Supplemental Data

Miller et al (2009) Unraveling Δ^{1}-Pyrroline-5-Carboxylate (P5C)/Proline Cycle in Plants by Uncoupled Expression of Proline Oxidation Enzymes

Supplemental Table 1

	Control				Drought				Rehydration			
Metabolite	wt		*p5cdh*		wt		*p5cdh*		wt		*p5cdh*	
	Resp[a] ratio	SD[b]	Resp ratio	SD	Resp ratio	SD	Resp ratio	SD	Resp ratio	SD	Resp ratio	SD
Ala	0.907	0.271	1.051	0.282	3.263	0.503	3.218	0.731	1.434	0.284	1.408	0.373
Asn	0.029	0.020	0.022	0.011	0.023	0.010	0.024	0.015	0.025	0.019	0.021	0.011
Arg	0.258	0.120	0.227	0.044	0.064	0.014	0.046	0.016	0.032	0.011	0.036	0.008
Glu	0.111	0.049	0.107	0.047	0.170	0.065	0.165	0.102	0.147	0.076	0.141	0.055
Gln*	1.269	1.082	0.967	0.173	2.178	0.083	2.479	0.464	1.263	0.707	1.107	0.312
Leu	0.282	0.047	0.318	0.062	0.263	0.028	0.217	0.032	0.427	0.042	0.425	0.097
Lys	0.178	0.011	0.222	0.022	0.094	0.018	0.072	0.013	0.116	0.034	0.147	0.025
Pro	0.118	0.027	0.044	0.016	13.709	3.380	15.305	4.413	0.288	0.078	8.842	3.053
Ser	0.480	0.096	0.493	0.084	0.469	0.097	0.475	0.070	0.524	0.061	0.467	0.072
Thr	0.047	0.002	0.047	0.004	0.081	0.007	0.080	0.010	0.086	0.013	0.076	0.011
Tyr	0.042	0.007	0.049	0.009	0.011	0.005	0.008	0.002	0.042	0.004	0.046	0.011
Val	0.384	0.064	0.429	0.073	0.514	0.027	0.570	0.064	0.518	0.055	0.545	0.103
Ornithine	0.627	0.211	0.584	0.098	0.238	0.191	0.118	0.029	0.145	0.025	0.189	0.114
Pyro-glu	3.689	1.076	3.756	0.398	5.906	0.867	4.854	0.612	5.558	1.592	4.421	1.213
Fructose	2.311	0.404	3.044	0.376	11.080	2.197	16.808	2.610	1.726	0.509	1.960	0.333
Galactose	0.264	0.060	0.316	0.031	0.260	0.044	0.303	0.019	0.196	0.023	0.229	0.044
Glucose	0.487	0.071	0.602	0.071	1.099	0.423	1.737	0.186	0.156	0.027	0.164	0.002
Trehalose	0.009	0.002	0.007	0.002	0.357	0.058	0.546	0.123	0.063	0.029	0.067	0.027
Malic acid	0.018	0.004	0.020	0.002	0.087	0.016	0.058	0.011	0.091	0.025	0.088	0.015

Metabolic profiling of leaves of *p5cdh* and WT plants grown under normal conditions or subjected to drought stress.

GC-MS analysis of polar extracts from shoots of 3-week-old *Arabidopsis* plants grown on vermiculite under normal conditions (control) or subjected to 3 days of drought and thereafter rehydrated for 24 h. Polar extracts of fresh samples (100 mg FW) were prepared and derivatized, as described in ''Experimental Procedures", prior to their GCMS analysis.

[a]Respective (Resp) ratio represents the ratio between the integrated peak area of a certain metabolite and that of the ribitol internal standard. The values of the Resp. ratio were normalized to milligram dry weight according to the average dry weight of the samples at each time point of the experiment. Peak areas were integrated using XCALIBUR V1.3 program (ThermoFinnigan). [b]SE, Standard error of 5 samples.

*Resp. ratio of the average of 3 repeats of Gln.

