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Proteomic and functional analysis of proline dehydrogenase 1 link proline catabolism to  
mitochondrial electron transport in *Arabidopsis thaliana*

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**ABSTRACT**

Proline accumulates in many plant species in response to environmental stresses. Upon relief from stress, proline is rapidly oxidized in mitochondria by proline dehydrogenase (ProDH) and then by pyrroline-5-carboxylate dehydrogenase (P5CDH). Two *ProDH* genes have been identified in the genome of the model plant *Arabidopsis thaliana*. To gain a better understanding of ProDH1 functions in mitochondria, proteomic analysis was performed. ProDH1 polypeptides were identified in *Arabidopsis* mitochondria by immunoblotting gels after 2D blue native – SDS/PAGE, probing them with an anti-ProDH antibody and analysing protein spots by MS. The 2D gels showed that ProDH1 forms part of a low-molecular-weight (70-140 kDa) complex in the mitochondrial membrane. To evaluate the contribution of each isoform to proline oxidation, mitochondria were isolated from wild type and *prodh1*, *prodh2*, *prodh1prodh2* and *p5cdh* mutants. ProDH activity was high for genotypes in which ProDH, most likely ProDH1, was strongly induced by proline. Respiratory measurements indicate that ProDH1 has a role in oxidising excess proline and transferring electrons to the respiratory chain.

**Summary statement:**

Molecular and biochemical analyses indicate a key role of proline dehydrogenase 1 in proline oxidation. ProDH1 binds to mitochondrial membranes, is part of small respiratory chain complex and delivers its electrons to the respiratory chain.

**Short title:** Proline dehydrogenase 1 in *Arabidopsis* mitochondria

**Keywords:** *Arabidopsis thaliana*; Electron transfer chain; Mitochondria; Proline dehydrogenase (ProDH); Proline

**Abbreviations:** ANT, adenine nucleotide translocator; AOX, alternative oxidase; CP, cytochrome pathway; BN, Blue-Native; DCIP, 2,6-dichlorophenolindophenol; ETC, electron transfer chain; ICDH, isocitrate dehydrogenase; MDH, NAD-malate dehydrogenase; P5C, pyrroline-5-carboxylate; P5CDH, pyrroline-5-carboxylate dehydrogenase; ProDH, proline dehydrogenase; RCR, respiratory control ratios; ROS, reactive oxygen species.

## INTRODUCTION

Proline is a proteinogenic amino acid that plays an essential role in primary metabolism, redox homeostasis, osmotic adjustment, protection against stress, and signaling in many prokaryotes, yeasts, mammals and plants (for review, see [1]). The biosynthesis of proline has been well documented, but less is known about its catabolism, a process which generates ATP, reactive oxygen species (ROS) and/or reducing power [2]. The first enzyme of proline oxidation, the flavin-dependent oxidation of proline to delta-1-pyrroline-5-carboxylate (P5C), is catalyzed by proline dehydrogenase (ProDH) [3–5]. P5C is then non-enzymatically converted into glutamate semialdehyde, which is oxidized to glutamate by delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH) [6]. In most bacteria a membrane-associated dehydrogenase known as Proline utilization A (PutA) combines ProDH and P5CDH activities in a single bifunctional enzyme, while in eukaryotes ProDH and P5CDH are separate monofunctional enzymes [2]. ProDH proteins in prokaryotes and yeasts are flavoenzymes that transfer electrons to the respiratory chain via ProDH-FAD complexes while P5CDH reduces  $\text{NAD}^+$  [7,8].

In response to drought, plants accumulate low-molecular-weight organic solutes, collectively known as osmolytes, that participate to the osmotic adjustment of the cell [9]. Among them, proline is widely distributed in higher plants and can be accumulated in considerable amounts of up to 80% of the amino acid pool in response drought [10] due to increased synthesis occurring both in the cytosol and chloroplasts [11] and decreased degradation [2]. Upon recovery from stress, the accumulated proline is oxidized in mitochondria. Proline oxidation activity in maize, wheat, barley, soybean and mung bean has been estimated by measuring L-proline dependent  $\text{O}_2$  consumption by isolated mitochondria [12,13]. Other abundant mitochondrial amino acids like glutamate and glycine can also be used as respiratory substrates by Arabidopsis mitochondria [14], but wheat mitochondria cannot support  $\text{O}_2$  consumption from glycine, arginine, serine or alanine [12]. This indicates that different plant species have different specificities for substrates providing electrons for mitochondrial respiration. It can be hypothesized that the first ProDH-catalyzed step of proline oxidation in plants produces  $\text{FADH}_2$  and the second P5CDH-catalysed step generates NADH. Both reducing equivalents could donate electrons to the mitochondrial electron transfer chain (ETC) [15]. P5C was indeed found to be used as a respiratory substrate in maize mitochondria [13] so electrons can be transferred from P5CDH to the ETC. However, the ability of ProDH to mediate direct electron transfer for mitochondrial respiration has not been demonstrated in plants.

In Arabidopsis, there are two ProDH isoforms that have 75% amino acid sequence identity, ProDH1 and ProDH2. *ProDH1* and *ProDH2* are both expressed at very low levels in vegetative

organs when *Arabidopsis* seedlings are cultivated under standard conditions and they are differentially regulated by various bZIP transcription factors [16,17]. Specific expression patterns have been found using *ProDH1* promoter-GUS reporter fusions. The *ProDH1* promoter directs abundant expression in flowers, particularly in pollen and stigmas, while *ProDH2* is preferentially expressed in vascular tissues and abscission zones [18]. *ProDH1* expression is repressed when plants are dehydrated and proline accumulates, but increases during recovery from drought stress, addition of exogenous proline or upon hypoosmolarity [3,5,19]. The *ProDH1* promoter contains a PRE motif that is involved in the transcriptional regulation of the gene [19]. Recently, *ProDH1* expression was shown to be regulated by the physical interaction of the *Arabidopsis* type-B response regulator 18 with bZIP63 *in vivo* [20]. *ProDH1* expression is induced in a *pi3k*-hemizygous mutant and in seedlings treated with LY294002, an inhibitor of phosphatidylinositol 3-kinase, demonstrating that this lipid signaling pathway enzyme is also involved in the regulation of proline catabolism [21]. *ProDH2* transcript accumulation can be induced by pathogen attack or UV stress (*Arabidopsis* eFP Browser) and in the presence of proline or NaCl [18].

In *Arabidopsis*, ProDH1 has been clearly demonstrated to be mitochondrial [3,22]. The subcellular location of ProDH2 is still under debate due to contradictory reports of ProDH-GFP fusion analyses [18,23]. However, neither ProDH1 nor ProDH2 have been identified in plant organelle proteomes. In comparison, P5CDH has been identified in both mitochondrial and chloroplast proteomes, so it may be a dual-targeted protein [24–27].

