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111Equation Chapter 1 Section 1PHYSIOLOGICAL CHARACTERIZATION AND PROLINE ROUTE GENES QUANTIFICATION UNDER LONG-TERM COLD STRESS IN CARRIZO CITRANGE.

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Keywords

Citrus, cold stress, ornithine, photosynthesis, proline route.

ABSTRACT

Low temperature is one of the abiotic stresses that most limits crop production, growth and distribution. Currently climate change weather alters temperature worldwide, which produces both high and low temperatures. This work aimed to know how citrus responds to long-term low temperatures after being submitted to 1°C day-night during six weeks. Physiological parameters were analyzed and it was obtained that photosynthesis and growth drastically decreased, water potential increased and osmotic potential did not change. Fv/Fm (maximum quantum yield of PSII) lowered, and degradation of chlorophylls a and b took place.

The proline concentration in leaves moderately increased. ~~Two synthesis gene expression from the glutamate route (P5CS1 and P5CS2) and one gene expression from the ornithine route (dOAT) were quantified~~ The expression of two genes from the glutamate synthesis route (P5CS1 and P5CS2) and one gene from the ornithine route (dOAT) was quantified. The results reveal that the dOAT transcripts from the proline synthesis gene underwent greater induction than P5CS1 and P5CS2. A marked increase in ~~from the degradative expression genes~~expression of proline degradative genes (PDH and P5CDH), a drop in starch, a decrease in the concentration of soluble sugars, and higher total nitrogen quantification in leaves were also observed. Citrus plants stopped growing, but mobilized reserve substances to survive ~~once~~cold stress ~~had passed~~conditions.

1. INTRODUCTION

Low temperature is one of the main stresses that limits crop growth, productivity and distribution. In the USA more economic losses are caused by freezing crops than any other abiotic stress (Attaway, 1997). In California, a loss of approximately 500 million dollars through lost and damaged fruit on approximately 450,000 ha of trees occurred in December 1990 (Attaway, 1997). Spain ranks among the top six international fresh producer countries, with a total production of 6512.6 thousand tonnes of citrus during the 2013 season, while it exported 3914 thousand tonnes (Citrus Fruit Statistics FAO 2018, http://www.fao.org/). Valencia is the most important citrus-producing region in Spain with 32% of Spanish production. In 2010 two consecutive frosts occurred in Valencia that caused production losses of 30%, which accounted for 142 million euros in losses according to AVA-Asaja (the Valencian Farmers' Association). Frosts have always appeared in our history, but climate change and global warming occur and reflect changes in the weather patterns in phenomena such as drought and frost, among others.

Citrus is considered a tropical and subtropical fruit that is generally vulnerable to frost (Sakai and Larcher, 2012). Overall the best fruit quality in Spain is obtained in areas where average temperatures range between 18°C and 28°C (minimum and maximum temperature, respectively), with variations for each species and variety. So if temperatures drop in winter beyond this temperature range, they can cause massive damage to crops. Physical damage has been studied. Temperatures below 0°C result in the formation of intra- and extracellular ice. The crystals that form in an extracellular

space can cause cellular dehydration (Taiz and Zeiger, 2010). The threshold temperature that kills young shoots is around -12°C (Nesbitt et al., 2002), although some citrus generally tolerate -10°C (Yelenosky, 1991). More serious fruit effects happen when internal flesh dries and open spaces between segments develop, which occur several days to a few weeks after the freezing event, depending on severity and duration. If freezing is severe, peel damage is observed in the form of brown staining and pitting (Obenland et al., 2003).