Supplemental Materials and Methods

Drought stress treatment

Drought stress was imposed on 3-week-old plants grown on MS-soaked vermiculite (60 ml MS per 20 g vermiculite) in round-shape (10 cm-diameter, 8 cm-height) transparent polypropylene cups by removing the lids for 3 days (reaching up to 80% water loss from the vermiculite in the cups). Plants were then rehydrated by adding the lost water volume to the cups and closing them for the recovery period.

Relative water content (RWC)

Shoot samples of 3-week-old WT and *p5cdh* plants (10 plants per sample in 5 replicates) collected during the dehydration (24 h, 72 h) and the consecutive 24 h rehydration periods, were placed in 2 ml tubes and immediately weighed to determine the net Fresh Weight (FW). Samples were then fully covered with ddH_2O and incubated in the dark for 4 hours, at 25^0C. The hydrated samples were weighed again to determine the Turgor Weight (TW), after carefully soaking the external water excess. Dry Weight values (DW) were measured after drying the samples at 80ºC for 16 h. RWC values were calculated using the following formula: RWC = (FW-DW)/(TW-DW)x100.

Vermiculite water retention measurement

In the drought experiments, water loss after the dehydration period from the MS-soaked vermiculite in growth cups with or without 3-week-old plants (described above) was estimated by weighing the cups. Changes in the vermiculite matric potential, reflecting the energy that must be exerted to extract the water, were measured using tensiometers (Soil moisture equipment corp., Santa Barbara, USA; Supplementary Fig. S6A) and expressed in megapascal units (1 centibar= 1 kiliopascal). The measurements were performed during 72 h drought stress in open cups and throughout the recovery period in closed cups.

Estimation of P5C stability and pH dependence-

P5C was applied as a 2,4-dinitrophenylhydrazine-hydrochloride (DNPH-Cl_2) salt that stabilizes P5C when crystallized (48). Our measurements (Supplemental Fig. S2) confirmed P5C instability. After 72 h incubation in MS medium (pH 5.8) about 30% (300 μM) of the original P5C amount (1mM) remained when applied as DNPH-Cl_2 salt, while only 10% P5C (100 μM) was detected after 72 h when pure P5C was applied. Hellmann et al. (2000) showed that WT *Arabidopsis* plants are much more sensitive to P5C (DNPH-Cl_2-P5C) than to Pro when both are externally applied. To verify the DNPH-Cl_2 effect, WT plants were exposed to 100 mM Pro or 1 mM P5C with or without DNPH-Cl_2 for 3 days (Supplemental Fig. S5). DNPH-Cl_2-P5C was found to be very toxic (Supplemental Fig. S5 C) but not pure P5C (about 100 μM remained after 72 h treatment) (Supplemental Fig. S5 D).

Pure P5C stock was prepared according to Mezl and Knox, 1976 (49). Stock of 10 mM DL-P5C was prepared by dissolving 2.6 mg DNPH-Cl_2-P5C in 0.75 ml 0.25 M HCl and then 0.75 ml acetophenone (Sigma) was added and mixed by shaking for 1 h until the 2,4-dinitrophenylhydrazine hydrochloride (orange) was completed extracted to the acetophenone phase. Pure 2,4-dinitrophenylhydrazine hydrochloride was produced by shaking 5 g of 2,4-dinitrophenylhydrazine (Sigma) with 10 ml HCl until the reagent-free-base (red) formed the hydrochloride derivative (yellow). It was then dissolved in 100 ml ethanol in a heated water bath, cooled and crystallized at room temperature and then washed with ether and dried at room temperature.