Although proline metabolism has been extensively investigated, our understanding of ProDH biochemistry is still limited. To characterize how ProDH participates in the resumption of growth after stress, we used an experimental system of *Arabidopsis* suspension cells and mutant seedlings to induce ProDH accumulation and activity. ProDH accumulation in mitochondria was investigated using proteomic methods. L-proline dependent O<sub>2</sub> consumption was analyzed in isolated mitochondria from wild type (WT) and proline catabolism insertion mutants to define the proteins involved in the electron transfer from L-proline to the respiratory chain in *Arabidopsis*. Analyses using a *p5cdh* knockout mutant demonstrated a direct electron transfer pathway from L-proline to the respiratory chain via the first step of proline catabolism. Investigations using *prodh1* and *prodh2* mutants provide evidence that the induced ProDH1 isoform is involved in transferring electrons to the *Arabidopsis* respiratory chain.

## EXPERIMENTAL

### Plant material and growth conditions

*Arabidopsis thaliana* L. Heynh. ecotype Col-0 T-DNA insertion lines *prodh1-3* (GABI\_308F08), and *prodh2-2* (GABI\_328G05), were obtained from the Center for Biotechnology, Universitaet Bielefeld, Germany [28]. T-DNA insertion lines *prodh1-4* (SALK\_119334) and *prodh2-3* (SALK\_918D08) were obtained from the Salk Institute, La Jolla, USA [29]. The presence of the T-DNA and zygosity of respective alleles were verified by PCR and sequencing of the T-DNA flanking sequences. For crosses between *prodh1-4* and *prodh2-2*, double *prodh1-4prodh2-2* F<sub>2</sub> seedlings were selected by PCR. Gene and T-DNA-specific primers are listed in Table S1. The mutant *p5cdh* T-DNA line (SALK\_018453) has been previously described [30].

Surface-sterilized seeds of *Arabidopsis* WT and mutant lines were sown on grids placed on solid half-strength Murashige and Skoog medium (0.8 % agar) in Petri dishes according to [31]. When they were 15-d-old, the *Arabidopsis* seedlings were transferred using the grids onto MS/2 liquid medium (for controls) or MS/2 liquid medium supplemented with 50 mM proline (for proline treatment) for 24 h. Sucrose was not added to the MS/2 medium in any of our experiments. For biochemical and proteomic analysis, up to 50 mM proline was used.

Suspension cell culture of *A. thaliana* (var. Columbia-0) was also used and maintained as described in [32]. Cells were cultivated at 24–26 °C under continuous shaking at 90 rpm. For ProDH induction, cells were incubated with 50 mM L-proline for 21 h.

### **Mitochondria isolation and subfractionation**

Mitochondria were isolated at 4°C from fresh leaves of 15-d-old *Arabidopsis* plantlets, 50 g of leaves for respiratory measurements and 130 g for purification on Percoll gradients for ProDH localization. Leaves were ground in a blender with three volumes of grinding buffer containing 0.3 M sucrose, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM Na<sub>4</sub>-pyrophosphate, 20 mM Na-ascorbate, 2 mM EDTA, 1% polyvinylpyrrolidone 40, 0.2 mM PMSF and 1% (w/v) BSA, pH 7.5) and filtered through Miracloth and Blutex filters. Contaminants were removed from the leaf extract by 2500 *g* centrifugation for 5 min then mitochondria in the supernatant were sedimented by centrifugation at 15000 *g* for 20 min. Crude mitochondrial pellets were resuspended in washing buffer (0.3 M sucrose, 10 mM TES buffer, 0.1% BSA fatty acid free, pH 7.5).

To prepare mitochondria for respiratory measurements, the crude mitochondrial pellets were washed with another cycle of the two-step differential centrifugation. BSA was then removed by several sedimentation steps at 15000 *g* in washing buffer without BSA.

To prepare mitochondria for protein localization, the crude mitochondrial pellets were purified on Percoll gradients by centrifugation at 15000 *g* for at least 45 min. Highly purified mitochondria at the 40%/30% interphase were carefully removed and diluted. Percoll was

eliminated by several 15-min centrifugations at 15000 *g* until pellets were well aggregated. BSA was then removed by repeated sedimentation in washing buffer without BSA as above. Mitochondrial protein concentration was determined according to [33] using BSA as a standard. Matrix and membrane fractions of Percoll-gradient purified mitochondria were separated as previously described in [34] with some modifications. Mitochondria were resuspended in sonication buffer (10 mM TES, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, pH 7.2) at 1 mg/mL protein concentration. Mitochondria (1.5 mL of suspension) were broken by three freeze-thaw cycles and four 16- s sonication steps using a Branson Sonifier 250. Intact mitochondria were sedimented by 7 min of centrifugation at 15000 *g* and discarded. The supernatant was then centrifuged for 100 min at 140000 *g*. The resulting pellet containing mitochondrial membranes was resuspended in sonication buffer. The soluble fraction corresponding to the contents of the matrix and inter-membrane space was precipitated with pure acetone. Proteins were then sedimented for 1 h at maximum speed in a bench centrifuge. Proteins resuspended from pellets were further analysed by Western blots. Enrichment of subfractions was analyzed by immunoblot experiments using antibodies directed against adenine nucleotide translocator (ANT), a protein marker of the inner mitochondrial membrane [34], and NAD<sup>+</sup>-isocitrate dehydrogenase (ICDH), a protein marker of the mitochondrial matrix (Agrisera, Sweden). For measurement of inner mitochondrial membrane integrity, NAD-malate dehydrogenase (MDH) activity was spectrophotometrically measured by following NADH oxidation at 340 nm. The assay was carried out using the following reaction mixture: 10 mM K<sup>+</sup>-phosphate buffer pH 8, 1 mM KCN, 0.2 mM NADH and 0.9 mM oxaloacetate. The non-latent MDH activity was measured in the same buffer supplemented with 0.3 M sucrose. For measurement of outer mitochondrial membrane integrity, Cytochrome c oxidase activity was measured as previously described [35]. Chlorophyll content was determined as previously described [36].

### **ProDH activity and protein assays**

ProDH activity was measured using a modified version of the 2,6-dichlorophenolindophenol (DCIP)-based assay [32], previously established by [37]. Soluble protein concentration was determined according to [33] using BSA as a standard. Sixty  $\mu$ g of mitochondrial protein were used for each assay in a final volume of 1 mL containing 5 mM MgCl<sub>2</sub>, 1 mM KCN, 0.06 mM DCIP, 0.5 mM FAD, 0.5 mM phenazine methosulfate, 150 mM L-proline and 200 mM Tris-HCl, pH 7.5. Two control assays were performed in parallel, either without L-proline as a substrate or with L-proline, but without mitochondrial protein. The background enzyme activity was less

than 2.2 nmol of reduced DCIP  $\text{min}^{-1} \text{mg protein}^{-1}$  and this value was subtracted from the value measured in the ProDH assays.