In this cold stress situation, plants usually respond to deal with the damage they have suffered. Osmoprotective substances begin to synthesise (sugars and sugar alcohols), along with glycine betaine and amino acids, like proline, to cope with water stress and to thus acclimatise (Megha et al., 2014). Proline is an amino acid that performs multiple functions in the cell, plays a key role in osmotic adjustment and has the increased ability to resist cellular dehydration. Indeed proline accumulation is capable of protecting plants against multiple abiotic stresses: drought, salinity, temperature extremes (heat, cold and freezing), waterlogging, heavy metal toxicity, nutritional imbalance, ozone and UV-B radiation (Kavi Kishor, 2004; Verbruggen and Hermans, 2008; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Anwar Hossain et al., 2014; Kaur and Asthir, 2015; Saibi et al., 2015)(P. B. Kavi Kishor, 2004; Verbruggen and Hermans, 2008; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Anwar Hossain et al., 2014; Kaur and Asthir, 2015; Saibi et al., 2015, P. B. Kavi Kishor, 2004; Verbruggen and Hermans, 2008; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Anwar Hossain et al., 2014; Kaur and Asthir, 2015; Saibi et al., 2015).

During osmotic stress, proline promotes the stabilization of subcellular structures and membranes, protein stabilization, denaturation protection of proteins, and the detoxification maintenance of cellular functions of reactive oxygen species (ROS) (Kavi Kishor, 2004; Szabados and Savouré, 2010).

Proline in plants is synthesized primarily from glutamate, which is reduced in the cytosol, first by glutamate-5-semialdehyde (GSA), by pyrroline-5-carboxylatesynthetase 1 and 2 (P5CS1 and P5CS2), and is spontaneously converted into pyrroline-5-carboxylate (P5C). P5C reductase (P5CR) reduces P5C in proline. Instead proline catabolism occurs in mitochondria via proline dehydrogenase (PDH) to produce P5C from proline and delta 1-pyrroline-5-carboxylate dehydrogenase (P5CDH) that converts P5C into glutamate. Alternatively, proline can also be synthesized from ornithine, which is transaminated in mitochondria by delta ornithine aminotransferase (dOAT) to produce GSA and P5C, which are then converted into proline (Hare and Cress, 1997; Szabados and Savouré, 2010; Anwar Hossain et al., 2014; Kaur and Asthir, 2015). Proline synthesis routes from glutamate by P5CS1 and P5CS2 and the ornithine route from dOAT are subjects of debate. In the present study we quantified the gene transcripts of the ~~biosynthesis~~ proline biosynthesis route (*P5CS1*, *P5CS2*, *P5CR* and *dOAT*) and degradation genes (*PDH* and *P5CDH*) to know what occurs in the proline route with long-term cold stress in citrus plants.

George Yelenosky group demonstrated that in Citrus (Rough Lemon, Sour orange and Citrus sinensis), the increase of ~~the~~ proline and leaf starch concentration could have a protective effect on Citrus plants at low temperatures (Kushad and Yelenosky, 1987; Vu and Yelenosky, 1992; Yelenosky, 1985). It would be, therefore, of much interest to

clarify what happens in synthesis-degradation proline route genes and in the physiological response to long-term cold stress in grafted citrus rootstocks to determine the influence of proline as a mechanism of adaptation. We chose Carrizo citrange grafted with the Valencia delta seedless orange variety. This rootstock is currently the most widely used, especially in Spanish citrus orchards, as more than 80% of plants are grafted on it. Valencia delta seedless is one of the most important varieties cultivated worldwide. Besides, one of the main aims of characterizing cold stress (1°C) is to increase cultivation areas to regions with a temperate climate as a measure against climate change and new biotic stresses that can attack these cultivars.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

The 18-month-old plants of the delta seedless variety grafted onto Carrizo citrange [*C. sinensis*(L.) Osbeck. x *Poncirus trifoliata* (L.) Raf.] were obtained from a nursery. Plants were grown individually in opaque plastic 4-L pots filled with a substrate composed of peat, coconut fiber, sand and perlite (40:25:25:10). Plants were irrigated twice weekly with the following basal nutrient solution (pH 6.0) at half strength of Hoagland solution: 5 mM $Ca(NO_3)_2$, 1.4 mM KNO_3, 2 mM $MgSO_4$, 0.6 mM H_3PO_4, 20 µM Fe-EDDHA, 7.6 µM $ZnSO_4 \cdot 7H_2O$, 0.50 µM $CuSO_4 \cdot 5H_2O$, 50 µM H_3BO_3, 0.50 µM MoO_3, 54 µM $MnSO_4 \cdot H_2O$. Plants were acclimated for 2 weeks before the experiments began under glasshouse conditions (26-28/16-18°C, 70-80% and a 16-hour photoperiod). Plants were selected according to uniformity of size and were separated into two groups. One group with

