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

Yao Zheng¹, Cécile Cabassa-Hourton¹, Holger Eubel², Guillaume Chevreux³, Laurent Lignieres³, Emilie Crilat¹, Hans-Peter Braun², Sandrine Lebreton^{1,*}, Arnould Savouré^{1,*}

1 Sorbonne Université, UPEC, CNRS, IRD, INRAE
Institute of Ecology and Environmental Sciences of Paris (iEES), 75005 Paris, France

2 Institute of Plant Genetics, Leibniz Universität Hannover, Germany

3 Université Paris Cité, CNRS, Institut Jacques Monod, F-75013 Paris, France

email addresses: yao-zheng@outlook.com, cecile.cabassa@sorbonne-universite.fr,
heubel@genetik.uni-hannover.de, guillaume.chevreux@ijm.fr, laurent.lignieres@ijm.fr, emi-
lie.crilat@sorbonne-universite.fr, braun@genetik.uni-hannover.de

* Corresponding authors: Sandrine Lebreton and Arnould Savouré
sandrine.lanfranchi@sorbonne-universite.fr, telephone: +33 1 44275902
arnould.savoure@sorbonne-universite.fr, telephone: +33 1 44272672

Running title

Evidence of a plant pyrroline-5-carboxylate metabolism complex

Number of table: 0

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Supplementary figures : 5

Supplementary tables : 2

Highlight

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

Abstract

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

Plant materials and growth conditions

Wild-type (WT) *Nicotiana benthamiana* (tobacco) plants were grown in growth chambers at 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 leaf infiltration.

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

Arabidopsis seeds were sown on soil and grown in growth chambers at 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. Dark-induced senescence (DIS) treatments were performed on excised rosette leaves of 4-week-old Arabidopsis as described before (Launay *et al.*, 2019).

Bimolecular fluorescence complementation (BiFC) assays

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

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

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

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

Transformation of Arabidopsis with transgenic GFP-fusion constructs

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

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

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

Protein extraction for BiFC controls

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

Extraction of crude mitochondria from Arabidopsis and Nicotiana

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

Fragmentation of Arabidopsis mitochondria for sub-mitochondrial fractionation

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

Western blot assays

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

Two-dimensional PAGE

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

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

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

Protein identification by mass spectrometry (MS) and complexome profiling

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

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

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

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

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

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

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

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

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

Affinity pull-down assays

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

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 disrupted by French Press at 1.5 kbar and centrifuged at 26,000 x g for 30 min at 4°C. Supernatants were incubated with equilibrated 100 µl Ni-agarose (Qiagen) or 100 µl GSH-agarose (ThermoFisher) for 2 h at 4 °C with end-over-end mixing. Supernatant used with Ni-agarose was first supplemented with 10 mM Imidazole and 0.15 M NaCl to achieve 0.3 M and to avoid non-specific interactions. After incubation, supernatants were discarded and resins were washed four times with 1 ml of lysis buffer, turning the tubes on a wheel for 5 min at 4°C between each washing step. The resins were resuspended in 1× Laemmli buffer, boiled for 10 min and centrifuged for 2 min at 10,000 × g to elute the dissociated proteins. Eluted proteins were subjected to SDS-PAGE and western blotting using anti-GST-tag (Proteintech) and P5CDH antibodies.

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Identification of a P5CDH-interacting complex by complexome profiling

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

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

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

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

ProDH1 and OAT co-immunoprecipitate with P5CDH-GFP

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

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

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

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

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

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

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

Discussion

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

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

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

Sub-mitochondrial fractionation and confocal imaging of fixed BiFC samples provided insights into the sub-cellular localization of ProDH1, P5CDH, and OAT in mitochondria. The western blot analysis of ProDH1 revealed that the signal was predominantly present in the membrane fraction, indicating its association with the mitochondrial membrane. The BiFC results of ProDH1 homo-dimer further supported this observation, as the YFP and mCherry fluorescence signals did not overlap. Previous studies have described the localization of ProDH1 in the matrix side of the mitochondrial inner membrane in both animals and plants (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.

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

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

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

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

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

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

Supplementary data

Table S1. List of primers used for cloning

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

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

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

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

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

Fig. S5. Complexome profiling for SDH1-1.

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Author Contributions

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

Conflict of interest

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

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Data availability

The data supporting the findings of this study are available from the corresponding author, 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