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Pyrroline-5-carboxylate metabolism protein complex detected in *Arabidopsis thaliana* leaf mitochondria

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1 Pyrroline-5-carboxylate metabolism protein complex detected in *Arabidopsis thaliana* leaf
2 mitochondria

3

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21

22 **Running title**

23 Evidence of a plant pyrroline-5-carboxylate metabolism complex

24

25

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34 **Highlight**

35 This work reports presence of a mitochondrial complex, which includes proline
36 dehydrogenase, pyrroline-5-carboxylate dehydrogenase and ornithine δ -aminotransferase, in
37 the model plant *Arabidopsis thaliana*, upon dark-induced leaf senescence.

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40

41 **Abstract**

42 Proline dehydrogenase (ProDH) and pyrroline-5-carboxylate (P5C) dehydrogenase (P5CDH)
43 catalyze the oxidation of proline into glutamate via the intermediates P5C and glutamate-
44 semialdehyde (GSA), which spontaneously interconvert. P5C and GSA are also intermediates
45 in the production of glutamate from ornithine and α -ketoglutarate catalyzed by ornithine δ -
46 aminotransferase (OAT). ProDH and P5CDH form a fused bifunctional PutA enzyme in
47 Gram-negative bacteria and are associated in a bifunctional substrate channelling complex in
48 *Thermus thermophilus*, but the physical proximity of ProDH and P5CDH in eukaryotes has
49 not been described. Here we report evidence of physical proximity and interactions between
50 *Arabidopsis* ProDH, P5CDH and OAT in the mitochondria of plants during dark-induced leaf
51 senescence when all three enzymes are expressed. Pairwise interactions and localization of the
52 three enzymes were investigated using bimolecular fluorescence complementation (BiFC)
53 with confocal microscopy in tobacco and sub-mitochondrial fractionation in *Arabidopsis*.
54 Evidence for a complex composed of ProDH, P5CDH, and OAT was revealed by co-
55 migration of the proteins in native conditions upon gel electrophoresis. Co-
56 immunoprecipitation coupled with mass spectrometry analysis confirmed the presence of the
57 P5C metabolism complex in *Arabidopsis*. Pull-down assays further demonstrated a direct
58 interaction between ProDH1 and P5CDH. P5C metabolism complexes may channel P5C
59 among the constituent enzymes and directly provide electrons to the respiratory electron chain
60 via ProDH.

61

62 **Keywords:** *Arabidopsis thaliana*; mitochondria, Ornithine δ -aminotransferase; Proline
63 dehydrogenase; Pyrroline-5-carboxylate dehydrogenase; proline catabolism;

64

65 **Abbreviations:** GSA: glutamate semialdehyde; OAT: Ornithine δ -aminotransferase; P5C:
66 pyrroline-5-carboxylate; P5CDH: P5C dehydrogenase; P5CR: P5C reductase; ProDH: Proline
67 dehydrogenase.

68

69 **Introduction**

70 Metabolism of the amino acid proline impacts many cellular processes beyond protein
71 synthesis, including stress responses, osmotic adjustment, protein chaperoning, reactive
72 oxygen species (ROS) signalling, redox homeostasis and energy metabolism (Szabados and
73 Savouré, 2010; Liu and Phang, 2012; Silao *et al.*, 2019; Alvarez *et al.*, 2021; Zheng *et al.*,
74 2021). For example, proline metabolism participates in both biotic (Cecchini *et al.*, 2011) and
75 abiotic (Hayat *et al.*, 2012) stress responses in plants and is critical in cancer reprogramming
76 in humans (Phang, 2019). Proline is also a respiratory fuel for insect flight, for instance in
77 *Apis mellifera* (honeybee) (Teulier *et al.*, 2016).

78 Proline can be synthesized from glutamate, which is first activated by phosphorylation and
79 then reduced to glutamate semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS)
80 using NADPH as cofactor. GSA is in nonenzymatic equilibrium with pyrroline-5-carboxylate
81 (P5C). P5C reductase (P5CR), using NAD(P)H as cofactor, reduces P5C to proline. When
82 plant cells recover from stress, proline is rapidly oxidized by the action of proline
83 dehydrogenase (ProDH), an FAD-dependent enzyme, to give P5C/GSA, which is then
84 oxidized by P5C dehydrogenase (P5CDH) using NAD as cofactor to produce glutamate.
85 Alternatively, proline can be synthesized from ornithine. The δ -amino group of ornithine is
86 transferred to α -ketoglutarate by ornithine δ -aminotransferase (OAT) forming P5C/GSA in
87 equilibrium, which may be converted either to proline by P5CR or to glutamate by P5CDH.
88 This alternative ornithine-derived proline biosynthesis pathway is likely to occur in bacteria,
89 yeast and animals, but it is not clear if it occurs in plants (Mesticelli *et al.*, 1979; Roosens *et*
90 *al.*, 2002; Funck *et al.*, 2008; Zheng *et al.*, 2023).

91 The cellular compartmentalization of enzymes involved in eukaryotic proline metabolism is
92 diverse (Szabados and Savouré, 2010). For proline anabolism in plants, P5CS and P5CR,
93 which are involved in proline anabolism, have been found in the cytosol (Szabados and
94 Savouré, 2010; Funck *et al.*, 2020). However, in humans, P5CS was observed in
95 mitochondria, while P5CR was detected in both mitochondria and cytosol (Fichman *et al.*,
96 2015; Phang, 2019). Proline catabolism has been detected in mitochondria from animals,
97 yeasts, and plants (Fichman *et al.*, 2015). ProDH is localized at the matrix-exposed surface of

98 the mitochondrial inner membrane in several species (Brunner and Neupert, 1969; Elthon and
99 Stewart, 1981; Cabassa-Hourton *et al.*, 2016). In *Zea mays* (maize), P5CDH was found to be
100 associated with the mitochondrial inner membranes (Elthon and Stewart, 1981), while in
101 animals, it is located in the mitochondrial matrix (Brunner and Neupert, 1969). OAT is
102 thought to be localized in the mitochondrial matrix in both animals (Doimo *et al.*, 2013) and
103 plants (Winter *et al.*, 2015).

104 There are interesting differences in the respective protein structures of ProDH, P5CDH and
105 OAT among species. In Gram-negative bacteria, the bifunctional enzyme known as proline
106 utilization A (PutA) has an N-terminal (N-ter) ProDH domain and a C-terminal (C-ter)
107 P5CDH domain. In some bacteria such as *Escherichia coli*, a DNA-binding domain is also
108 present in the N-ter part of the bifunctional enzyme, which enables EcPutA to act as a
109 transcriptional repressor of proline utilization genes (Gu *et al.*, 2004). In some bacteria such
110 as *Thermus thermophilus*, ProDH and P5CDH are monofunctional enzymes, which is also the
111 case in eukaryotes (Tanner, 2008; Servet *et al.*, 2012). OAT is thought to act as a
112 monofunctional enzyme in all kingdoms (You *et al.*, 2012; Anwar *et al.*, 2018).

113 The oligomeric states of ProDH, P5CDH and OAT are notably diverse. Dimeric and
114 tetrameric structures were observed for PutA in bacteria and for ProDH in *Th. thermophilus*
115 and *Trypanosoma cruzi*. A recent discovery is that ProDH proteins form oligomers via their
116 N-terminal α helix. Dimeric, tetrameric and hexameric states have been described for
117 monofunctional P5CDH (Srivastava *et al.*, 2012; Luo *et al.*, 2013; Pemberton and Tanner,
118 2013; Korasick *et al.*, 2019). Monomeric, dimeric, and tetrameric structures have also been
119 reported for OAT (Sekhar *et al.*, 2007; Jortzik *et al.*, 2010; Stránská *et al.*, 2010; Montioli *et*
120 *al.*, 2017).

121 The proximity of proline metabolism enzyme activities may have functional consequences.
122 Dimerization of PutA in *Geobacter sulfurreducens* and *Bradyrhizobium japonicum* enables
123 substrate-channelling to sequester P5C/GSA inside PutA (Srivastava *et al.*, 2010; Singh *et al.*,
124 2014). Similarly, in *Th. thermophilus*, monofunctional ProDH and P5CDH have been
125 reported to interact with each other, forming a similar substrate channel into PutA (Sanyal *et*
126 *al.*, 2015). Interestingly, Ren *et al.* (2018) have shown that a mitochondrial protein, DFR1,
127 physically interacts with ProDH and P5CDH to regulate the catabolic activity pathway in
128 *Arabidopsis* upon drought and cold stresses. Some studies have suggested that the
129 intermediate P5C, most probably in the GSA form, which is shared by proline and ornithine
130 metabolism, is toxic for the cell (Deuschle *et al.*, 2004; Lerma-Ortiz *et al.*, 2016). Indeed,

131 exogenous proline supply has been found to be lethal in both yeast and *Arabidopsis thaliana*
132 (*Arabidopsis*) *p5cdh* mutants due to its ability to induce a rapid increase in reactive oxygen
133 species (ROS) production (Deuschle *et al.*, 2004). P5C/GSA has been reported to have several
134 detrimental effects, including the deactivation of the OAT cofactor pyridoxal phosphate
135 (Farrant *et al.*, 2001), inhibition of respiration, induction of a burst of superoxide anions in
136 mitochondria (Nishimura *et al.*, 2012), and involvement in the apoptosis signaling pathway
137 (Maxwell and Davis, 2000). Consequently, the channelling of P5C/GSA could serve as a
138 mechanism to prevent its toxicity while tightly regulating proline catabolic flux, thereby
139 protecting cells from futile cycling between proline biosynthesis and catabolism. It has been
140 suggested that eukaryotic proline catabolic enzymes may form complexes, as ProDH has been
141 identified as one component of an uncharacterized protein complex in proline-treated
142 *Arabidopsis* (Cabassa-Hourton *et al.*, 2016). However, currently, there is limited evidence
143 regarding the interaction between proline catabolic enzymes and OAT.