19

127~~six~~twelve treated plants (cold) were cultured for 6 weeks in a Versatile Environmental
128Test Chamber, (MLR-350, *Sanyo*) with a temperature range between 1ºC and 2°C day
129and night, and a photoperiod of 16 h light/8 h darkness (500 µmol m^{-2} s^{-1}, 400-700 nm)
130and~~.~~ ~~R~~ relative humidity was maintained at approximately 80%. Six ~~P~~ plants ~~were~~ were
131used to monitoring physiological parameters and six to sampling plant tissues. Cold
132treated plants were irrigated once weekly with the previous nutrient solution. There were
133twelve plants in the control group (Ct~~): six plants used for initial plant growth and~~
134~~measures,)~~ ~~and six plants~~ maintained under glasshouse conditions during the
135experiment.
136At the beginning of the assay (three plants for initial plant growth ~~of Ct group~~) and after
1376 weeks of treatment, plants were removed from pots, rinsed with distilled water, and
138divided into leaves, stems and roots. These organs were fresh-weighed (FW). Fresh
139samples of organs were taken for analytical and molecular determinations, and the
140remaining parts were reweighed after being lyophilized (TelstarLyoAlfa6, Barcelona,
141Spain) for 3 days until constant dry weight (DW). ~~Dry weight from samples at 2 and 4~~
142~~weeks was calculated with RWC% and was added for RGR calculations.~~ The relative
143growth rate (RGR) per~~in~~ 1 week was calculated according to (Pitman, 1988):

144 $$RGR(week-1)=\left[\frac{\ln DW\,6-\ln DW\,0}{t6-t0}\right]$$

145 where DW0 and DW6 are the dry mass of shoot and root tissues of the plant at the
146beginning of the experiment (t0) and harvesting time at 6 weeks (t6).
147

207
21

2.2. Photosynthetic activity

The net CO_2 assimilation rate (ACO_2, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), substomatal CO_2 concentration (Ci µmol CO_2 mol^{-1}) and stomatal conductance (gs, mmol H_2O m^{-2} s^{-1}) of single attached leaves was measured outdoors between 10 am and 11.30 am on a sunny day, which allowed measurements to be taken under relatively stable conditions. Photosynthetically active radiation (PAR) on the leaf surface was adjusted to a photon flux density of 1,000 µmol photons m^{-2} s^{-1}. Closed gas exchange (*CIRAS-2*, *PP-systems*, Hitchin, UK) was used for measurements. Leaf laminae were fully enclosed within a PLC 6 (U) universal leaf autocuvette in a closed circuit model and were kept at 25±0.5°C with a leaf-to-air vapour deficit of about 1.7 Pa. The air flow rate through the cuvette was 200 mL min^{-1}. Measurements were taken weekly on the two youngest fully expanded leaves on all six trees. The average value of the two leaves was considered representative of each individual plant.

2.3. Fluorescence measurements

CFI (Chlorophgyll Fluorescence Image) (Fv/Fm) was measured at the end of the experiment in a portable fluorometer (PAM-2100 Walz, Effeltrich, Germany). The average of two leaves per plant were darkened for 30 min prior to taking measurements. Minimum (dark) fluorescence F_o, was obtained upon excitation of leaves with a weak beam from a light-emitting diode. Maximum fluorescence (F_m) was determined following a 600 ms pulse of saturating white light. The variable fluorescence (F_v) yield was calculated as $F_m - F_o$. Further information on CFI (Chlorophyll Fluorescence Imagine) measurements can be obtained from (Calatayud et al., 2013).