### **Respiratory measurements**

Oxygen consumption by isolated mitochondria (equivalent to 300  $\mu\text{g}$  of mitochondrial protein) was measured using a Clark-type  $\text{O}_2$  electrode system (Hansatech, United Kingdom) in 1 ml of reaction buffer (0.3 M sucrose, 5 mM  $\text{KH}_2\text{PO}_4$ , 10 mM TES, pH 7.2, 10 mM KCl, 2 mM  $\text{MgSO}_4$ , 0.1 % BSA fatty acid free) stirred at 25°C. Final concentrations of respiratory substrates used in assays were 1 mM NADH, 10 mM succinate, 6 mM L- or D-proline, and 6 mM hydroxyproline. Alternative oxidase (AOX) capacity was measured in the presence of 1 mM KCN and by adding 1 mM DTT and 5 mM pyruvate to fully reduce and activate the enzyme. Cytochrome-dependent  $\text{O}_2$  uptake, termed cytochrome pathway (CP), was specifically inhibited by 1 mM cyanide while the alternative oxidase pathway was inhibited by 0.1 mM n-propyl gallate. Respiratory control ratios (RCR) and phosphorylation yields (ADP/O ratios) were measured as previously described in [38]. State 3 rates are oxidation rates under phosphorylating conditions (i.e. in the presence of ADP) and state 4 rates are oxidation rates under non-phosphorylating conditions when all ADP has been converted to ATP. RC is the ratio of the state 3 rate to the state 4 rate, which indicates the ability of the respiratory chain to readjust its electron flow according to the ADP content. The ADP/O ratio is the ratio between ADP and the quantity of molecular oxygen necessary to convert all the ADP added to ATP and thus represents the phosphorylation yield of the chain. CP represents the electrons flow directed to the cytochrome pathway in state 4 in the absence of AOX activity. AOX enzymes were fully reduced and activated by the presence of cyanide, pyruvate and DTT thus providing a measure of AOX capacity.

### **ProDH immunodetection**

Mitochondria were resuspended in Laemmli buffer containing freshly added  $\beta$ -mercaptoethanol. Proteins were separated by SDS-PAGE on 11% (v/v) polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad) in a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 30 min at 135 mA using transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Equal protein loading and integrity of protein samples were checked by Ponceau S staining of the nitrocellulose membrane. For immunodetection, the nitrocellulose membrane was incubated in TBS containing 0.1% Tween 20 with 5% non-fat dry milk for 30 min at room temperature and then in TBS (Tween 20) with 1:1000 anti-ProDH antibody overnight at 4°C. The antibodies used were those obtained from rabbits immunized with



AtProDH1 as described in [39]. The ECL prime chemiluminescence detection kit (GE Healthcare, France) was used for immunodetection. The sensitivity of the anti-ProDH antibody was tested using recombinant His-tagged AtProDH1 or AtProDH2 proteins produced in *E. coli*. Purified proteins were quantified, then diluted and immunodetected after SDS-PAGE.

### **Two-dimensional gel electrophoresis and identification of ProDH1 by MS**

Molecular identification of ProDH of Arabidopsis was carried out using mitochondria isolated from either Arabidopsis seedlings (grown as described above) or from Arabidopsis cell cultures (see [32]). Mitochondrial membrane proteins were separated by 2D blue native (BN) – SDS/PAGE according to [40] using the experimental parameters given by [41]. The 2D gels were either Coomassie-stained [42], or used for Western blots and immunodetection (as described above). Tryptic digestion of the selected proteins was carried out as described by [41]. The extracted peptides were analyzed by tandem MS using a micrOTOF Q-II mass spectrometer (Bruker, Bremen, Germany) as described previously [41]. Additionally, ProDH1 was detected in isolated mitochondria by means of shotgun mass spectrometry using a Q-Exactive instrument coupled to a Dionex Ultimate3000 UPLC (both by Thermo Fisher Scientific, Dreieich, Germany) as described in Fromm et al. 2016 [43].

### **Statistical analysis**

The statistical significance of respiration and ProDH activity assays were investigated using unpaired Student's *t*-tests to compare mean values of independent groups ( $P < 0.05$  or  $P < 0.01$  as stated). Differences in ProDH activity in wild-type and *prodh* mutants incubated with or without proline were analyzed using Mann and Whitney test, where  $P < 0.05$  was considered significant.

## **RESULTS**

### **ProDH1 is associated with low-molecular-weight complexes in Arabidopsis mitochondria**

In plants, ProDH is a very scarce protein even when it is induced after relief from stress. Only a few ProDH peptides have been reported in shotgun analyses of global Arabidopsis proteomes [44]. ProDH1 is known to be a mitochondrial protein, but it has not been identified in any organelle-based proteome projects. In order to identify ProDH in mitochondria, we used an Arabidopsis cell suspension culture recently developed by [32] in which ProDH can be strongly induced by addition of 50 mM L-proline. Proteins from mitochondria purified on Percoll density gradients were separated by 2D BN/ SDS PAGE. On a Western blot of the 2D gel of

mitochondrial proteins from proline-treated cells, several spots with apparent molecular mass of ~54-55 kDa in the second dimension were revealed using an anti-ProDH antibody (Figure 1). The oligomeric state of ProDH is not clear because the proteins producing the positive signal migrate in the range of 70 kDa to 140 kDa in the first dimension (BN). Several exposure times were used to compare the signals between the 2D gel blot from untreated and proline-treated mitochondrion samples (Figure 1B). The intensity of the signal produced by a 5-s exposure of the blot of mitochondrial proteins from proline-treated cells was similar to the 300-s exposure of the corresponding blot from non-treated cells. This indicates an estimated 60-fold difference in ProDH content between the two samples. Additional experiments were carried out using mitochondria isolated from non-treated and proline-treated *Arabidopsis* seedlings. Very similar spot patterns were observed in 2D BN/SDS PAGE, but the intensity of chemiluminescence signals for blots of mitochondrial proteins from seedlings was much lower than for those from cultured cells (Supplemental Figure 1). Indeed to obtain similar ProDH signals, much longer exposure times (min rather than s) were needed, indicating that ProDH is expressed at much higher concentrations in cell cultures than in seedlings.

Four spots recognized by the anti-ProDH antibody and differentially expressed between proline-treated and control mitochondrial samples were further analyzed by MS (Figure 2). Within these four spots, peptides of ProDH1 were detected (Figure 2C). A total of 7 different peptides were identified that are well distributed in the ProDH1 protein (Figure 2D). These results demonstrate that the anti-ProDH antibody does recognize ProDH1 and that this isoform is located in mitochondria of proline-treated plant cells.