144 *Arabidopsis* possesses two distinct and non-redundant isoforms of ProDH, ProDH1 and
145 ProDH2, which share 75% sequence identity (Servet *et al.*, 2012). Additionally, *Arabidopsis*
146 has one isoform each of P5CDH and OAT (Deuschle *et al.*, 2001; Funck *et al.*, 2008). Among
147 these enzymes, P5CDH is abundantly expressed in various organs of *Arabidopsis* under
148 normal growth conditions (Deuschle *et al.*, 2001; Funck *et al.*, 2010). The expression of
149 ProDH1, which encodes the main isoform of ProDH (Funck *et al.*, 2010), and OAT is
150 specifically regulated during different developmental stages and under stress conditions
151 (Kiyosue *et al.*, 1996; Funck *et al.*, 2010). However, it appears that P5CDH exerts the most
152 significant influence among the three enzymes as it is required for maintaining ProDH
153 activity to support proline catabolism and to prevent the toxicity of P5C by diverting it away
154 from OAT (Zheng *et al.*, 2023).

155 Our objective was to investigate the potential colocalization and interaction between
156 *Arabidopsis* ProDH, P5CDH, and OAT enzymes, considering their shared intermediate, P5C.
157 To test the binary interactions among these enzymes, we conducted bimolecular fluorescence
158 complementation (BiFC) assays in *Nicotiana benthamiana*. Proline catabolism can be induced
159 in excised *Arabidopsis* leaves undergoing dark-induced senescence (DIS), where proline acts
160 as an alternative respiratory substrate in response to energy deprivation (Chrobok *et al.*, 2016;
161 Launay *et al.*, 2019). Therefore, we selected DIS as the experimental condition to explore the
162 potential colocalization of ProDH, OAT, and P5CDH in *Arabidopsis* through subcellular
163 fractionation of proteins. To further investigate protein-protein interactions, we employed

164 two-dimensional (2D) blue native (BN) / SDS-PAGE, complexome profiling, co-
165 immunoprecipitation (co-IP), and affinity pull down assays. Our findings provide compelling
166 evidence that a eukaryotic mitochondrial complex, consisting of ProDH1, P5CDH, and OAT,
167 is formed during proline catabolism.

168

169 **Materials and methods**

170 *Plant materials and growth conditions*

171 Wild-type (WT) *Nicotiana benthamiana* (tobacco) plants were grown in growth chambers at
172 22°C with 16-h light/8-h dark cycles with 80-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light for 4 weeks until used for
173 leaf infiltration.

174 *Arabidopsis thaliana* (Arabidopsis) experiments were carried out using the Columbia-0 (Col-
175 0) ecotype. The *prodh1prodh2* double mutant was previously obtained by crossing the
176 respective homozygous single knockout mutants *prodh1-4* (SALK_119334) and *prodh2-2*
177 (GABI_328G05) as described by Cabassa-Hourton *et al.* (2016). The *oat-1* (SALK_033541)
178 and *p5cdh-2* (SALK_018453) single knockout T-DNA insertion mutants have been described
179 previously by Funck *et al.* (2008) and Deuschle *et al.* (2004) respectively.

180 Arabidopsis seeds were sown on soil and grown in growth chambers at 22°C with 16-h
181 light/8-h dark cycles with 80-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light for 4 weeks. Dark-induced senescence
182 (DIS) treatments were performed on excised rosette leaves of 4-week-old Arabidopsis as
183 described before (Launay *et al.*, 2019).

184

185 *Bimolecular fluorescence complementation (BiFC) assays*

186 Arabidopsis *ProDH1* (AT3G30775), *P5CDH* (AT5G62530), *OAT* (AT5G46180) and *SHMT2*
187 (AT5G26780) cDNAs were inserted in the enhanced yellow fluorescent protein (eYFP)
188 vectors pSPYNE173 and pSPYCE(M). pSPYNE173 encodes the N-ter part of eYFP and a
189 Myc epitope tag while pSPYCE(M) encodes eYFP C-ter part and an HA epitope tag (Waadt
190 *et al.*, 2008). All constructs were verified by automated DNA sequencing. *Agrobacterium*
191 *tumefaciens* strain GV3101 was transformed with each plasmid as described in Valsecchi *et*
192 *al.* (2013). mCherry (mt-rk, CD3-991) plasmid was used as a mitochondrial matrix marker
193 (Nelson *et al.*, 2007). Wild-type (WT) *Nicotiana benthamiana* (tobacco) was infiltrated with
194 different combinations of the enzyme-eYFP constructs with the mCherry control as described
195 in Valsecchi *et al.* (2013). Infiltrated tobacco plants were kept in a growth chamber at 22°C
196 with 16-h light/8-h dark cycles.

197 After three-day infiltration, fluorescence was analyzed in two to three leaves from each of the
198 three independently transformed plants and images (8-bit) were collected using a Leica $\times 40$
199 oil immersion objective with an inverted Leica laser scanning confocal microscope TCS SP5
200 II (Leica Microsystems, Heidelberg, Germany). Analyses were performing by triplicate for all
201 constructs, and gave similar results. Infiltrated samples were fixed with fixation buffer (4%
202 (w/v) formaldehyde, 1 \times PBS buffer, 0.01% (v/v) Tween) before acquisition of optical
203 sections with z-spacing steps of 0.08 μm using a Leica $\times 63$ oil immersion objective with the
204 TCS SP5 II Leica confocal microscope. Confocal images of fixed tobacco samples were
205 deconvoluted with the Huygens 3.7 software (Scientific Volume Imaging, Hilversum, the
206 Netherlands).

207 For both fresh and fixed samples, fluorescence of YFP and mCherry was excited using a laser
208 at 514 nm (15-20% laser power) and 561 nm (10-15% laser power), respectively. Detection
209 ranges were set at 520-564 nm for YFP and 590-650 nm for mCherry and the same gain was
210 chosen for all images. All images were captured in a temperature-controlled room at 21°C.

211 The possible detection of a mCherry signal in the YFP detector was carefully assessed.
212 Spectra acquisition ranging from 524 to 740 nm was made at 514 nm excitation. The spectra
213 signatures revealed that the peak of mCherry responding at 514 nm (corresponding in terms of
214 intensity to less than half that of YFP signal) begins only from 575 nm in emission. However,
215 the confocal acquisitions carried out to observe YFP signal use a detection window ranging
216 from 524 nm to 564 nm. Therefore, the minor mCherry signal did not interfere with the YFP
217 signal.

218

219 *Transformation of Arabidopsis with transgenic GFP-fusion constructs*

220 The cDNA sequence of Arabidopsis *P5CDH* (AT5G62530) was modified by site-directed
221 mutagenesis to remove the *BsaI* restriction site, and cloned with the addition of an
222 enterokinase recognition and cleavage site into the pAGM1287 entry plasmid (MoClo Tool
223 Kit, Addgene) (Weber *et al.*, 2011) using the primers described in Supplementary Table
224 S1. TurboGFP (tGFP), a bright dimeric green fluorescent protein (GFP) derived from the GFP
225 of *Pontellina plumata*, was used as a protein tag. The sequence of the tag tGFP was cloned
226 from the pICSL50016 plasmid (MoClo Plant Parts Kit, Addgene) (Engler *et al.*,
227 2014). The C-ter fusion construct *P5CDH-tGFP* was cloned into the destination plasmid
228 pICH86988 (MoClo Tool Kit, Addgene) by Golden Gate Modular cloning to obtain the
229 *35S:P5CDH-tGFP* final construct.

230 The sequence of the mitochondrial targeting signal of mCherry (MTS), used for BiFC
231 experiments was synthesized by GeneCust in the entry plasmid pICH41258 (MoClo Tool Kit,
232 Addgene). The cDNA sequence of tGFP was amplified from the plasmid pICSL80005 using
233 the primers described in Supplementary Table S1, and cloned into the entry plasmid
234 pICH41264 (MoClo Tool Kit, Addgene). Both sequences were then cloned by Golden Gate
235 Modular cloning in the destination plasmid pICH86988 in order to generate a *35S:MTS-tGFP*
236 fusion construct.

237 Vectors were verified by sequencing and then used to transfect *A. tumefaciens* GV3101.
238 Agrobacterium was then used to transform *p5cdh-2* with *35S:P5CDH-tGFP* construct and
239 Col-0 with *35S:MTS-tGFP* construct by the floral-dip method (Bent, 2006). Transformants
240 were selected on MS/2 media with 75 µg/ml kanamycin and transferred to soil for
241 propagation until homozygous lines (*35S:P5CDH-tGFP/p5cdh* and *35S:MTS-tGFP/Col*) were
242 obtained. Functional complementation of the *p5cdh* phenotype by *35S:P5CDH-tGFP* was
243 checked using homozygous seedlings grown in the presence of proline (Supplementary Fig.
244 S1).