2.4. Leaf chlorophyll concentration

The leaf chlorophyll (Chl, μg g^{-1} DW) concentration was measured at the end of experiment according to (Moran and Porath, 1980). Samples from the two youngest fully expanded leaves per plant were lyophilized, pulverized in a refrigerated mill (IKA A10, Staufen, Germany) and stored at -80°C. The lyophilized material (0.5 g) was incubated in 6 mL N,N-dimethylformamide at 4°C for 72 h and centrifuged at 4,000 rpm and 4°C for 15 min (Eppendorf Centrifuge 5810R, AG, Hamburg, Germany). The supernatant was left for 1 h in the presence of Na_2SO_4 and absorbance was measured at 664 and 647 nm in the same sample (Lambda 25, PerkinElmer, Shelton, CT, USA). Chlorophyll a, b and total chlorophyll was calculated with (Moran, 1982).

2.5. Proline route phylogenetic analysis

All the putative genes that encode to proline biosynthesis enzymes on the *Citrus* route were consulted for *Arabidopsis thaliana* from (Szabados and Savouré, 2010), and were checked on The Arabidopsis Information Resource web (TAIR) https://www.arabidopsis.org/. A tBLASTtn was carried out for *Citrus clementina* in the International Citrus Genome Consortium, Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) (Goodstein et al., 2011) .

For the phylogenetic analysis, P5CS, P5CR, dOAT, PDH and P5CDH proteins from *Arabidopsis thaliana* and from other species were aligned using ClustalW algorithm (opening = 10, extension = 0.2). The *Arabidopsis* proteins most similar to P5CS, P5CR, dOAT, PDH and P5CDH were also included as external controls. Five phylogenetic trees were elaborated for each enzyme using the Maximum Likelihood method and

tested using Bootstrap method with 1000 replicates. Both alignment and phylogenetic analysis were performed using MEGA version 6 (Tamura et al., 2013).

2.6. Proline

The free proline concentration (Pro, mg g^{-1} DW) in leaves was determined according to Bates et al. 1973. Samples from two expanded leaves per plant were collected biweekly, frozen and lyophilized. Then 0.025 g were weighed and homogenized (Vortex) in 1.5 mL of sulphosalicylic acid (3%) for 1 min, centrifuged at 14,000 rpm for 5 min (Eppendorf Centrifuge 5810R, AG, Hamburg, Germany) and the supernatant was stored at 4°C. An aliquot (0.2 mL) was incubated with 0.5 mL of sulphosalicylic acid (3%), 0.7 mL of reactive ninhydrin acid reagent (ninhydrin 0.4%, phosphoric acid 6 M, glacial acetic acid 60%) and 0.6 mL of glacial acetic acid (99%) in a dry bath at 100°C for 1 h (Thermostatic Bath BD, Bunsen SA, Humanes, Spain). Samples were cooled in an ice bath for 15 min and absorbance was measured at 520 nm (Lambda 25, PerkinElmer, Shelton, CT, USA).

2.7. RNA isolation and quantitative real-time RT-PCR

Leaf samples were obtained biweekly, collected in liquid N_2 and stored at -80°C. Total RNA was isolated from 100 mg of plant tissue using the RNase Plant Mini Kit (Quiagen) with RLT-β-mercaptoethanol (SigmaAldrich) buffer. Contaminant genomic DNA was removed with the RNase-Free DNase kit (Qiagen, CA, USA) by on-column digestion, according to the manufacturer's instructions. Purified RNA (2 µg) was reverse-transcribed with SuperScript® III Reverse Transcriptase (RT) (Life Technologies, Carlsbad, CA, USA) in a total volume of 10 µL. First-strand cDNA was 50-fold diluted and 2 µL were used as a template for the quantitative real-time RT-PCR in a final

volume of 20 µL. Quantitative real-time PCR was performed in a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using TB Greenpremix Ex Taq (TliRNaseH plus) (Takara Europe, S.A.S, Saint Germain en Laye, FR). The PCR protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, and 1 min at 60°C. The specificity of the reaction was assessed by the presence of a single peak on the dissociation curve and through size estimations of the amplified product by agarose electrophoresis. All specific primers (Table 1) were tested before PCR reaction and obtain an efficiency=2 (Pfaffl, 2001). The CiclevActin and CiclevUBC4 transcripts, amplified with specific primers, were used as the reference genes (Agüero et al., 2014; Estornell et al., 2016) and single-factor ANOVA and linear regression analyses of CT values to examine variation in our reference genes was realized (Brunner et al., 2004). The normalization factor of reference genes was calculated by the geometric mean of the values of both genes (Brunner et al., 2004). The relative expression was measured by the relative standard curve procedure with 5 points of dilutions (Pfaffl, 2001). The results were the average of three independent biological replicates with three technical replicates per biological sample.