### **ProDH is associated with mitochondrial membrane fraction in *Arabidopsis***

Proline oxidation activity was shown by [13] to be associated with the inner mitochondrial membrane in *Zea mays*. To pinpoint ProDH1 localization in *Arabidopsis* cells, mitochondria were purified using Percoll density gradient ultracentrifugation from 15-d-old WT seedlings treated for 24 h with 50 mM L-proline. Mitochondrial Percoll fraction showed less than 2 % contamination with peroxisomes (Supplemental Figure 2). In addition chlorophyll was not detectable in the mitochondrial band of the 30 % - 40% interphase of the Percoll gradient indicating non measurable chloroplast contamination in the purified mitochondria fraction used for matrix and membrane fractionation. NAD-MDH activity in purified mitochondria was  $9.5 \pm 1.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$  protein while the non-latent activity measured in 0.3 M sucrose medium represented at most 5% of it. Cytochrome c oxidase activity in the presence of 0.04% Triton X-100 was around  $1.25 \pm 0.25 \mu\text{mol min}^{-1} \text{mg}^{-1}$  protein while the remaining activity in the absence

of Triton X-100 reached at most 5% of it. Altogether, outer and inner membrane integrity assessed by cytochrome c oxidase and NAD-MDH activities, respectively, indicates 95% integrity at least of the purified mitochondria (30-40% Percoll interphase).

Mitochondrial membranes were separated from the soluble fraction and tested for the presence of ProDH1 in Western blots. Enrichment of the two fractions was assessed using antibodies against the integral membrane protein ANT and the matrix-localized NAD<sup>+</sup>-ICDH. Figure 3A shows a ProDH1-specific signal in the membrane fraction, confirmed by the controls showing enrichment of ANT in the membrane fraction (Figure 3B) and ICDH in the soluble fraction (Figure 3C). These results demonstrate a physical association between ProDH1 and mitochondrial membranes in Arabidopsis.

### **Molecular characterization of *prodh* mutants**

To assess the biochemical and physiological function of ProDH, we genetically characterized allelic T-DNA insertion mutants of *ProDH1* and its counterpart *ProDH2* (Figure 4). We confirmed that T-DNA insertions in *prodh1-4* and *prodh2-2* mutants are located in introns as previously described by [18] (Figure 4A). However, in contrast to what was indicated by [18], we discovered that in *prodh1-3* mutants the T-DNA was inserted in an exon, which is also in agreement with the SIGnAL insertion sequence database (<http://signal.salk.edu>) and in *prodh2-3* mutants the T-DNA was in the 5' non-coding region (Figure 4A). For all experiments involving mutants, the homozygosity of lines was confirmed by PCR genotyping (Figure 4B).

We investigated the impact of *prodh* mutations on ProDH protein content. WT, *prodh1-4*, *prodh2-2* and double mutant *prodh1-4 prodh2-2* seedlings were treated with L-proline for 24 h, which triggered ProDH accumulation in WT (Supplemental Figure 3, Figure 4C). With the anti-ProDH1 antibody, Western blots of proteins from non-treated seedlings show faint signals at around 54 kDa that were not found in the *prodh1prodh2* double mutant. A very strong signal was observed in WT and the two *prodh2* mutant lines treated with exogenous L-proline. However, treatment with L-proline did not trigger ProDH signals in either of the two *prodh1* lines (Figure 4C and D).

While we have demonstrated by MS that the anti-ProDH antibody specifically recognizes proline-inducible AtProDH1, it is possible that it also recognizes ProDH2. Bacterially produced recombinant ProDH1 and ProDH2 proteins were serially diluted and blotted using the same method as for the plant protein blots. In these conditions the anti-ProDH1 antibody detects as little as 1 ng of ProDH1 or 5 ng of ProDH2 (Supplemental Figure 4). As a control for a competition assay, the anti-ProDH1 antibody was pre-incubated with an excess of recombinant

ProDH1 before being used for Western blots of proline-treated and non-treated seedling extracts. The ProDH signal was not visible in these conditions (Supplemental Figure 3). These results suggest that the 54-kDa inducible signal observed in WT and *prodh2* mutant lines can be attributed to ProDH1 isoform.

In standard growth-medium, wild-type and *prodh* mutants displayed similar root growth (Supplemental Figure 5). The presence of proline did not affect WT plants. However, in the presence of 5 and 10 mM proline, primary root growth of the *prodh2-2* mutant was slightly lower at 4 d but later became identical to WT (Supplemental Figure 5B). *prodh1-4* and *prodh1prodh2* mutants had more striking phenotypes: primary root growth of these mutants was clearly inhibited during development in the presence of proline, 46% and 43% less growth for *prodh1* and for *prodh1prodh2* after 12 d on 10 mM proline, respectively. The development of the aerial parts was not affected by proline.

### **L-proline treatment triggers ProDH1 activity and increases respiration rate**

ProDH activity was assayed in mitochondria from proline-treated seedlings using a modified version of the DCIP-based assay [32]. ProDH activity of 29.9 nmol reduced DCIP min<sup>-1</sup> mg protein<sup>-1</sup> was measured in mitochondria isolated from proline-treated WT (Figure 5). ProDH activity was higher in *prodh2* than in WT. In contrast, ProDH activity was 80% lower in *prodh1* than in WT and was not detectable in *prodh1prodh2* double mutants (Figure 5). Thus, the amount of ProDH activity in mitochondria from seedlings corresponds well with the overall ProDH protein content detected on Western blots (Figure 4), and in particular that induction of ProDH1 leads to high ProDH activity.

Proline and some other amino acids can act as respiratory substrates. We measured standard respiration parameters in mitochondria isolated from proline-treated and non-treated seedlings (Supplemental Figure 6, Table 1) to test whether they are affected by the observed induction of ProDH1 (Supplemental Figure 3). NADH and succinate were used in combination as respiratory substrates according to [38] to define the intrinsic mitochondrial parameters as described in Materials and methods. In controls not treated with proline, WT mitochondria displayed a state-3 O<sub>2</sub> consumption rate of 144.5 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>. When ADP had been used up, the state-4 rate was 72.3 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> (Table 1). The respiratory control ratio (RCR) of 2.0 indicates a relatively good capability to readjust electron flow. Coupling between respiration and phosphorylation was normal in these mitochondria with an ADP/O ratio of 1.4 with NADH and succinate as substrates (Table 1). The flow of electrons directed to the CP can be estimated by specifically inhibiting cytochrome c oxidase with cyanide. WT mitochondria

displayed a high CP capacity of 96% of total electron flow (Table 1). AOX capacity was low in WT mitochondria with a mean value of 13.3 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> (Table 1).

Mitochondria from proline-treated seedlings displayed higher respiratory rates than non-treated plants in both phosphorylating states, 166 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> in state 3 and 83.4 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> in state 4 (Table 1). RCR was similar to that observed in non-treated mitochondria indicating a normal capacity to readjust electron flow in the presence of ADP. By contrast, the ADP/O ratio was lower (1.0 versus 1.4), indicating that more electrons had to be transferred to oxygen to produce the same amount of ATP (Table 1). This partial uncoupling may explain the higher respiratory rates observed in proline-treated mitochondria. Proline catabolism is known to induce ROS production [45–47]. We found AOX capacity in WT was 40% higher in mitochondria from proline-treated seedlings compared to non-treated ones, such that the contribution of the CP pathway was only 85% of total electron flow (Table 1).