245

246 *Protein extraction for BiFC controls*

247 Two to three tobacco leaves infiltrated as for BiFC from independently transformed plants
248 were harvested separately, frozen with liquid nitrogen and ground to fine powder. Frozen
249 powder (100-200 mg) was suspended in 2 volumes of extraction buffer (50 mM DTT, 8 M
250 urea, 5% (w/v) SDS, 40 mM Tris-HCl pH 6.8, 0.1 mM EDTA, 0.4 mg/ml bromophenol blue)
251 supplemented with 1× protease inhibitor cocktail (Merck). Samples were shaken three times
252 for 5 min at room temperature after 5-min intervals on ice. The samples were then centrifuged
253 at 18000 × g for 10 min at 10 °C. Supernatants were transferred into new tubes for SDS-
254 PAGE and western blots.

255

256 *Extraction of crude mitochondria from Arabidopsis and Nicotiana*

257 Crude mitochondria were freshly prepared from 3 g of mature rosette leaves from 4-week-old
258 Arabidopsis after 4 or 5 days of DIS or from three day-infiltrated tobacco leaves as described
259 before (Launay *et al.*, 2019). The mitochondrial protein concentration was determined with
260 the Lowry assay (Lowry *et al.*, 1951) using BSA as a standard.

261

262 *Fragmentation of Arabidopsis mitochondria for sub-mitochondrial fractionation*

263 Mitochondrial matrix and membrane proteins were separated as described before (Cabassa-
264 Hourton *et al.*, 2016) with some modifications. Arabidopsis mitochondria samples (3 mg)
265 were resuspended in 3 ml of sonication buffer (10 mM TES, 1 mM EDTA, 1 mM EGTA and
266 0.2 mM PMSF, pH 7.2). Mitochondria (1.5 ml of suspension) were broken by three freeze-
267 thaw cycles and four 16-s sonication steps using an ultrasonic apparatus (Bioblock Scientific
268 Vibracell 75041). Intact mitochondria were sedimented by centrifugation at $15000 \times g$ for 7
269 min at 4°C and removed. The supernatant was then ultra-centrifuged at $140000 \times g$ for 100
270 min at 4°C (Beckman Coulter Optima™ MAX ultracentrifuge, MLS-50 swinging rotor).
271 The resulting pellet containing mitochondrial inner and outer membranes was resuspended in
272 sonication buffer. The soluble fraction contained proteins of the mitochondrial matrix and
273 inter membrane space. Samples were analysed by SDS-PAGE and western blots.

274

275 *Western blot assays*

276 Protein samples were separated by SDS-PAGE at 100 V at room temperature using either
277 manually cast polyacrylamide gels (8.75% separation gel and 4% stacking gel,
278 acrylamide/bis-acrylamide 29:1, 30% solution, Sigma-Aldrich) or the 7.5% TGX Stain-
279 Free™ FastCast™ Acrylamide Kit (Bio-Rad). Separated proteins in gels were transferred to
280 nitrocellulose or PVDF-LF membranes (Bio-Rad) in a Trans-Blot Semi-Dry Electrophoretic
281 Transfer Cell (Bio-Rad) for 1 h at 100 V using transfer buffer (25 mM Tris-HCl, 192 mM
282 glycine and 20% (v/v) ethanol, pH 8.3). Equal protein loading and integrity of protein
283 samples were checked by Ponceau S staining or Stain-Free channel by ChemiDoc (Bio-Rad).
284 Immunodetection was performed using purified rabbit antibodies directed against either
285 ProDH1 (Cabassa-Hourton *et al.*, 2016), P5CDH (Launay *et al.*, 2019), or OAT (Zheng *et al.*,
286 2023). The quality and purity of mitochondrial protein fractions were checked by western
287 blots using alternative oxidase (AOX; Agrisera) and glycine decarboxylase (GDC; Agrisera)
288 antibodies as controls for mitochondrial membrane and matrix fractions, respectively. Primary
289 antibody binding was detected using either chemiluminescent secondary antibodies (1:1000
290 dilution) and the ECL prime system (GE Healthcare) or using StarBright Blue 520 or
291 StarBright Blue 700 conjugated Goat Anti-Rabbit fluorescent secondary antibodies (1:2500
292 dilution) and the ChemiDoc system (Bio-Rad).

293

294 *Two-dimensional PAGE*

295 Separation of proteins from crude mitochondria was performed by 2D BN / SDS PAGE as
296 described by Wittig *et al.* (2006). Mitochondrial protein samples were solubilized using 1%
297 (w/v) digitonin solubilization buffer (30 mM HEPES, 150 mM potassium acetate, 10% (v/v)
298 glycerol, pH 7.4) to a final protein concentration of 10 µg/µl, incubated for 20 min on ice,
299 then centrifuged (10 min, 18300 × g, 4°C). Insoluble material was discarded and supernatants
300 were analysed.

301 For the BN PAGE, ~1000 µg of mitochondrial protein was solubilized per sample. The
302 resultant supernatant was supplemented with 1 µl of Coomassie Blue loading buffer (750 mM
303 aminocaproic acid, 5% (w/v) Coomassie blue 250 G) per 20 µl. Samples were directly loaded
304 onto 4.5–16% (top to bottom) acrylamide gradient gels (acrylamide 4K-Solution 40%, 32:1)
305 manually cast and electrophoresed using the PROTEAN II xi 2-D cell casting system (Bio-
306 Rad). The same system was used for the second dimension of Tricine SDS-PAGE (Schägger,
307 2006) with 10% acrylamide separation and 8% acrylamide stacking gels. After
308 electrophoresis in both dimensions, gels were either used for western blot or stained with
309 colloidal Coomassie blue (Schägger, 2006) for gel cutting.

310 For the first dimension of clear native PAGE, ~250 µg of mitochondrial protein was
311 solubilized per sample. The resultant supernatant was mixed 3:1 (v/v) with 4 × native PAGE
312 loading buffer (200 mM Tris-HCl pH 6.8, 40% (v/v) glycerol, 0.4% (w/v) bromophenol blue).
313 Samples were directly loaded onto a native 4-15% (top to bottom) acrylamide gradient gel
314 (Mini-PROTEAN TGX Stain-Free precast gel, Bio-Rad) and electrophoresed using the Mini-
315 PROTEAN Tetra cell (Bio-Rad). The second dimension of SDS-PAGE was done using either
316 the TGX Stain-Free™ FastCast™ Acrylamide Kit (7.5% acrylamide, Bio-Rad) or manually
317 cast 8.75% acrylamide gels electrophoresed with the Mini-PROTEAN Tetra cell (Bio-Rad).
318 After 2D electrophoresis, gels were either used for western blot or kept in water for gel
319 cutting.

320

321 *Protein identification by mass spectrometry (MS) and complexome profiling*

322 Gel spots (diameter 1 mm) were cut out from gels after 2D BN/SDS-PAGE or 2D
323 native/SDS-PAGE using sheared 100-µl disposable plastic pipettes. Gel slices (width 1 mm)
324 were cut out from gels after one dimensional (1D) BN-PAGE or native PAGE using a blade,
325 which was thoroughly washed with ethanol for each cut (Senkler *et al.*, 2017). Gel pieces
326 were prepared for MS analyses by tryptic in-gel digestion and peptide extraction, as described
327 before (Senkler *et al.*, 2017).

328 For complexome profiling analysis, a lane of BN gel was dissected into gel slices and proteins
329 of all slices were identified by label free quantitative mass spectrometry (Schröder *et al.*
330 2022). Abundance profiles of proteins along the BN gel lane were analyzed by the NOVA
331 software package (Giese *et al.*, 2015) using the intensity based absolute quantification (IBAQ)
332 values from MS data, which were normalized using maximum normalization and clustered
333 using the Pearson Correlation distance function.

334

335 *Co-immunoprecipitation (Co-IP) and LC-MS/MS analyses*

336 Co-IP experiments were carried out with mitochondrial proteins isolated from either
337 *35S:P5CDH-tGFP/p5cdh* or *35S:MTS-tGFP/Col* leaves for control after 4 days of DIS. The
338 Co-IP assays were repeated three times with *35S:MTS-tGFP/Col* leaves for control and four
339 times with *35S:P5CDH-tGFP/p5cdh* leaves and similar results were obtained.

340 ~800 µg of 1% (w/v) digitonin-solubilized proteins were incubated with equilibrated 50 µl
341 tGFP-Trap agarose (Chromotek) for 2 h at 4 °C with end-over-end mixing. tGFP-Trap
342 Agarose was first saturated with 1% (w/v) BSA then equilibrated in IP buffer (50 mM
343 NaH₂PO₄, 150 mM NaCl, 0.5 mM EDTA, 5% glycerol, pH 7.5) supplemented with 0.1 mM
344 PMSF, protease inhibitor cocktail (Sigma) and 0.1% (w/v) digitonin before use.