Table 1. List of the primers used for quantitative real-time PCR.

At Gene	Gene Name	Ciclev Gene[a]	Primers
AT2G39800	*P5CS1*	Ciclev10030839	FOR 5' CCCTTGGGTGTTCTCTTGATTG 3' REV 5' CTTGTGCAGAATTGCATTTGA 3'
AT3G55610	*P5CS2*	Ciclev10011176	FOR 5' ATGTGCGTGCTGCTATTGACCA 3' REV 5'CCAAATCGTGCTCCATCACAGAA 3'
AT5G14800	*P5CR*	Ciclev10005607	FOR 5' TCTGCTGTAGGTGAGGCTGC 3' REV 5' CAAACAATTTCTCATCCGCTCGC 3'
AT3G30775	*PDH1*	Ciclev10011584	FOR 5' ATCTGCCAAGTCTCTGCCTC 3' REV 5' GCTTCCACGGGAGATTAAATGA 3'

AT5G38710	*P5CDH*	Ciclev10019562	FOR 5' ATCACCTTGGACAGCAGAGC 3' REV 5' CCATATAGAGCGCACCCATCA 3'
AT5G46180	*dOAT*	Ciclev10015647	FOR 5' GAACCAATTCAAGGAGAGGCTG 3' REV 5' CGAACTTCTTCCCAATCAGAGG 3'
ATUBC4	*UBC4*	Ciclev10009771	FOR 5' TGGACGCTTCAGTCTGTTTG 3' REV 5' TCGTCAATCACCCCTTCTTT 3'
β-ACTIN	*ACTIN*	Ciclev10025866	FOR 5' CAGTGTTTGGATTGGAGGATCA 3' REV 5' TCGCCCTTTGAGATCCACAT 3'

a Code refers to the transcript name in the database available in the International Citrus Genome Consortium (https://phytozome.jgi.doe.gov/pz/portal.html).

2.8. Protein nitrogen and total nitrogen quantification

The samples for protein nitrogen and total nitrogen (%N in DW) were quantified in leaf samples at the end of the experiment in expanded leaves at 6 weeks. Next 250 mg of powdered lyophilized leaves were taken. For the protein nitrogen samples, previous precipitation with 5% trichloroacetic acid for 15 min and ice bath, filtered through paper filter, was carried out. From this point, nitrogen was quantified together in both samples, protein nitrogen and total nitrogen, by the Semi-Micro kjeldahl method with digestion, distillation and titration of samples according to (Bremner, 1965).

2.9. Leaf water potential components

Measurements were taken with two leaves per plant biweekly. Leaves were excised at 10.30 am and were immediately placed in a plastic bag. Determinations were then made. The leaf water potential (ψ_W, MPa) was measured in a Scholander-type pressure chamber (Soil-moisture Equipment Corp., Santa Bárbara, CA, USA), equipped with a binocular microscope to observe end points. The osmotic potential (ψ_π, MPa) was measured with an osmometer (Wescor, Logan, USA). Briefly, leaves were tightly wrapped in aluminium foil, frozen in liquid nitrogen and stored at −80°C. The 0.5 mL

microcentrifuge tube with a hole and leaves sample was inserted into a 1.5 mL Eppendorf tube. Leaf sap was extracted by centrifuging for 5 min at 14,000 rpm, as modified from Callister et al. 2006. Osmolyte content (mmol kg^{-1}) was converted into MPa using the Van't Hoff equation.

