*, $p < 0.05$) (Supports Figure 5).