Generation of transgenic tobacco and Arabidopsis plants

The full-length 1.53 kb *MsProDH1* cds (12) was PCR amplified with forward 5'GAACAACAACAAACAACAAACATCTTTGACCGGTTGATGGCCACCAGAG 3' and reverse 5'ATCAACTCATCCAACAAATTTAAAGCACAGAAGC 3' primers, using

ExTaq polymerase (Takara). The amplified fragment was cloned downstream to the CaMV 35S promoter in the pPCV702 binary vector and introduced into *Agrobacterium tumefaciens* GV3101::pMP90RK strain. Transgenic tobacco and *Arabidopsis* plants were generated by leaf disc transformation or by floral dipping, respectively as previously described (12, 46).

Proline oxidation assay

Samples of 1g drought-stressed tobacco leaves, frozen in liquid nitrogen, were ground by pre-cooled pestle and mortar. After adding 2 ml extraction buffer [50 mM Tris-HCl, pH 7.5, 3 mM EDTA, pH 7.5, 600 mM KCl, 7 mM $MgCl_2$, 10 mM DTT, 0.1% Triton-X-100, 1% Protease Inhibitor Cocktail (Roche)], the extract was centrifuged (16000 x g) at 4°C, for 20 minutes. The supernatant was loaded onto PD10 column, pre-washed 4 times with 4 ml Activity Buffer (150 mM Na_2C0_3-HCl, pH 10.3) in the cold room. Proteins were eluted in Activity Buffer and stored at 4°C until used. The reaction mixture (1 ml) contained 200 µg proteins, 500 µl Activity Buffer, 1.5 mM NAD^+ and increasing concentrations of L-Proline (ranging from 0 mM to 75 mM). Reduction of NAD^+ to NADH was measured at 340 nm (Ultrospec 2000) during 4 min incubation at 25°C. NADH concentration was calculated using Beer-Lambert equation and NADH extinction coefficient of 6.220 mM^{-1} cm^{-1}.

Real-Time Quantitative RT-PCR

Real-time PCR reactions (qRT-PCR) were performed in an optical 96-well plate using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Scoresby, Victoria, Australia) and SYBR Green I for monitoring dsDNA synthesis. For all PCR reactions the following standard thermal profile was used: 50°C for 2 min; 95°C for 15 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. The real-time PCR data were analyzed using the SDS 2.0 software (Applied Biosystems). C_T (threshold cycle) values for *ProDH* cDNAs were normalized to the C_T values of cyclophilin (*At2g36130*), used as an internal standard. The following primers were used for amplification: 5'-TGGCGAACGCTGGTCCTAATACA-3' and 5' CAAAAACTCCTCTGCCCCAATCAA-3' for cyclophilin cDNA; 5'-CGATACAACACCGCCACAAAA-3' and 5'-TTGTCGATGATGGCGGTTT-3' for *MsProDH1* cDNA; 5'-TGGCATGGTTTTAGGGCTTG-3' and 5'-ACGCTTCTCACGCGCTCAG-3' for *AtProDH1* and *AtProDH2*. The primers were designed using the Primer Express 2.0 software (Applied Biosystems). The size of amplified products was about 100 bp. The real-time PCR data were analyzed using the comparative CT-method with appropriate validation experiments performed beforehand (Applied Biosystems, User Bulletin #2, http://home.appliedbiosystems.com/). Every experiment was repeated three times with cDNA template prepared from the same batch of plants.