345 After incubation, the supernatant was discarded. Beads were washed 4 times with 500 µl of IP
346 buffer supplemented with 0.1% (w/v) digitonin, turning the tubes on a wheel for 5 min at 4°C
347 between each washing step. The beads were resuspended in 1 × Laemmli buffer, boiled for 10
348 min and centrifuged for 2 min at 10000 × g to elute the dissociated proteins. Eluted proteins
349 were subjected to SDS-PAGE and western blotting or analyzed by LC-MS/MS as described
350 below.

351 A 1:1 (v/v) mixture of acetonitrile and 50 mM NH₄HCO₃ was used to distain gel plugs.
352 Proteins in gel pieces were then digested overnight with either sequencing-grade trypsin
353 (Promega) or chymotrypsin (ThermoFisher Scientific) at 37°C in 25 mM NH₄HCO₃ buffer
354 (20 µL final volume with 0.2 µg trypsin or 0.25 µg chymotrypsin). The resulting peptides
355 were loaded and desalted on Evotips (Evosep, Odense, Denmark) according to the
356 manufacturer's procedure.

357 Samples were analyzed on an Orbitrap Fusion mass spectrometer (ThermoFisher Scientific,
358 Waltham, MA, USA) coupled with an Evosep One system (Evosep, Odense, Denmark)
359 operating with the 30SPD method developed by the manufacturer. Briefly, the method is
360 based on a 44-min gradient and a total cycle time of 48 min with a C18 analytical column

361 (0.15 × 150 mm, 1.9 μm beads, ref EV-1106) equilibrated at room temperature and operated
362 at a flow rate of 500 nl/min with H₂O/0.1% formic acid as solvent A and acetonitrile/0.1%
363 formic acid as solvent B. The mass spectrometer was operated in data-dependent MS/MS
364 mode. Peptide masses were analyzed in the Orbitrap cell in full ion scan mode, at a resolution
365 of 120,000, a mass range of *m/z* 350-1550 and an automatic gain control (AGC) target of
366 400,000. MS/MS was performed in the top speed 3s mode. Peptides were selected for
367 fragmentation by higher-energy C-trap dissociation with a normalized collisional energy of
368 27% and a dynamic exclusion of 60 s. Fragment masses were measured in an ion trap in the
369 rapid mode, with an AGC target of 10,000. Monocharged peptides and unassigned charge
370 states were excluded from the MS/MS acquisition. The maximum ion accumulation times
371 were set to 100 ms for MS and 35 ms for MS/MS acquisitions respectively.
372 Raw data processing was done using the PEAKS Studio X_Pro software (Bioinformatics
373 Solutions Inc., Waterloo, ON, Canada). MS/MS spectra were searched against the UniprotKB
374 protein database of *A. thaliana* (UP000006548, release 2020_05, 39345 Entries). A maximum
375 of two missed cleavages were allowed, and precursor and fragment mass tolerances were set
376 to respectively 15 ppm and 0.5 Da. Post-translational modifications (PTMs) were included
377 with carbamidomethyl (C) as fixed and deamidation (NQ), and oxidation (M) as variable. The
378 following variable PTMs were included in a second-round search using the PEAKS PTM
379 module: acetylation (Protein N-term), acetylation (K), methylation (K, R), dimethylation (K,
380 R), trimethylation (K, R) and phosphorylation (STY). The maximum of variable PTMs per
381 peptide was set to 3 and MS/MS spectra were filtered at 1% FDR. Coverage and total
382 intensities of the detected peptides were used to qualitatively assess the presence of
383 differential (i.e. P5CDH interacting) proteins between D0 and D4 conditions.

384

385 *Affinity pull-down assays*

386 *Arabidopsis ProDH1* and *P5CDH* cDNAs were inserted in the pGEX-6P1 and pCDF-Duet
387 vectors respectively, using the primers described in Supplementary Table S1. Primers were
388 designed to remove mitochondrial targeting peptides of ProDH1 and P5CDH. These vectors
389 were compatible for coexpression of multiple genes in *E. coli* (Tolia and Joshua-Tor, 2006).
390 Vectors were verified by sequencing and then used to transform BL21(C41) *E. coli* cells.
391 Overexpression of His-P5CDH alone or with either GST-ProDH1 or GST was achieved in
392 auto-inducing media (Studier, 2005) for 20 h at 16°C.

393 Cells were collected, resuspended in lysis buffer (50 mM NaH₂PO₄, 0.15 M NaCl, 5% Glycerol) supplemented with 0.1 mM PMSF and protease inhibitor cocktail (Sigma). Cells were
394 disrupted by French Press at 1.5 kbar and centrifuged at 26,000 x g for 30 min at 4°C. Super-
395 natants were incubated with equilibrated 100 µl Ni-agarose (Qiagen) or 100 µl GSH-agarose
396 (ThermoFisher) for 2 h at 4 °C with end-over-end mixing. Supernatant used with Ni-agarose
397 was first supplemented with 10 mM Imidazole and 0.15 M NaCl to achieve 0.3 M and to
398 avoid non-specific interactions. After incubation, supernatants were discarded and resins were
399 washed four times with 1 ml of lysis buffer, turning the tubes on a wheel for 5 min at 4°C
400 between each washing step. The resins were resuspended in 1× Laemmli buffer, boiled for 10
401 min and centrifuged for 2 min at 10,000 × g to elute the dissociated proteins. Eluted proteins
402 were subjected to SDS-PAGE and western blotting using anti-GST-tag (Proteintech) and
403 P5CDH antibodies.
404

405

406

407 **Results**

408 *Bimolecular fluorescence complementation (BiFC) assays indicate potential interaction among*
409 *ProDH1, P5CDH and OAT in plant mitochondria*

410 To test for putative interaction between Arabidopsis ProDH1, OAT, and P5CDH, a series of
411 BiFC assays was set up. ProDH1, OAT, and P5CDH were each fused in frame with either
412 YFP_N or YFP_C at the C-ter, thus keeping the mitochondrial targeting signals of the three
413 enzymes intact at the N-ter of the fusions. *N. benthamiana* leaves were transformed with all
414 possible combinations of the transgenic fusion constructs by infiltration. A binary plasmid
415 encoding a mCherry-labelled mitochondrial matrix marker was also used as a control (Nelson
416 *et al.*, 2007) (Fig. 1, Supplementary Fig. S2 and Supplementary Fig. S3).

417 Yellow fluorescence signals were observed in *N. benthamiana* leaves transiently transformed
418 with the following combinations of fusion constructs: ProDH1-YFP_N and P5CDH-YFP_C,
419 ProDH1-YFP_N and OAT-YFP_C, OAT-YFP_N and P5CDH-YFP_C (Fig.1), as well as P5CDH-
420 YFP_N and OAT-YFP_C (Supplementary Fig. S2). Additionally, the YFP signals from these
421 four co-infiltrations exhibited colocalization with the mitochondrial marker signals (Fig. 1,
422 Supplementary Fig. S2). In order to verify the specificity of the signal, leaves were co-
423 infiltrated with a mixture of *A. tumefaciens* transformed with BiFC plasmids carrying the full-
424 length cDNA of either *ProDH1*, *P5CDH* or *OAT* and the full-length cDNA of the isoform 2
425 of *serine hydroxymethyltransferase (SHMT2)*, which is also targeted to the mitochondrion.

426 While SHMT2 expression and mitochondrial localization were confirmed (Fig. 2A), no
427 fluorescence signal was detected with ProDH1, P5CDH, or OAT (Fig. 2B).
428 Consistently, no fluorescence was detected when leaves were co-infiltrated with a mixture of
429 *A. tumefaciens* transformed with BiFC plasmids carrying the full-length cDNA of either
430 *ProDH*, *P5CDH* or *OAT* and empty plasmids (Supplementary Fig. S2). In addition, no YFP
431 fluorescence was detected in tobacco leaves infiltrated with P5CDH-YFP_N and ProDH1-YFP_C
432 (Supplementary Fig. S2) or with OAT-YFP_N and ProDH1-YFP_C (data not shown). These
433 unexpected results were not due to the lack of protein expression since fusion proteins were
434 detectable in all tested BiFC assays, though in variable amounts (Supplementary Fig. S3). All
435 these results thus indicate that ProDH1, P5CDH, and OAT exhibit a high degree of proximity,
436 facilitating their specific interactions with each other in pairs in mitochondria by forming
437 hetero-oligomers.