### **L-proline provides electrons to the respiratory chain**

L-proline oxidation in mitochondria isolated from Arabidopsis leaves was tested and compared with standard respiration (Table 1). Specific L-proline-dependent respiration was detected in mitochondria from proline-treated WT plants (27.9 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> for the CP pathway and up to 36.4 for the AOX capacity) but not in non-treated plants. Proline-related substrates like D-proline (Supplemental Figure 6C) or L-hydroxyproline (data not shown) did not lead to any respiratory chain-dependent O<sub>2</sub> consumption even in mitochondria isolated from L-proline-treated plants. Thus, only mitochondria containing high ProDH levels displayed L-proline respiration.

Can ProDH mediate electron transfer to the mitochondrial respiratory chain? To address this question, proline-dependent mitochondrial respiration was investigated using a *p5cdh* knockout mutant [30] to avoid any electron transfer from this enzyme. State 3 rates were similar in WT and *p5cdh* mitochondria from non-treated plants but RCR was significantly lower in *p5cdh* mitochondria (1.8 versus 2.0) because of higher state 4 rates (78.6 versus 72.3) (Table 1). The ADP/O ratio was also lower and AOX capacity higher (16.5 versus 13.3) in non-treated *p5cdh* mutant line (1.2 versus 1.4), which indicates that mitochondria were less well coupled under control conditions. After proline treatment *p5cdh* mitochondria contained more ProDH than WT (Supplemental Figure 7). The mitochondrial ADP/O ratio in *p5cdh* mutant did not change in response to proline. AOX capacity was also higher in the non-treated *p5cdh* mutant. Proline treatment induced an even greater increase in the AOX capacity of the *p5cdh* mitochondria (36.6 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>). The partial uncoupling and higher AOX capacity observed in non-

treated *p5cdh* suggest that these plants behaved as stressed plants. The apparently contradictory *p5cdh* response to proline treatment (i.e. a large increase in AOX capacity, but no change in ADP/O ratio) may indicate that mitochondrial membrane properties are modified to decrease proton leakage, which compensates for the increased uncoupling by the AOX capacity, thus maintaining the same uncoupling status. L-Proline-dependent O<sub>2</sub> uptake was observed in *p5cdh* mitochondria isolated from proline-treated plants, but the rates were significantly lower compared to WT mitochondria (about 17.9 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> in *p5cdh* versus 27.9 in WT for CP and 19.0 in *p5cdh* versus 36.4 in WT for the AOX capacity). The lower L-proline-dependent oxidation rates measured in the *p5cdh* mutant could be due to the absence of electron transfer from P5CDH to ETC. As *p5cdh* mutants deficient in the second step of L-proline oxidation can use L-proline as sole respiratory substrate, it follows that ProDH itself can deliver electrons to the respiratory chain.

### **ProDH1 isoform is essential for L-proline-dependent respiration**

We wanted to know which ProDH isoform is the main provider of electrons to the mitochondrial respiratory chain in mitochondria isolated from proline-treated Arabidopsis seedlings. We therefore measured respiratory parameters of mitochondria isolated from different *prodh* mutants either treated or not treated with exogenous L-proline. When tested with NADH and succinate as respiratory substrates, all mutant lines behaved like WT with similar increases in the respiratory parameters observed in response to proline (Table 1). AOX capacity values were very high in both mutant lines (between 29 to 45.6 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>). When plants were treated with proline, AOX capacity did not increase significantly in the *prodh1* mitochondria and it decreased in *prodh2* lines. ADP/O ratios were similar in WT and *prodh2* mitochondria but *prodh1* mitochondria had significantly lower ADP/O ratios (1.18 in non-treated and 0.82 in proline-treated plants). This is consistent with the particularly high AOX capacity (up to 45.6 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>) measured in *prodh1* mitochondria.

The *prodh1* mutant mitochondria did not support any L-proline-dependent respiration, whether proline was added to plants or not. In contrast, *prodh2* mitochondria isolated from proline-treated plants displayed L-proline-dependent respiration (up to 23.3 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> for the CP and 18.3 nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> for the AOX pathway). Thus, L-proline dependent respiration was only observed in mitochondria from WT and *prodh2* mutant lines treated with proline, i.e. mitochondria containing high levels of induced ProDH1. These experiments demonstrate that electron transfer from L-proline to the ETC is dependent on ProDH1.

## DISCUSSION

Physiological, biochemical and proteomic analyses have been carried out to get better insights into the role of ProDH1. The use of a specific ProDH antibody allowed, for the first time, to investigate ProDH content in different mutants. One important step in ProDH characterization was the generation of a viable *prodh1prodh2* double mutant. We could demonstrate that ProDH1 is linked to mitochondrial membrane and is part of a low-molecular-weight (70-140 kDa) complex. Proline-dependent respiration analysis indicated a key role of ProDH1 in proline oxidation. Finally, protein separation by 2D BN/SDS PAGE in combination with immunoblot and protein analysis by MS allowed the identification of ProDH1 peptides in a mitochondrial fraction.

### **ProDH forms part of a low-molecular-weight complex in mitochondria**

Proteomic, biochemical and physiological approaches were used to analyze the role of ProDH1 in Arabidopsis. With exogenous proline treatment, ProDH can be substantially induced in suspension cells or seedlings [32]. Western blot experiments carried out on purified mitochondria demonstrated a physical interaction between PRODH1 and the mitochondrial membranes (Figure 3). Using 2D BN/SDS PAGE we detected ProDH as a 70 to 140 kDa signal in the first native gel dimension (Figure 1), indicating that ProDH might dimerize or binds to another protein, an issue to be further investigated. ProDH migrates as a smear in the first BN gel dimension between 70 and 140 kDa as is commonly observed in BN gels because protein separation takes place under very mild conditions. Possibly this causes lipids or detergent molecules to stick to proteins making them smear. In the second denaturing dimension, the ProDH signal runs at 54 kDa, as expected. Overall, seven unique ProDH peptides were identified from gel spots by MS, which cover different regions of the protein (Figure 2). All peptides exactly match the predicted amino acid sequence of ProDH1. This confirms the ability of the anti-ProDH antibody to recognize AtProDH1. ProDH2 peptides were not identified in the spots. We conclude that ProDH1 is by far the most prominent ProDH isoform in mitochondria under the conditions tested. It forms part of a low-molecular-weight complex associated with the mitochondrial membrane.