Mitochondrial Superoxide detection in roots by MitoSox staining

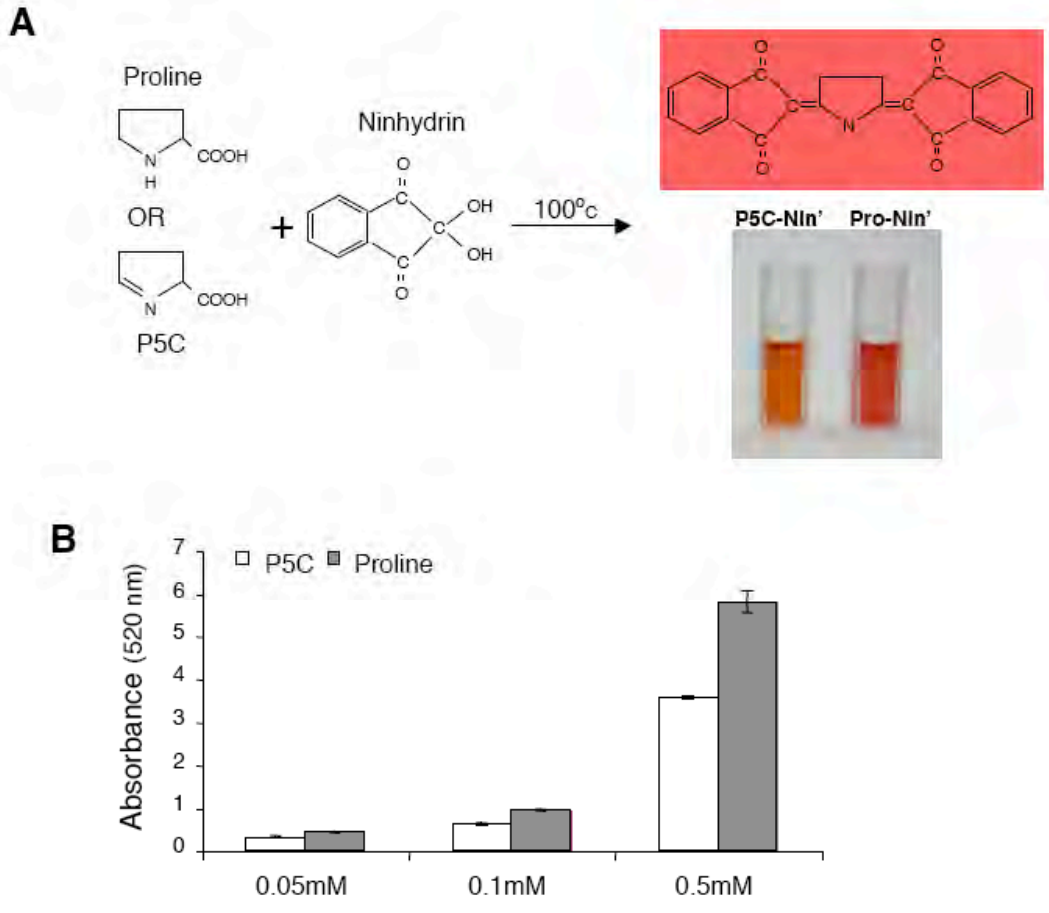
WT and *p5cdh* were germinated in 3 ml liquid MS in 6-well plates under constant agitation and under constant light (~50 µE). Five days old seedlings were treated with 100 mM Pro, 100 µM PQ or 100 mM Pro + 100 µM PQ in the dark for 24 h. In additional experiments, light-grown seedlings were treated 100 mM Pro or with MS medium (control) for 24 h.

MitoSox-Red is a newly developed superoxide indicator (Invitrogen, Mito-HE), specific to mitochondria of living cells. MitoSox is an ethidium-base dye that binds to DNA and upon oxidation by O_2.- becomes highly fluorescent (59). Since MitoSox does not enter the nuclei under normal conditions, its mitochondrial staining is considered specific to ROS presence.

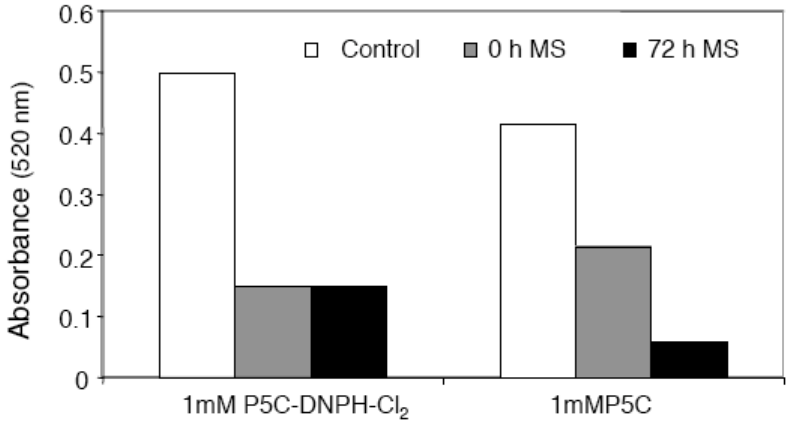
MitoSox-Red (5 µM, Molecular Probes-Invitrogen) and MitoTracker-Deep Red (250 nM, Molecular Probes-Invitrogen) were added to each solution for 25 and 40 min respectively, under the same experimental condition. Fluorescence was detected in roots using a Confocal microscope (Olympus, IX 81 FV 1000, Olympus, Tokyo) with Ex/Em of 515/580 and