2.10. Soluble sugars and starch

Soluble sugars and starch were measured at the end of experiment in the lyophilized and milled leaves (100 mg DW). Soluble sugars (SU) and starch (ST) were analyzed by a colorimetric method based on (McCready et al., 1950). Samples were mixed with heated ethanol and centrifuged. The liquid part contained the SU and the precipitate contained the ST. For the SU and ST determinations, anthrone-acid solution was added and samples were placed in a boiling water bath. The results were read at 630 nm (Lambda 25, PerkinElmer, Shelton, CT, USA).

2.11. Statistical analyses

For the statistical analyses, all the resulting values were the mean of six independent plants per treatment. The RT-PCR values were the mean and the standard deviation of three biological replicates and three technical replicates each. Data were submitted to an analysis of variance (ANOVA) using Statgraphics Centurion, 16.1 version (Statistical Graphics, Englewood Cliffs, NJ, USA), prior to testing for normality, homogeneity and no interaction in ANOVA multifactor analysis. When the ANOVA showed a statistical effect, means were separated by least significant differences (LSD) at $P < 0.05$.

3. RESULTS

3.1. Plant growth

Plant growth (Table 2) was seriously affected by cold conditions. The total dry weight of the control plants increased by 59.6% compared to the cold-treated ones by the end of the experiment. This response affected all plant organs. In the cold-treated plants, leaves underwent lower biomass (71.1% lower than the control ones). Interestingly, the growth of other more lignified organs, like stems and roots, also reduced significantly by 43.0%

and 48.5%, respectively, in the cold-treated plants.

Table 2. Plant growth (in g DW) in total plants, leaves, stems and roots, and the relative growth rate (RGR, in g DW, week-1) measured in the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 6 weeks. The values are the means ±SE of six biological replicates (n=6). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001, ns, non-significant.

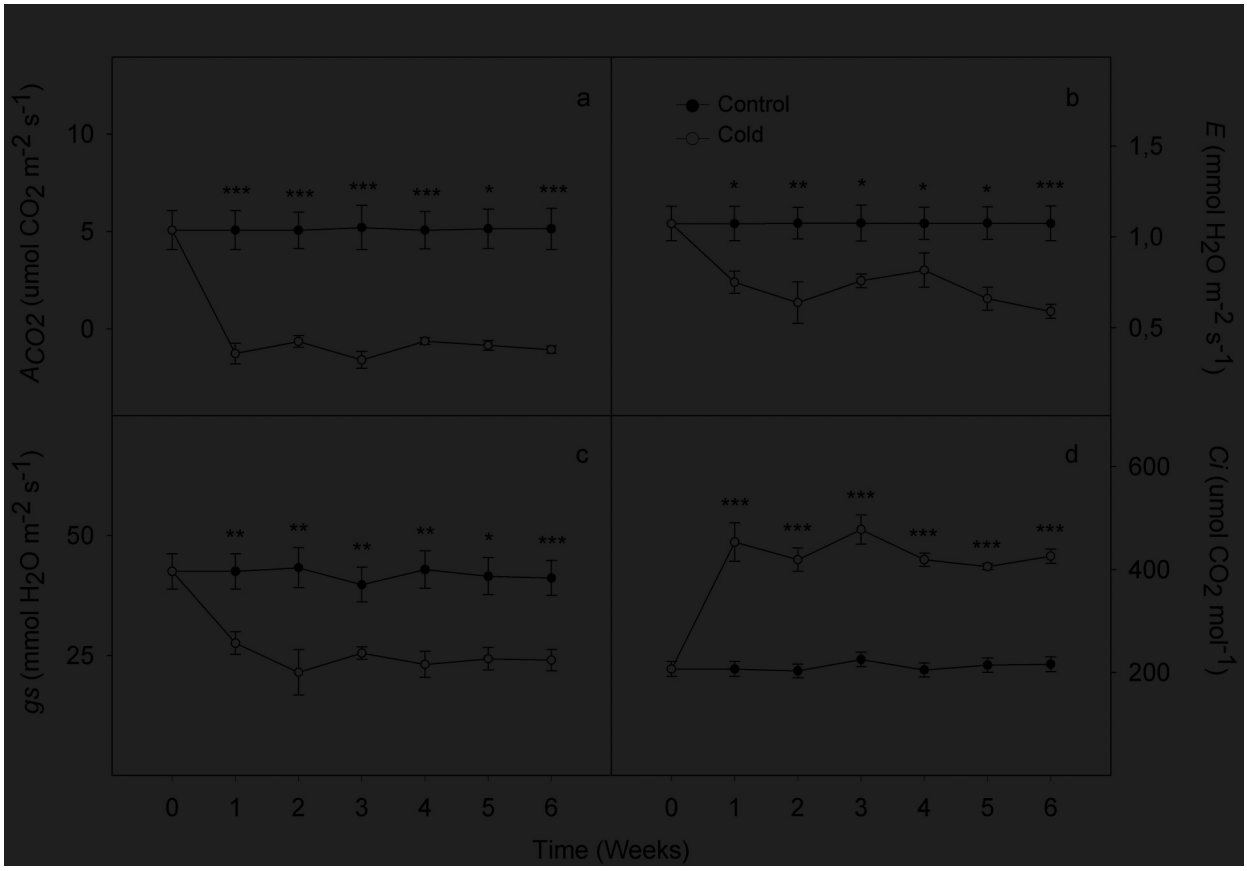
Biomass (g DW)	Control	Cold	
Total plant	59.89 ± 2.78	24.19 ± 1.25	***
Leaves	29.73 ± 0.62	8.59 ± 0.99	***
Stems	26.02 ± 0.67	14.82 ± 0.92	***
Roots	30.33 ± 3.84	15.60 ± 1.01	***
RGR (g DW $week^{-1}$)	0.11	0.00	***