Dehydrogenases are important in mitochondrial catabolism [15, 48], because they transfer electrons either to NAD(P)<sup>+</sup> or FAD, forming NAD(P)H and FADH<sub>2</sub>, respectively. NADH-producing enzymes are often localized in the mitochondrial matrix or attached to the inner mitochondrial membrane. NADH is re-oxidized by complex I of the respiratory chain or

alternative NADH dehydrogenases, which are especially abundant in plant mitochondria. In contrast,  $FAD^+/FADH_2$  is normally tightly bound to dehydrogenases. It is thought that electrons from  $FADH_2$  are often directly transferred to ubiquinone, which requires tight linkage of the corresponding enzymes to the inner mitochondrial membrane. Like other FAD-containing mitochondrial dehydrogenases, we propose that ProDH is tightly linked to the mitochondrial membrane. The occurrence of two separate carriers for proline in wheat mitochondria supports the model that proline enters the matrix of the mitochondria to be oxidized [49]. ProDH is the first enzyme involved in proline catabolism and most likely transfers electrons directly to ubiquinone as proline is oxidized. In a second step, oxidation of P5C (catalyzed by P5CDH) leading to the formation of glutamate and its conversion into  $\alpha$ -ketoglutarate (transamination step catalyzed by glutamate dehydrogenase), which represents an intermediate of the tricarboxylic acid cycle, contribute to the NADH pool and finally delivery of electrons to the ETC. It is known that electron entry pathways into the respiratory chain of plant mitochondria can vary depending on the biochemical status of the plant cell [15]. The precise mode of action of the 70-140 kDa ProDH complex during electron transfer from proline should be addressed by future research.

### **Proline is a respiratory substrate for ProDH1**

It is well known that proline is an essential energy substrate for insect flight muscles [50,51] and trypanosomatids [52]. The accumulation of proline is a good way of storing energy because the total oxidation of proline can generate reductants that support mitochondrial ETC and the formation of 30 ATP equivalent [53]. The use of *prodh* mutants, including a viable *prodh1prodh2* double mutant, allowed us to investigate the role of each ProDH isoform in mitochondrial respiration (Table 1). *prodh1* exhibited less respiration indicating that ProDH1 is likely to be essential in L-proline-dependent respiration in mitochondria. L-proline-dependent respiration was nevertheless weak compared to respiration driven by canonical substrates like NADH or succinate and it was highly dependent on uncoupling AOX activity. It is thus unlikely that L-proline is oxidised as the sole energy substrate for resuming growth after stress in plants. Proline oxidation might rather help in increasing respiratory rates and regulating redox balance and ROS generation to promote plant recovery from water stress and repair stress-induced damage.

It was shown by [18] that *ProDH2* is induced by proline but repressed by sucrose at least at the transcript level. In isolated *prodh1* mitochondria treated with proline, a weak ProDH activity was measured, which might be explained by the presence of ProDH2 (Figure 5). However, no



proline-inducible ProDH2 isoform was detected in the *prodh1* mutant in response to proline addition (Figure 4). Although the anti-ProDH antibody allows the detection of very small amounts of ProDH2 (Supplemental Figure 4) under our experimental conditions, ProDH2 was either not present or present in such small amounts that it could not be detected in Western blots. In addition, mitochondria from proline-treated *prodh1* mutants did not show any measurable proline-dependent respiration (Table 1). These findings suggest firstly that ProDH2 is not at all abundant in mitochondria even in the presence of proline, and secondly that ProDH2 cannot deliver electrons to the ETC. More importantly ProDH2 did not compensate for the lack of ProDH1 under our experimental conditions and hence may play a role different from that of ProDH1.

The *p5cdh* mutant was reported by [30] to be unable to completely oxidize <sup>14</sup>C-labeled proline into glutamine or glutamate but could accumulate P5C. This is consistent with our results where mitochondria isolated from *p5cdh* seedlings displayed slightly higher ProDH1 content (Supplemental Figure 7) and some L-proline-dependent respiration (Table 1), although at lower rates than in WT seedlings. This indicates that ProDH1 is able to oxidize proline and provide electrons to the ETC even when P5CDH is lacking. The lower rates of respiration observed in *p5cdh* could be explained by the absence of electron transfer from P5CDH to the ETC. It has indeed been demonstrated that plant P5CDH is a soluble enzyme that can function without ProDH [54]. The fact that ProDH and P5CDH can function independently is in contrast to the analysis of the structure of the bifunctional PutA enzyme, which revealed a dynamic tunnel system that allows a substrate channeling between the ProDH and P5CDH active sites [55]. A detailed characterization of the interaction between ProDH and P5CDH in plants will be the next step to understand the mechanism of proline oxidation into glutamate.

Hydroxyproline, a proline derivative, is known to serve as substrate for the vertebrate ProDH2 [56]. However, plant and animal ProDH2 isoforms are not homologous as they have evolved different functions [2]. As shown by oxygen consumption measurements, hydroxyproline could not be used as a respiratory substrate for mitochondria isolated from either WT or *prodh* mutants. The tyrosine 540 residue was found to be an important in enforcing the preference for proline over hydroxyproline as the substrate [56]. Both Arabidopsis ProDH1 and ProDH2 protein sequences show high sequence similarity to vertebrate ProDH1 including tyrosine residues in analogous positions [2]. It is therefore unlikely that any plant ProDH could use hydroxyproline as a substrate. We found that unlike ProDH1, ProDH2 does not participate in a detectable level of L-proline respiration (Table 1). In this respect the function and location of plant ProDH2 should be further investigated especially in relation to biotic stresses.

### **ProDH1 and ProDH2 have distinct roles**

The high levels of proline accumulated under stress, as well as possible movement of proline between different organs of the plant and cyclic proline synthesis and degradation, suggest that the flux of proline into and out of specific compartments is likely to be an important determinant for stress tolerance [57]. Oxidation of proline in mitochondria may provide reducing power and nitrogen sources for growth. Our data demonstrated that ProDH did not accumulate at all in the *prodh1prodh2* double mutant in response to proline addition (Figure 4). To our surprise, this double mutant did not show an obvious developmental phenotype. It grew in a similar way to WT and produced viable seeds, which suggests that proline degradation is not essential for vegetative and reproductive plant growth. The presence of another enzyme able to oxidize proline is a possible explanation. However <sup>14</sup>C proline feeding studies gave no evidence for an alternative proline degradation pathway in Arabidopsis [30].

The use of *prodh* mutants in the investigation of proline hypersensitivity demonstrated the key role of ProDH1 in primary root length growth (Supplemental Figure 5), which is in accordance with previous published work [22,58]. Under control conditions, the roots of plants lacking *prodh1* were indistinguishable from the wild type, indicating that ProDH1 is not involved in growth *per se*, which is consistent with previous report [56]. The fact that *prodh2* behaved differently for its primary root growth points to different and non-redundant functions of the two ProDH isoforms [18]. When proline is in excess, the P5C-proline cycle, involving ProDH and P5C reductase, is activated to deliver electrons to the mitochondrial ETC [45]. The sensitivity of both *prodh1* and *prodh1prodh2* mutants to exogenously supplied proline cannot be explained by the activation of this cycle because ProDH, which is a key component of this P5C-proline cycle, is absent in *prodh* mutant. In addition, the fact that *prodh1prodh2* is sensitive to excess of proline suggests that proline toxicity is not mediated by P5C in these conditions.