635/680 for MitoSox-Red and MitoTracker-Deep, respectively and with DAPI staining as a control for nuclear localization.

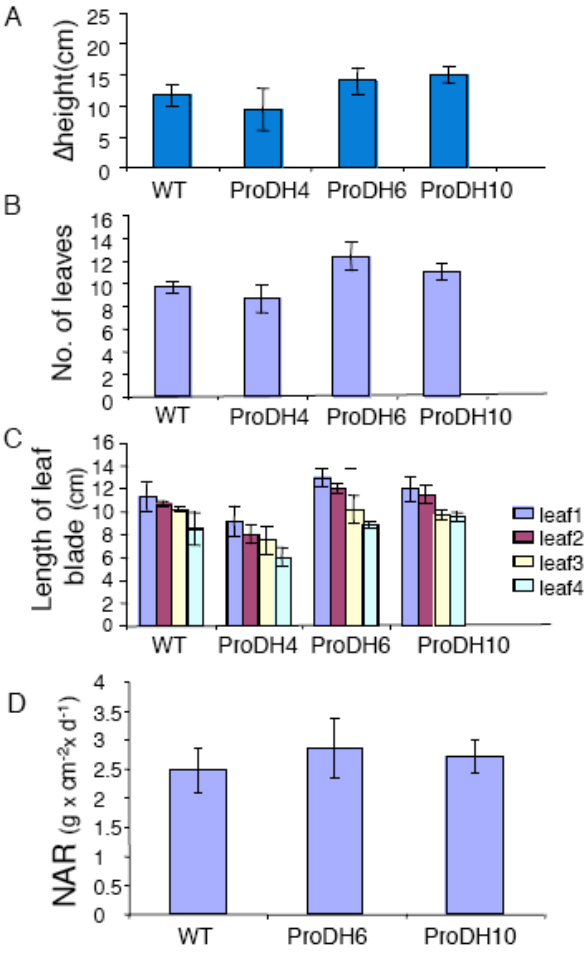
Supplemental Figures



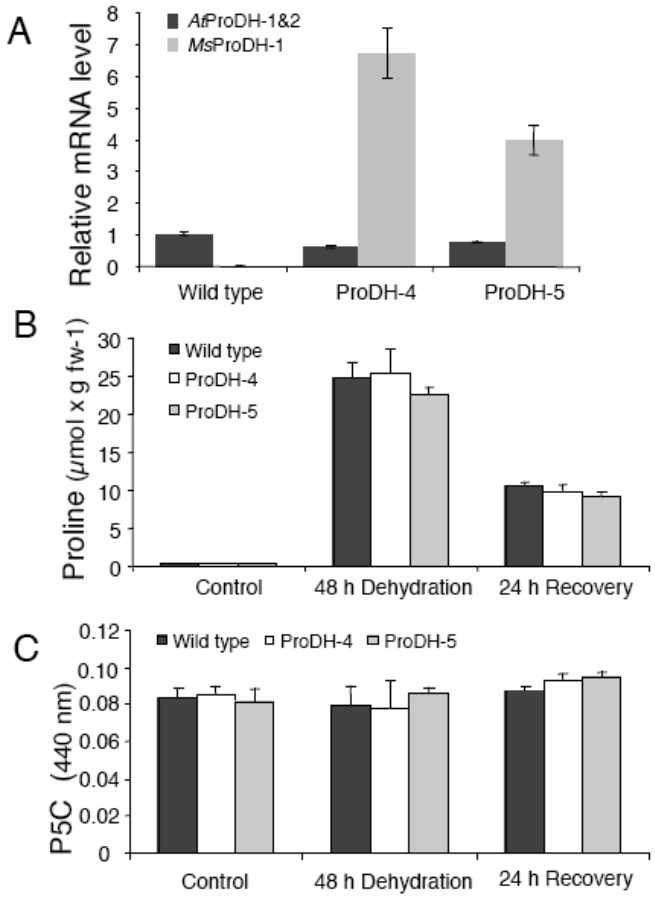
Supplemental Figure S1. Pro and P5C form complexes with ninhydrin.
Pro and P5C have almost identical structure and can similarly react with ninhydrin (Nin'). A. Scheme of the ninhydrin reaction based on Troll and Lindsley (1) (left) and the colored products of Pro (red) and P5C (orange). B. The average absorbance of increasing concentrations of each compound (at 520 nm). Standard deviation represents 3 replicates.