438 *N. benthamiana* leaves co-infiltrated with complementing combinations of YFP constructs
439 were fixed with formaldehyde to immobilize mitochondria, which are highly dynamic in
440 living cells, in order to localize the fluorescence at the sub-mitochondrial level. This step
441 made it possible to acquire high-resolution images of mitochondria by z-spacing confocal
442 microscopy, such that the optical signals between mitochondrial matrix and inner membrane
443 were distinguishable (Fig. 3). Confocal images of leaves overexpressing *ProDH1-YFP_N* and
444 *P5CDH-YFP_C* exhibited clear patchy patterns of fluorescence indicating that ProDH1 and
445 P5CDH interacted in the mitochondrial membrane. For leaves over-expressing *ProDH1-YFP_N*
446 and *OAT-YFP_C* or *OAT-YFP_N* and *P5CDH-YFP_C*, the fluorescence signal suggests that the
447 interacting proteins were localized in both the matrix and membranes of mitochondria, which
448 however had an atypical shape (Fig. 3). Localization of homo-oligomers of the three enzymes
449 was also assessed. In leaves where P5CDH-YFP_N and P5CDH-YFP_C were over-expressed,
450 the fluorescence signals of YFP and mCherry co-localized, indicating a preferential
451 localization of homo-oligomeric P5CDH in the mitochondrial matrix (Fig. 3). Conversely, in
452 leaves over-expressing ProDH1-YFP_N and ProDH1-YFP_C or OAT-YFP_N and OAT-YFP_C,
453 the fluorescence exhibited a patchy pattern, which is indicative of a localization within the
454 mitochondrial membrane. However, in co-infiltrated Nicotiana cells, the mitochondria
455 displayed elongated and/or tubular shapes, posing challenges in accurately determining their
456 respective submitochondrial localization.

457 These results show that the sub-mitochondrial localization of these proteins is more complex
458 than previously described.

459

460 *ProDH1, P5CDH and OAT are present in different sub-mitochondrial fractions during DIS in*
461 *Arabidopsis thaliana*

462 To investigate the sub-mitochondrial localization, leaves of Arabidopsis Col-0,
463 *prodh1prodh2*, *oat*, and *p5cdh* plants were examined after 5 days of DIS. DIS is a condition
464 in which the three P5C metabolism enzymes are expressed in WT to initiate proline
465 catabolism (Kiyosue *et al.*, 1996; Funck *et al.*, 2010; Launay *et al.*, 2019; Zheng *et al.*, 2023).
466 Crude mitochondria from leaves were fragmented and the proteins fractionated according to
467 their solubility. To assess the quality and purity of the mitochondrial sub-fractions, markers
468 such as alternative oxidase (AOX) and glycine decarboxylase complex (GDC) were utilized.
469 AOX is a marker for the mitochondrial inner membrane (Millenaar and Lambers, 2003),
470 while GDC serves as a marker for the mitochondrial matrix (Douce *et al.*, 2001). AOX signal
471 was exclusively detected in membrane fractions, indicating a good separation between the
472 mitochondrial membrane and the soluble fractions. Conversely, GDC was only found in the
473 soluble fractions, further supporting the distinct separation between the mitochondrial
474 membrane and soluble fractions (Fig. 4).

475 ProDH1 was primarily localized in the mitochondrial membrane fraction, indicating its
476 predominant presence in the membranes. In contrast, OAT was predominantly localized in the
477 soluble fraction, suggesting its presence inside the matrix. P5CDH was observed in both
478 fractions, indicating a distribution in both the mitochondrial membrane and matrix. These
479 findings are fully consistent with the BiFC results from *N. benthamiana* for ProDH1, but only
480 partially consistent for P5CDH and OAT (Fig. 3). The partial inconsistency observed may be
481 attributed to the variations in the physiological states of the leaves used in the experiments.
482 The submitochondrial localizations of ProDH1, P5CDH, and OAT remained consistent across
483 the different genetic backgrounds of the P5C metabolism mutants investigated. This suggests
484 that the localization of these proteins is not influenced by the specific genotypes. Taken
485 together, these findings provide direct molecular evidence of a differential but overlapping
486 sub-mitochondrial localization of ProDH1, P5CDH, and OAT in the mitochondrial membrane
487 and matrix. It is important to note that in the *p5cdh* mutant, the abundance of ProDH1 and
488 OAT is comparatively lower.

489

490 *Proteomic evidence for interactions between ProDH1, P5CDH and OAT during DIS in Arabidopsis*
491 *leaves*

492 To confirm the reliability of BiFC findings of endogenous interactions, 2D BN/SDS-PAGE
493 were performed using digitonin-solubilized crude mitochondria extracted from Arabidopsis
494 Col-0 leaves that were placed in DIS conditions. Two parallel gels were utilized for the
495 experiment. After electrophoresis, one gel was stained with Coomassie blue (Fig. 5A) and the
496 other was used for transfer onto a nitrocellulose membrane. Membrane was then sequentially
497 probed with primary antibodies directed against ProDH1, OAT, and P5CDH (Fig. 5B-C).
498 Multiple signals for each protein of interest were detected. Thirteen protein spots, recognized
499 by either anti-ProDH, anti-P5CDH or anti-OAT sera, were excised from the Coomassie blue
500 stained gel (Fig. 5A) undergo mass spectrometry (MS) analysis (Fig. 5C-D). ProDH1 was
501 detected in 12 of the spots while OAT was detected in 5 spots (spots 5, 6, 9, 12, and 13).
502 P5CDH was present in 3 spots (spots 2, 3, and 4). These findings provided confirmation
503 regarding the specificity of the immunoblotting technique employed.

504 In the first dimension (1D) of electrophoresis under BN conditions, signals corresponding to
505 ProDH1 were detected over a wide range of gel area, spanning from 70-300 kDa. In the
506 second dimension of the electrophoresis (2D) in the presence of SDS, ProDH1 was detected
507 at ~55 kDa, as expected. For OAT, two distinct signals were observed in 1D, one ranging
508 from 70-150 kDa and the other around 150-200 kDa (Fig. 5B). Interestingly, all detected
509 P5CDH signals in 1D were located around ~300 kDa. Molecular masses of these three
510 proteins detected in 1D were higher than the theoretical molecular mass of their
511 corresponding monomer. This disparity is likely attributed to homo- or hetero-oligomerization
512 of the proteins.

513 In 1D, the signals for ProDH1 overlapped with the signals for OAT in the range 70-150 kDa
514 and with the signals for P5CDH at approximately 300 kDa (Fig. 5B-C). This suggests the
515 possibility of interactions between ProDH1 and both OAT and P5CDH during DIS.

516 The co-migration of ProDH1 with both OAT and P5CDH in the 1D gel suggests the
517 possibility of their interactions. However, it is important to consider the potential that these
518 interactions may occur within a larger complex, which could dissociate during 1D
519 electrophoresis due to the electrical repulsion caused by the negative charge of Coomassie
520 blue (Eubel *et al.*, 2005). To explore this possibility further, 2D native/SDS-PAGE was
521 performed using protein from crude mitochondria extracted from Col-0 DIS leaves (Fig. 6). In
522 native PAGE where no Coomassie blue is used, proteins are separated based on their net
523 charge, size, and native structure, preserving the integrity of protein complexes during
524 electrophoresis. The patterns observed for P5CDH and OAT after 2D clear native/SDS-PAGE

525 are consistent with the results obtained from BN/SDS-PAGE (Fig. 5). One notable difference
526 is the expanded signal of ProDH1 in the native 1D gels, suggesting that ProDH1 may form
527 additional protein complexes. However, the three proteins co-migrated to the same position in
528 1D, indicating that they could potentially be part of the same protein complex. This supports
529 the possibility of interactions among ProDH1, OAT, and P5CDH within a larger protein
530 complex.

531

532 *Identification of a P5CDH-interacting complex by complexome profiling*

533 To investigate whether ProDH1, P5CDH, and OAT interact within Arabidopsis mitochondria
534 during DIS, complexome profiling was employed. Our approach was based on
535 complementing *p5cdh* with a *35S:P5CDH-tGFP* construct. The underlying idea was that
536 P5CDH-interacting proteins should exhibit a different migration pattern in *35S:P5CDH-*
537 *tGFP/p5cdh* background, compared to WT P5CDH in Col-0. Crude mitochondria were
538 extracted from both Col-0 and *35S:P5CDH-tGFP/p5cdh* leaves undergoing DIS. First, the
539 transformation and complementation of *p5cdh* with *P5CDH-tGFP* construct was confirmed
540 phenotypically *in vivo*. While *p5cdh* mutant seedlings were unable to grow on MS media
541 supplemented with proline 25 mM, *p5cdh* seedlings carrying the *35S:P5CDH-tGFP* construct
542 showed a WT phenotype in the presence of proline (Supplementary Fig. S1).

543 Next, mitochondria were isolated from both genotypes, and mitochondrial proteins were
544 extracted and separated using 1D clear native PAGE. Gel lanes corresponding to each
545 genotype were divided into 30 slices (1 mm), which were then subjected to tryptic digestion
546 and peptide identification by label-free quantitative MS. Based on the relative abundance of
547 their constituent peptides (as estimated based on iBAQ values) in different gel slices,
548 migration patterns for ProDH1, P5CDH and OAT were determined for the two genotypes (Fig.
549 7).