Based on our data, it is clear that proline cannot be oxidized in mitochondria when ProDH1 is lacking or present at very low levels. Thus the presence of a high amount of proline in *prodh1* may affect mitochondrial homeostasis as indicated by the strong increase in AOX capacity in these mutants. The results from the proline-treated plants are characteristic of an oxidative stress response. As AOX is one of the uncoupling systems of plant mitochondria, the high AOX capacity could partially explain the low ADP/O ratio observed in these mitochondria (Table 1). In addition, an excess of proline might differentially regulate the expression of key genes involved in amino acid metabolism, cell wall and photosynthesis [22,59], which reinforces the idea that proline metabolism is tightly regulated to maintain plant homeostasis. As suggested by

[18], our results points to different and non-redundant functions of the two ProDH isoforms in Arabidopsis.

### **AUTHOR CONTRIBUTION**

CCH, KS, AG and SP performed the experiment on mitochondrial respiration. CCH, PS, MBJ, KS and SL were involved in the biochemical characterization of ProDH, EC, DLDV, TG and LR in the molecular analysis of the *prodh* mutants, JK, PS and HE in proteomic analyses. CA and PC provided helpful feedback on the experimental design and an early draft of the paper. HPB and AS formulated the original problem, provided direction and guidance and wrote the manuscript.

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## FIGURE LEGENDS

### **Figure 1 ProDH1 is strongly induced upon proline treatment of Arabidopsis suspension cells.**

Total mitochondrial proteins of cells incubated without or with proline for 21 h were separated by 2D blue native – SDS/PAGE. (A) Proteins were either Coomassie stained (top) or blotted onto nitrocellulose (bottom). Blots were developed using an antibody directed against ProDH from Arabidopsis. Molecular masses of standard proteins are given to the left (in kDa), identities of the OXPHOS complexes are indicated above the gels. I+III<sub>2</sub>: supercomplex formed of complexes I and dimeric complex III; I: complex I; V: complex V; III<sub>2</sub>: dimeric complex III; F<sub>1</sub>: F<sub>1</sub> part of complex V; IV: complex IV; II: complex II. The lipoamide dehydrogenase (L protein of the glycine dehydrogenase complex; mtLPD) is indicated on the gels. (B) Immune signals from corresponding areas of blots from (A) visualized after 2, 5, 120 and 300 s.

### **Figure 2 Identification of ProDH1 by MS.**

Total mitochondrial protein of proline-treated Arabidopsis cells was separated by 2D Blue native – SDS/PAGE and Coomassie-stained (A). Four gel spots were cut out from the gel at positions corresponding to ProDH signals obtained on a parallel immunoblot (B) and analyzed by MS (C). Peptides identified within spots 1-4 are reported. (D) Amino acid sequence of ProDH1 with the location of the corresponding peptides.

### **Figure 3 ProDH is associated with mitochondrial membranes.**

Mitochondria were purified from 15-d-old seedlings treated for 24 h with 50 mM L-proline by Percoll density gradient ultracentrifugation. Proteins in mitochondrial membrane (MB) and soluble (S) fractions were then separated by SDS PAGE (10 μg of each fraction per lane). Western blots were probed with antibodies that recognize (A) ProDH, (B) adenine nucleotide translocator (ANT) or (C) NAD<sup>+</sup>-isocitrate dehydrogenase (ICDH) and developed with chemiluminescence reagents (left panels). Blots were stained with Ponceau S (right panels). M, molecular weight makers.

### **Figure 4 Molecular characterization of the Arabidopsis *prodh* mutants.**

(A) Map of T-DNA insertion loci in four mutant lines with the primer references used for genotyping. Primers are listed in Supplemental Table S1. *prodh1-3* and *prodh1-4* carried inverted T-DNA tandem repeats. Grey box, promoter; white boxes, 5' and 3' non-coding

regions; black boxes, exons; dashed lines, introns.

(B) Genomic DNA was extracted from either Col-0 (WT), *prodh1-3*, *prodh1-4*, *prodh2-2* or *prodh2-3* seedlings and PCR products were separated in a 0.8 % agarose gel. M, molecular weight makers in kb.

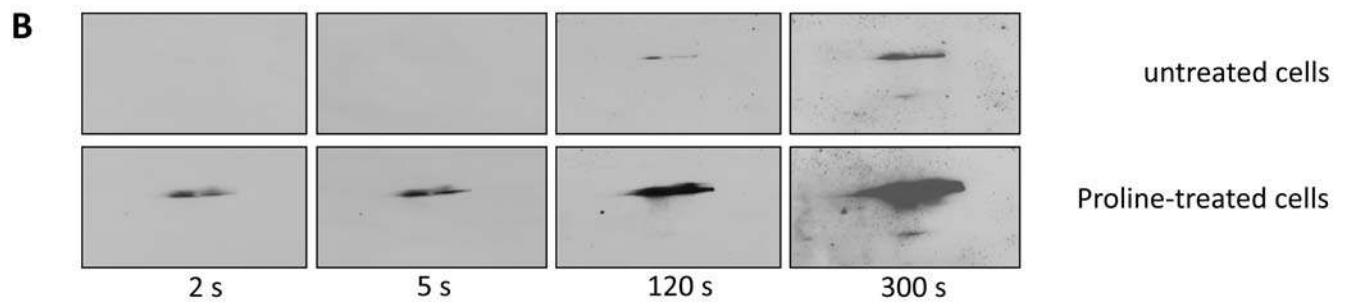
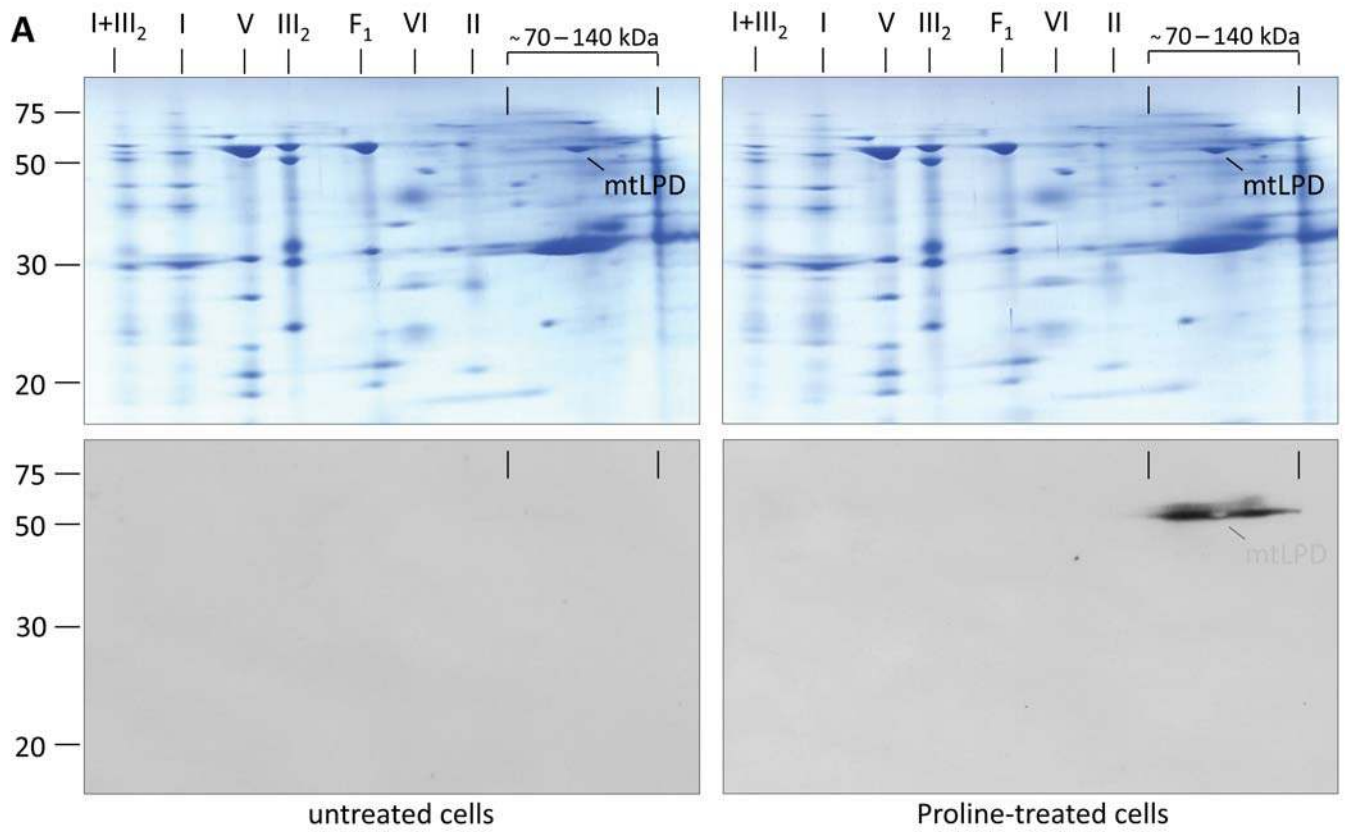
(C) and (D) Western blots of proteins from 15-d-old seedlings non-treated (NT) or treated with 50 mM L-proline (Pro) for 24 h. Proteins were separated by SDS PAGE and blots were probed with anti-ProDH antibody.