Supplemental Figure S2. Stability of P5C in MS medium. Purified P5C (1 mM) or P5C-hydrazine hydrochloride double salt (1 mM) were dissolved in 25 mM HCl (Control, pH<2) or in MS medium (pH 5.8). P5C concentrations were determined by the ninhydrin assay immediately or after 72 h. The results show that under low acidic conditions both P5C and P5C-hydrazine hydrochloride are relatively stable. At pH 5.8, P5C is less stable than P5C-hydrazine hydrochloride after 72 h.



Supplemental Figure S3. ProDH-OE and WT tobacco plants exhibit similar growth rates under salinity stress. Six-week-old plants, grown on soil, were irrigated with 300 mM NaCl. After 5 weeks, shoot elongation (A), number of newly added leaves (B) and blade length of the first 4 leaves that developed during the salinity stress (C) were measured. Net Assimilation Rate (NAR) of WT and ProDH-OE plants during 21 d of drought is shown in D, expressed in dry weight gained per cm^2 of leaf area per day (D).

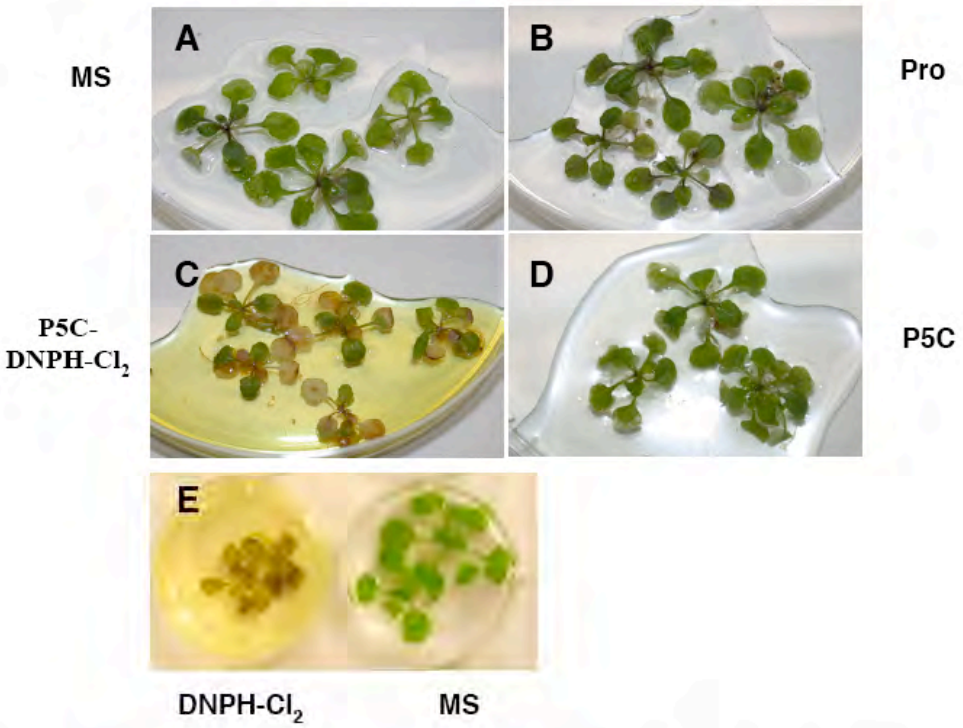


Supplemental Figure S4. Changes in Pro and P5C levels in WT and *MsProDH*-overexpressing *Arabidopsis* plants in response to dehydration.

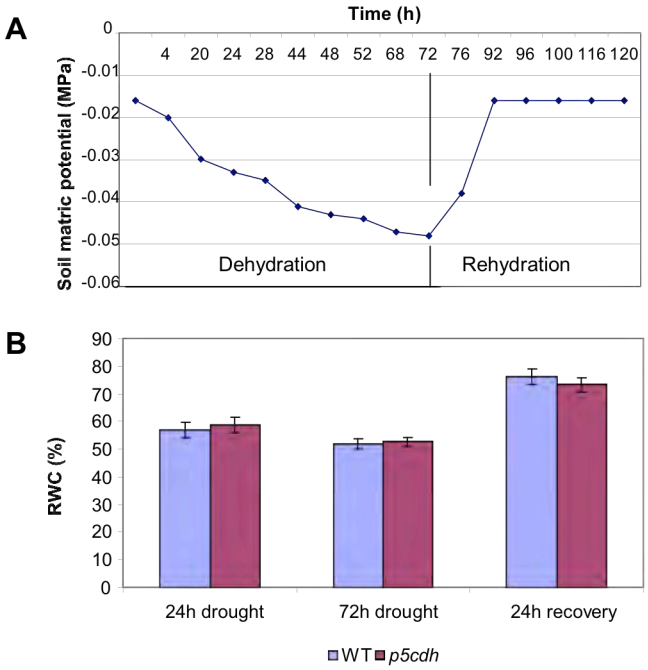
Three-week-old WT and *p5cdh* plants, grown on vermiculite, were dehydrated for 48 h and thereafter rehydrated for additional 24 h by adding the water lost by evaporation. A. Real time qPCR results showing the relative steady state levels of the endogenous *AtProDH1* and *AtProDH2* mRNAs and ectopic *MsProDH* mRNA after 48 h dehydration, in shoots of WT and *MsProDH*-overexpressing plants. B. Pro levels accumulated in shoots during dehydration and after recovery. C. P5C levels in shoots during 48 h water stress and after recovery.

Because commercially produced P5C is no longer available, we were not able to convert the optical density units of P5C absorbance to concentration values. Standard deviation represents 4 biological replicates, each comprised of pooled shoots of 15 plants.

No significant differences (ANOVA) in Pro and P5C values were found among lines in each treatments.



Supplemental Figure S5. Hypersensitivity of plants to the hydrazine salt of P5C. Two-week-old *Arabidopsis* plants were transferred to MS liquid media with no supplements (A), with 100 mM Pro (B), with 1 mM P5C as 2,4-dinitrohydrazine hydrochloride double salt (C) or with purified P5C (D). Plants were photographed after 72 h incubation. An additional control showing 2,4-dinitrohydrazine hydrochloride (1 mM for 48 h) damage (E - left side) compared to MS-grown plants (E-right side).



Supplemental Figure S6. Soil matric potential and shoot relative water content of WT and *p5cdh* plants during drought and recovery. A. Changes in the vermiculite martric potential, reflecting the energy that must be exerted to extract water from the soil, during 72 h dehydration and consecutive rehydration. B. Relative water content (RWC) of WT and *p5cdh* shoots of 3-week-old plants exposed to 72 h drought stress and 24 h rehydration.