550 The migration profiles for ProDH1 and OAT exhibited several peaks, while P5CDH showed
551 fewer peaks in comparison (Fig. 7), which agrees with the findings from the native/SDS-
552 PAGE results (Fig. 6). The three proteins co-migrated at different positions, particularly in the
553 gel slice number 18 in Col-0 (Fig. 7A). In mitochondria extracted from *35S:P5CDH-*
554 *tGFP/p5cdh* leaves, the migration of P5CDH was clearly affected and shifted as anticipated
555 (Fig. 7E). Importantly, the migration of ProDH1 and OAT was also shifted, confirming the
556 interaction among these three proteins within the mitochondria of plants expressing the
557 functional P5CDH fusion protein. Specifically, the peak observed in the gel slice 18 in Col-0

558 was shifted to slice 17 in P5CDH-tGFP/*p5cdh* samples. It should be noted that the abundance
559 of P5CDH-tGFP is high due to its overexpression, resulting in some alteration of the ratios
560 between P5CDH, ProDH1, and OAT. Therefore, the peak of the overexpressing P5CDH-
561 tGFP in the slice 16 may correspond to homo-oligomerization of P5CDH-tGFP. Slice 17
562 likely indicates the interaction between a portion of P5CDH-GFP with ProDH1 and OAT.
563 The migration profile of ProDH1 was more affected than that of OAT. Compared to Col-0,
564 the migration pattern of ProDH1 was altered in *P5CDH-tGFP/p5cdh* samples in slices 9-16,
565 indicating that the change in molecular mass of P5CDH-GFP influenced the migration of
566 ProDH1. As a control, the specificity of these shifts was confirmed by identifying several
567 proteins with identical migration patterns in both genotype (Supplementary Fig. S4). Notably
568 some other proteins had also altered electrophoretic mobilities in *P5CDH-tGFP/p5cdh*
569 samples, e.g., succinate dehydrogenase subunit SDH1-1 of respiratory chain complex II
570 (Supplementary Fig. S5).

571

572 *ProDH1 and OAT co-immunoprecipitate with P5CDH-GFP*

573 To further investigate the interaction between ProDH1, P5CDH and OAT,
574 immunoprecipitation experiments were performed using digitonin-solubilized mitochondria
575 isolated from *35S:P5CDH-tGFP/p5cdh* after 4 days of DIS (D4). Proteins were
576 immunoprecipitated with tGFP-Trap agarose beads (Fig. 8A). The immunoprecipitated
577 proteins were then separated by SDS gel electrophoresis, transferred onto a PVDF membrane,
578 and probed with α -ProDH1.

579 A major band corresponding to ProDH1 was observed in both the input and eluted fractions,
580 confirming that the interaction between ProDH1 and P5CDH during DIS. To verify that the
581 interaction was specific to P5CDH and not an artefact resulting from a direct interaction of
582 ProDH1 with tGFP, transgenic plants expressing tGFP targeted to the mitochondria were
583 utilized. The mitochondrial targeting signal (MTS) of *mCherry*, which was used for BiFC
584 experiments, was cloned in frame with the cDNA of *tGFP* into a binary vector in order to
585 transform wild-type plants. Mitochondria were isolated from *35S:MTS-tGFP/Col-0* plants
586 after 4 days of DIS (D4) and then solubilized with digitonin. While ProDH1 was present in
587 the input fraction, as expected after 4 days of DIS, no ProDH1 was immunoprecipitated by
588 the tGFP-Trap agarose beads (Fig. 8A). The two bands observed in the eluted fractions were
589 attributed to nonspecific signals, as they were also present in co-immunoprecipitation
590 experiments performed on mitochondria extracted from non-treated *35S:P5CDH-tGFP/p5cdh*

591 leaves (D0), where ProDH1 was not present (Fig 8B). The presence of ProDH1 in
592 immunoprecipitated mitochondrial proteins at D4 and the absence in non-senescent
593 mitochondria (D0) were confirmed by MS (Fig. 8C, Supplementary Table S2).

594 Membranes with immunoprecipitated fractions were also probed with antibodies raised against
595 OAT (Fig. 8A). A signal corresponding to OAT was revealed in protein samples of
596 *35S:P5CDH-tGFP/p5cdh* but not in *35S:MTS-tGFP/Col-0* mitochondria at D4. The presence
597 of OAT in the D4 fraction and the absence in the non-senescent mitochondria were also
598 confirmed by MS (Fig. 8C). Taken together, these results further demonstrate the interaction
599 between ProDH1, P5CDH, and OAT during DIS *in vivo*.

600 Ren *et al.* (2018) previously demonstrated the inhibitory effect of a mitochondrial protein
601 called DFR1 on proline oxidation under drought and cold stresses. They showed that DFR1
602 could effectively interact with both ProDH1 and P5CDH, thereby restraining their enzymatic
603 activities. In our investigation, we sought to ascertain whether DFR1 was co-
604 immunoprecipitated with P5CDH-tGFP. However, our analysis yielded no identifiable
605 peptides corresponding to DFR1 among the immunoprecipitated mitochondrial proteins at D4.
606 Moreover, DFR1 did not emerge in the complexome profiling dataset (Supplementary Table
607 S2).

608 To go deeper into the direct interaction between ProDH1 and P5CDH, both proteins were
609 genetically modified by fusing them with different tags. ProDH1 was fused with a GST-tag,
610 resulting in GST-ProDH1, and P5CDH was similarly tagged with a His-tag, creating His-
611 P5CDH. Subsequently, these genetically modified proteins were co-expressed within *E. coli*
612 cells and subjected to a purification process employing affinity chromatography with GSH-
613 agarose resins. As a control, we also conducted affinity chromatography on GSH-agarose
614 resins using crude extracts from bacteria expressing His-P5CDH alone, as well as those co-
615 expressing GST and His-P5CDH. When P5CDH was co-expressed with GST-ProDH1 in *E.*
616 *coli* cells, a distinctive band corresponding to His-P5CDH was observed in the eluted
617 fractions from the GSH-agarose resin (Fig. 9). In contrast, no such band was observable in the
618 eluted fractions obtained from the GSH-sepharose resin when His-P5CDH was overexpressed
619 independently in *E. coli* cells or co-expressed with GST (Fig. 9).

620 This pull-down assay provides robust confirmation of a direct and specific interaction
621 between ProDH1 and P5CDH, shedding light on the intricacies of their functional partnership.

622

623 **Discussion**

624 ProDH, P5CDH and OAT are enzymes involved in P5C metabolism and share a common
625 intermediate P5C/GSA, which may be toxic for the cell when its metabolism is not correctly
626 regulated. Interestingly, in prokaryotes, ProDH and P5CDH have been observed to be in close
627 proximity or physically interact. Based on this observation, we hypothesized that the
628 coordination of the activities of these three eukaryotic P5C metabolism enzymes might rely
629 on their physical interaction. In this study, we used a combination of transgenic, imaging,
630 biochemical, and proteomic approaches to investigate this hypothesis. Our findings provide
631 evidence that ProDH1, P5CDH, and OAT can indeed interact and form a protein complex, at
632 least during DIS in leaves when all three enzymes are expressed.

633

634 *ProDH1, P5CDH, and OAT interact in mitochondria during DIS.*

635 Our study offered compelling evidence on the presence of ProDH1, P5CDH, and OAT within
636 the mitochondria during DIS. To investigate the potential interactions among these proteins,
637 we conducted BiFC assays using *Nicotiana* as our experimental model. These assays not only
638 confirmed the co-localization of these proteins within the mitochondria but also revealed their
639 proximity, facilitating interactions both among themselves and with each other. The
640 interaction between ProDH1, P5CDH, and OAT was further demonstrated by conducting Co-
641 IP on *Arabidopsis* mitochondria. It is important to acknowledge that the possible involvement
642 of protein intermediates should not be overlooked, as they could potentially play a significant
643 role in facilitating these interactions. Notably, the research conducted by Ren et al. (2018)
644 offered some evidence of the physical interaction between DFR1 and ProDH/P5CDH, leading
645 to the inhibition of their activities. However, Co-IP and complexome analyses did not reveal
646 the presence of DFR1 associated to ProDH, P5CDH or OAT during DIS. The combination of
647 BiFC and *in vivo* GST pull-down assays strongly supports a direct interaction between
648 ProDH1 and P5CDH.

649 Sub-mitochondrial fractionation and confocal imaging of fixed BiFC samples provided
650 insights into the sub-cellular localization of ProDH1, P5CDH, and OAT in mitochondria. The
651 western blot analysis of ProDH1 revealed that the signal was predominantly present in the
652 membrane fraction, indicating its association with the mitochondrial membrane. The BiFC
653 results of ProDH1 homo-dimer further supported this observation, as the YFP and mCherry
654 fluorescence signals did not overlap. Previous studies have described the localization of
655 ProDH1 in the matrix side of the mitochondrial inner membrane in both animals and plants
656 (Brunner and Neupert, 1969; Elthon and Stewart, 1981; Cabassa-Hourton *et al.*, 2016).

657 Interestingly, our BiFC results showed that P5CDH homo-oligomers were localized in the
658 matrix in plant cells, while P5CDH interacting with ProDH1 showed a stronger association
659 with membranes. This dual localization of P5CDH was also observed in sub-mitochondrial
660 fractions evaluated by immunoblotting and in a *prodh1prodh2* mutant, indicating that ProDH
661 alone cannot be responsible for the P5CDH interaction with membranes.