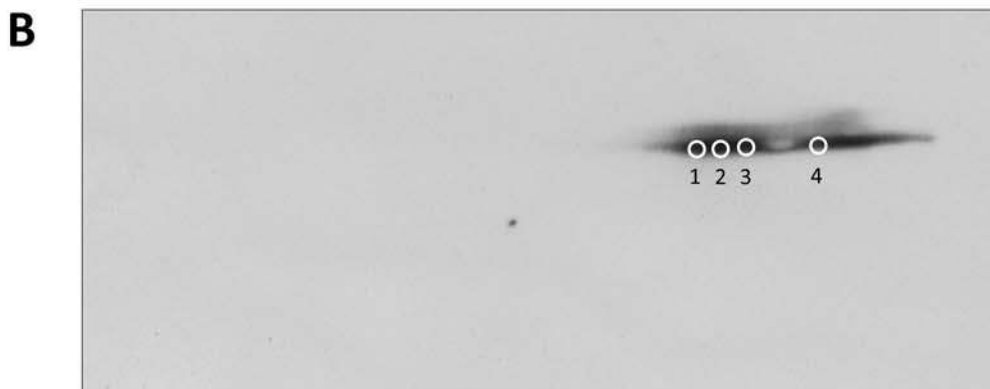
(C) Total protein or mitochondrial fractions from WT, *prodh1-4*, *prodh2-2* or double mutant *prodh1-4prodh2-2* (p1xp2).

(D) Mitochondrial proteins isolated from WT, *prodh1-3* and *prodh2-3*.

**Figure 5 ProDH activity in WT and in *prodh1-4*, *prodh2-2* and *prodh1-4prodh2-2* (p1xp2) mutants treated with 50 mM proline for 24 h.**

Crude mitochondria were isolated from 15-d-old plants. ProDH activity was measured using 2,6- dichlorophenolindophenol (DCIP) as an electron acceptor. Standard errors are based on at least three biological replicates.  $P < 0.05$ , Mann-Whitney U test, a: *prodh* mutant versus WT in non-treated condition (Control); b: *prodh* mutant versus WT in proline-treated condition; c: proline-treated seedlings versus non-treated ones for each genotype.





**C**

spot	accession	protein	Mass	MASCOT	peptides	Seq.cov.
1	AT3G30775	ProDH1	54.9	279	6	13.6
2	AT3G30775	ProDH1	54.9	222	5	9.2
3	AT3G30775	ProDH1	54.9	224	6	10.8
4	AT3G30775	ProDH1	54.9	186	5	9.2

**D**

MATRLLRITNF	IRRSYRLPAF	SPVGPPTVTA	STAVVPEILS	FGQQAPEPPL
HHPKPTEQSH	DGLDLSQAR	LFSSIP TSDL	LRSTAVLHAA	AIGPMVDLGT
WVMSSK LMDA	SVTRGMVLGL	VKSTFYDHFC	AGEDADAAAE	RVRSVYEATG
LKGM LVYGVE	HADDAVSCDD	NMQQFIRTIE	AAKSLPTSHF	SSVVVKITAI
CPISLLKRVS	DLLRWEYKSP	NFKLSWKLKS	FPVFSESSPL	YHTNSEPEPL
TAEERELEA	AHGRIQEICR	KCQESNVPLL	IDAEDTILQP	AIDY MAYSSA
IMFNADKDRP	IVYNTIQAYL	RDAGERLHLA	VQNAEKENVP	MGFKLV RGAY
MSSEASLADS	LGCKSPVHDT	IQDTHSCYND	CMTFLMEKAS	NGSGFGVVLA
THNADSGR LA	SRKASDLGID	KQNGKIEFAQ	LYGMSDALS F	GLKRAGFNVS
KYMPFGPVAT	AIPYLLRRAY	ENRGMMATGA	HDRQLRMEL	KRRLIAGIA

spot 1 R.GMVLGLVK.S K.LMDASVTR.G K.ITAICPISLLK.R R.SVYEATGLK.G R.LFSSIP TSDLLR.S K.ANSGSGFGVV LATHNADSGR.L	spot 3 R.GMVLGLVK.S K.LMDASVTR.G R.IQEICR.K K.ITAICPISLLK.R R.SVYEATGLK.G R.LFSSIP TSDLLR.S
spot 2 K.LMDASVTR.G R.IQEICR.K K.ITAICPISLLK.R R.SVYEATGLK.G R.LFSSIP TSDLLR.S	spot 4 K.LMDASVTR.G R.IQEICR.K K.ITAICPISLLK.R R.SVYEATGLK.G R.LFSSIP TSDLLR.S