662 Interactions between P5CDH and OAT could take place in the membrane or matrix
663 compartments, as both proteins were detected in these two compartments in the current study.
664 OAT was found in the mitochondrial matrix in both animals (Doimo *et al.*, 2013) and plants
665 (Winter *et al.*, 2015). However P5CDH has been reported to associate with mitochondrial
666 inner membrane in *Zea mays* (Elthon and Stewart, 1981) and the parasitic protozoan *Tr. cruzi*
667 (Mantilla *et al.*, 2015), but in the mitochondrial matrix in mouse (Brunner and Neupert,
668 1969). As no transmembrane domain has been identified in P5CDH, it may rely on post-
669 translational modifications, possibly triggered by DIS, to promote binding to the membrane.
670 In particular, acylation could increase hydrophobicity of P5CDH and anchor P5CDH within
671 the mitochondrial membrane (Chamberlain and Shipston, 2015). Interestingly, using biotin
672 switch isobaric tagging to select S-acylated proteins from Arabidopsis, Hemsley (2015)
673 identified 694 putative S-acylated proteins affecting diverse cellular processes, among them
674 P5CDH. Further studies will be necessary to confirm that P5CDH can be anchored in
675 mitochondrial membranes through S-acylation.

676 It was not surprising to observe homo-oligomerization since different oligomerization states
677 have been previously described for ProDH1, P5CDH and OAT in several species (White *et al.*
678 *et al.*, 2007; Luo *et al.*, 2013; Montioli *et al.*, 2017). Some combinations of fusion proteins
679 involving ProDH1-YFP_C did not interact in the BiFC assays in tobacco leaves even though
680 they were expressed and the corresponding hetero-oligomers were formed in reciprocal assays
681 (Supplementary Fig. S2, Supplementary Fig. S3). This might be due to the structure of
682 ProDH1-YFP_C, possibly preventing its interaction with P5CDH-YFP_N and OAT-YFP_N. The
683 observed variability in submitochondrial localization could reflect differential protein-protein
684 interactions that would target the proteins differently to the membrane or to the matrix,
685 possibly depending on cell physiology.

686

687 *ProDH, P5CDH, and OAT can form a complex with other partners during DIS*

688 The interaction between ProDH1 and P5CDH reported in this study is in line with the Rosetta
689 Stone hypothesis, which suggests that monofunctional homologs of fused bifunctional

690 proteins in different organisms are likely to interact (Marcotte *et al.*, 1999). This finding is
691 further supported by the observation of a bi-enzyme complex of ProDH and P5CDH in *Th.*
692 *thermophilus* (Sanyal *et al.*, 2015). Kinetic data and surface plasmon resonance experiments
693 were used to propose a model where one dimer of P5CDH is associated with two monomers
694 of ProDH. However, no *in vivo* interactions between ProDH and/or P5CDH with OAT have
695 been reported in any organism so far. The absence of these interactions in the mitochondrial
696 complexome of Arabidopsis (<https://gelmap.de/1275>; Senkler *et al.*, 2017) can be attributed to
697 low expression levels of ProDH and OAT under normal growth conditions (Zheng *et al.*,
698 2023). Fuchs *et al.* (2020) have recently estimated the copy number of ProDH1, OAT and
699 P5CDH among 1000 mitochondrial proteins per single mitochondrion from cultured
700 heterotrophic Arabidopsis cells. The data showed that the copy number for ProDH1 was only
701 26 compared to those of OAT and P5CDH with 1274 and 4771 copies, respectively. During
702 DIS, a remarkable 200-fold increase in ProDH1 transcript level was observed at D3 compared
703 to D0, according to the study conducted by Zheng *et al.* (2023). In contrast, OAT and P5CDH
704 transcript levels exhibited 8-fold and 5-fold increases, respectively, at the same timepoint.
705 Even if all the transcripts were translated into proteins, the abundance of ProDH1 would still
706 be lower in comparison to that of P5CDH and OAT. These findings indicate that only a minor
707 fraction of P5CDH and to a lesser extent OAT can interact with ProDH1, as suggested by
708 native/SDS-PAGE data where the three proteins co-migrate only at one position in 1D
709 electrophoresis.

710 Native/SDS-PAGE and proteomic data suggest that ProDH1, P5CDH, and OAT could be
711 components of a larger complex. However, the widely spread pattern of ProDH1 and OAT on
712 2D native gels may be due to dissociation of the complex during electrophoresis. While it is
713 possible that only homo-oligomers dissociated, some evidence suggests that a high-
714 molecular-mass complex involving these three enzymes and other partners may exist in
715 mitochondria.

716 Co-IP experiments with P5CDH-tGFP revealed the presence of different subunits of succinate
717 dehydrogenase, particularly the SDH1-1 subunit mainly at D4 of DIS, and SDH2-1 and
718 SDH2-2 at D0. Interestingly, in native PAGE, SDH1-1 migrates similarly to ProDH1,
719 P5CDH, and OAT. Furthermore, the migration pattern of SDH1-1 was altered in *P5CDH-*
720 *tGFP/p5cdh* samples, as was the case for ProDH1 and OAT. These results support the
721 hypothesis that a P5C metabolic complex composed of ProDH1, P5CDH and OAT interacts
722 with complex II of the respiratory chain in Arabidopsis mitochondria.

723 Although further investigation is necessary, this hypothesis is consistent with other findings.
724 For instance, Hancock *et al.* (2016) showed that ProDH can be co-immunoprecipitated with
725 both SDH1 and SDH2 subunits in mouse mitochondria using the membrane-permeable
726 crosslinker DSP. P5CDH was also found to co-migrate with complex II subunits in *Tr. cruzi*
727 (Mantilla *et al.*, 2015). In addition, succinate has been found to be a non-competitive inhibitor
728 of ProDH in cultured human colorectal cancer cells. Interestingly, the respiratory rate of
729 complex II in Arabidopsis is significantly decreased in the *prodh1prodh2* double mutant
730 compared with WT (Launay *et al.*, 2019), suggesting that the interaction with complex II may
731 contribute to binding ProDH, P5CDH, and OAT to the inner mitochondrial membrane.
732 The ProDH1-P5CDH-OAT complex has a tendency to dissociate during electrophoresis,
733 suggesting a high dissociation constant (K_D). However, *in vitro* reconstitution assays with
734 recombinant enzymes are needed to confirm this observation. Post-translational modifications
735 such as succinylation, acetylation, and phosphorylation of P5CDH and ProDH1 in mice Park
736 *et al.*, 2013; Rardin *et al.*, 2013; Zhou *et al.*, 2013; Bian *et al.*, 2014), and acetylation of
737 P5CDH in plants (König *et al.*, 2014) can modulate the dynamic behavior of these
738 interactions, supporting the likelihood of a high K_D . Phosphorylation of all three proteins has
739 also been reported in plants (Wang *et al.*, 2020), with related phosphopeptides present in the
740 PhosPhAt database (Heazlewood *et al.*, 2008).
741 The functional significance of the ProDH1-P5CDH-OAT interaction during DIS remains
742 unknown. However, decreased ProDH1 and OAT activities in *p5cdh* mutants suggest that
743 these interactions are essential for complete activation of the P5C metabolic pathway (Zheng
744 *et al.*, 2023). The association of enzymes catalyzing sequential reactions can channel
745 intermediates (Sweetlove and Fernie, 2018) and coordinate and regulate arginine and proline
746 metabolisms. The ProDH1-P5CDH-OAT complex could also help channel the unstable and
747 potentially toxic P5C/GSA to the appropriate enzyme, producing NADH and FADH₂
748 concomitantly and impacting the mitochondrial electron transfer chain. While substrate-
749 channeling has been documented in PutA (Srivastava *et al.*, 2010; Singh *et al.*, 2014) and the
750 monofunctional ProDH-P5CDH complex of *Th. Thermophilus* (Sanyal *et al.*, 2015), it has not
751 yet been reported for OAT.
752 Further investigation using fluxomics approaches is required to determine the physiological
753 role of the interactions in the P5C metabolism complex detected in plants. Overall, our
754 findings demonstrate that ProDH1, P5CDH, and OAT can interact in the mitochondria of

755 senescing Arabidopsis leaves. This interaction may be important in coordinating the P5C
756 metabolism pathway and regulating arginine and proline metabolisms.

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759 **Supplementary data**

760 **Table S1. List of primers used for cloning**

761

762 **Table S2. LC-MS/MS data from gel slice analyses**

763

764 **Fig. S1. Proline sensitivity of *p5cdh* was complemented in *35S:P5CDH-tGFP/p5cdh*
765 **plants.****

766

767 **Fig. S2. Controls and additional BiFC assays in *Nicotiana benthamiana*.**

768

769 **Fig. S3. Protein expression of BiFC-infiltrated *Nicotiana benthamiana* leaves.**

770

771 **Fig. S4. Migration pattern of selected mitochondrial proteins which are not affected by
772 the presence of P5CDH-tGFP.**

773

774 **Fig. S5. Complexome profiling for SDH1-1.**

775

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779

780 **Author Contributions**

781 S.L. and A.S. conceived the research plans and supervised the experiments; Y.Z. performed
782 most of experiments; C.C.-H. and S.L. performed the experiments on Co-IP; H.E., L.L. and
783 H-P.B. performed the MS experiments and analyzed the data; L.L. provided technical
784 assistance to G.C.; E.C. prepared gene constructs and transformed plants; Y.Z. drafted the
785 manuscript, which was completed by A.S. and S.L with contributions from all the authors.

786

787 **Conflict of interest**

788 The authors declared that they have no conflicts of interest in relation to this work.

789

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795

796 **Data availability**

797 The data supporting the findings of this study are available from the corresponding author,
798 Arnould Savouré, upon request.

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Figure legends

Fig. 1. BiFC assays with ProDH1, P5CDH, and OAT in *Nicotiana benthamiana*.

Representative confocal analyses of living epidermal cells of *N. benthamiana* showing transient expression of the infiltrated plasmid combinations indicated schematically on the left of each panel. The size of each cassette is proportional to the corresponding CDS length. YFP panels show YFP fluorescent signals; mCherry panels indicate the position of mitochondria; bright field panels show the cellular structure of the tobacco epidermal cells under white light; and merged panels show merged images from the three channels. Bars = 10 μ m.

Fig. 2. BiFC assays of the interaction of either ProDH1, P5CDH, and OAT with the mitochondrial SHMT2 in *Nicotiana benthamiana*.

SHMT2 is a protein localized in mitochondria that was used as a negative control for BiFC. (A) Crude mitochondria were isolated from tobacco leaves infiltrated with different combinations of plasmids as indicated in the diagrams of the constructs and on the top of the membranes. Same amounts of mitochondrial proteins were loaded into SDS-PAGE gels (as shown by Stain Free Technology) and blotted onto PVDF membranes. Proteins were revealed using antibodies against MYC or HA tags. (B) The different combinations of ProDH1, P5CDH, and OAT with SHMT2 did not show any YFP fluorescence signal. mCherry signal revealed mitochondria. Bars = 10 μ m.

Fig. 3. BiFC assays with ProDH1, P5CDH, and OAT in fixed epidermal cells of *Nicotiana benthamiana*.

Z-spacing confocal analyses coupled with image deconvolution of fixed epidermal cells of *N. benthamiana* infiltrated with the plasmid combinations indicated on the left. YFP signals, which indicate the localization of protein interaction, are yellow. mCherry signals from the matrix of mitochondria are blue. The overlap between YFP and mCherry signals are shown in white. Bars = 1.5 μ m.

Fig. 4. Sub-mitochondrial localization of ProDH1, P5CDH, and OAT in *Arabidopsis thaliana* during DIS

Crude mitochondria were isolated from 4-week-old *A. thaliana* plants of the indicated genotypes after 5 days of DIS. Crude mitochondria were subjected to sonication and ultracentrifugation to separate mitochondrial membranes from matrix. The same amounts of protein were loaded into each well of SDS-polyacrylamide gels which were blotted onto nitrocellulose membrane. Blots were probed with antibodies against alternative oxidase (α -AOX), glycine decarboxylase complex (α -GDC), α -ProDH1, α -P5CDH and α -OAT. ME, membrane fraction containing proteins from the mitochondrial inner and outer membrane; SO, soluble fraction containing proteins from the mitochondrial intermembrane space and matrix.

Fig. 5. Proteomic analysis of ProDH1, P5CDH, and OAT in mitochondrial fractions of *Arabidopsis thaliana* using 2D BN/SDS-PAGE, immunoblotting and MS

Crude mitochondria were extracted from Col-0 leaves of *A. thaliana* after 5 days of DIS and solubilized by 1% (w/v) digitonin. Proteins were separated by 2D BN/SDS PAGE, loading 1 mg of mitochondrial protein on each of two gels run in parallel. After electrophoresis, one gel was stained with Coomassie blue (A), and the other was used for western blotting (B-C). The masses of standard proteins are given on the top and the right of each gel image (in kDa). Primary antibodies against ProDH1 and OAT were first incubated with the membrane (B) and then P5CDH antibodies (C), as labelled. Gel spots excised from the Coomassie blue stained gel subjected to MS analysis are indicated by circles and numbered on the gel (A) and the membrane (C). iBAQ values obtained by MS analysis are shown in (D) for the abundance of ProDH1, OAT, and P5CDH peptides detected. N.d., not detected.

Fig. 6. ProDH1, P5CDH, and OAT expressed during DIS in *Arabidopsis thaliana* co-migrate on 2D clear native/SDS gels

Crude mitochondria were extracted from Col-0 leaves of *Arabidopsis* after 5 days of DIS, and solubilized by 1% (w/v) digitonin. Mitochondrial protein (250 µg) was separated by 2D clear native/SDS-PAGE, and the proteins transferred to a PVDF membrane, which was sequentially incubated with antibodies raised against ProDH1 (blue), OAT (cyan), and P5CDH (yellow) and analyzed using Image J. In the last right panel, the white rectangle indicates the co-migration of ProDH1, OAT and P5CDH to the same position in 1D electrophoresis.

Fig. 7. Comparative complexome profiling of mitochondria from two *Arabidopsis* lines

Crude mitochondria extracted from Col-0 and P5CDH-tGFP/*p5cdh* *Arabidopsis* leaves after 5 days of DIS were solubilized with 1% (w/v) digitonin and separated by 1D clear native-PAGE (130 µg of mitochondrial protein). After electrophoresis, gels were visualized using either the Stain-free channel for total protein (B) or the StarBright Blue 520 channel (λ_{exc} : 488 nm; λ_{em} : 520 nm) for the GFP signal of the P5CDH-GFP protein (C). Gels were then blotted onto a membrane, which was probed with first an antibody against P5CDH and then with a StarBright Blue 700 fluorescent secondary antibody. Membranes were visualized using the StarBright B700 channel (D). Gel slices were cut as indicated in (B-D); gel slices 5 to 23 were used for label-free MS analysis. The iBAQ values of ProDH1, P5CDH, and OAT in Col-0 (A) and P5CDH-tGFP/*p5cdh* (E) are visualized in the alignments shown in parts B-D of the Figure.

Fig. 8. Co-immunoprecipitation (Co-IP) of ProDH1 and OAT with P5CDH-tGFP

Co-IP using tGFP agarose beads from mitochondrial extracts at D4 (A) or D0 (B) of DIS from *35S:P5CDH-tGFP/p5cdh* and *35S:MTS-tGFP/col* leaves. Western blots of mitochondrial protein extracts (Input) and eluted fractions were probed with antibodies raised against ProDH1 and OAT. IBAQ values obtained by mass spectrometry analysis of Co-IP from mitochondrial extracts from either D0 or D4 *35S:P5CDH-tGFP/p5cdh* leaves are shown in (C).

Fig. 9. Co-purification of P5CDH with ProDH1 on GSH-agarose.

ProDH1-GST and His-P5CDH were co-overexpressed in BL21(C41) *E. coli* cells. Total protein extracts (Input) were loaded onto GSH-agarose. After washing, proteins were eluted with Laemmli buffer. Inputs and eluted proteins from GSH-agarose (AP:GSH-Ag) were revealed by western blot analysis using antibodies raised against P5CDH or GST. Total protein extracts from bacteria over-expressing free GST and His-P5CDH were used as negative control.

Supplementary data

Fig. S1. Proline sensitivity of *p5cdh* was complemented in *35S:P5CDH-tGFP/p5cdh* plants.

Seeds of different genotypes (as indicated in the Figure) were sterilized, stratified at 4°C for 72h and grown onto a MS/2 medium supplemented without (A) or with 25 mM proline (B) for 7 days.

Fig. S2. Controls and additional BiFC assays in *Nicotiana benthamiana*.

YFP channel shows YFP fluorescent signals; mCherry channel indicates the location of mitochondria; bright field channel shows the cellular structure of tobacco epidermal cell under white light; merged channel shows picture merged with other three channels. Bar = 10 μm.

Fig. S3. Protein expression of BiFC-infiltrated *Nicotiana benthamiana* leaves.

(A) Representative diagrams of constructs used for BiFC assays. Cassettes are presented in 5' to 3' direction. The size of each cassette is based on their nucleic acid sequence length. Gray box, insertion of the CDS fragment of each construction; Myc, c-myc epitope tag; HA,

hemagglutinin epitope tag; YFP_N, N-terminal fragment of eYFP; YFP_C, C-terminal fragment of eYFP.

(B-F) Total proteins were extracted from tobacco leaves infiltrated with different combinations of plasmids indicated on the top of each western-blot. Same amount of proteins was loaded into SDS-PAGE gels. Proteins were then transferred onto a nitrocellulose membrane and revealed using antibodies raised against c-Myc or HA tags. Constructs with positive YFP signal are labelled with yellow backgrounds. The theoretical molecular weight of B4-ProDH1 is 73.7 kDa; B5-ProDH1, 65.6 kDa; B4-OAT, 70.9 kDa; B5-OAT, 62.8 kDa; B4-P5CDH, 80.5 kDa; B5-P5CDH, 72.4 kDa.

Fig. S4. Migration pattern of selected mitochondrial proteins which are not affected by the presence of P5CDH-tGFP.

The iBAQ values of each protein in Col-0 and P5CDH-tGFP/*p5cdh* in different gel slices are shown.

Fig. S5. Complexome profiling for SDH1-1.

The iBAQ value of ProDH1, P5CDH, OAT, and Succinate dehydrogenase 1-1 (SDH1-1) in Col-0 (A) and in P5CDH-tGFP/*p5cdh* (B) are shown. The iBAQ values of P5CDH is shown on the right y-axis, iBAQ values of the other proteins are shown on the left y-axis.

Table S1. List of primers used for cloning

Table S2. LC-MS/MS data from gel slice analyses