The seriously impaired plant growth of the cold-treated plants was reflected by the RGR (relative growth rate) value, which was calculated weekly. While the control plants grew at a rate of 0.11 g DW/week, this value was 0.0 g DW/week-1 in the plants grown under the cold conditions. Finally, the percentage of water in the different plant tissues was not significant (data not shown).

3.2. Photosynthesis parameters

The photosynthetic rate (ACO_2) measure (Fig. 1a) drastically dropped early in the cold-treated leaves compared to the control ones, with a value that came close to 0 µmol

297CO_2 m^{-2} s^{-1} for all the test points. The evapotranspiration (*E*) and stomatal conductivity 298(*gs*) parameters (Fig. 1b and c) paralleled the *ACO₂* pattern and strongly reduced from 299the very beginning of the experiment. Six weeks after cold treatment, the *E* value 300lowered by 45.1% as a result of low temperatures, and went from 1.07 mmol H_2O m^{-2} 301s^{-1} in the control plants to 0.59 mmol H_2O m^{-2} s^{-1} in the cold-treated ones. At that point, 302*gs* also reduced by 41.6% in the cold-treated leaves from 41.1 mmol H_2O m^{-2} s^{-1} to 24.0 303mmol H_2O m^{-2} s^{-1} in the control and cold-treated plants, respectively. Finally, the internal 304CO_2 concentration (Ci) (Fig. 1d) of the leaves grown under the cold conditions increased 305compared with the control ones. This rise, from 200 µmol mol^{-1} to about 400 µmol mol^{-1}, 306was noted 1 week after the experiment began and continued for up to 6 weeks.



307

308Fig. 1. Gas exchange parameters [(a) photosynthetic rate (ACO_2, in µmol CO_2 m^{-2} s^{-1}), (b) 309evapotranspiration (E, in mmol H_2O m^{-2} s^{-1}),(c) stomatal conductance (gs, in mmol H_2O m^{-2} s^{-1}) and (d) 310internal CO2 concentration (Ci, in µmol CO_2 mol^{-1})] measured weekly in the fully expanded leaves of the 4415

Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 0, 2, 4 and 6 weeks. The values are the means of six biological replicates (n=6). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001.

3.3. Fluorescence and chlorophylls

The fluorescence and chlorophylls measurements are shown in Table 3, and both were taken at the end of the experiment. The Fv/Fm value lowered by about 32% in the cold-treated citrus plants compared to their controls (Fig. 2).

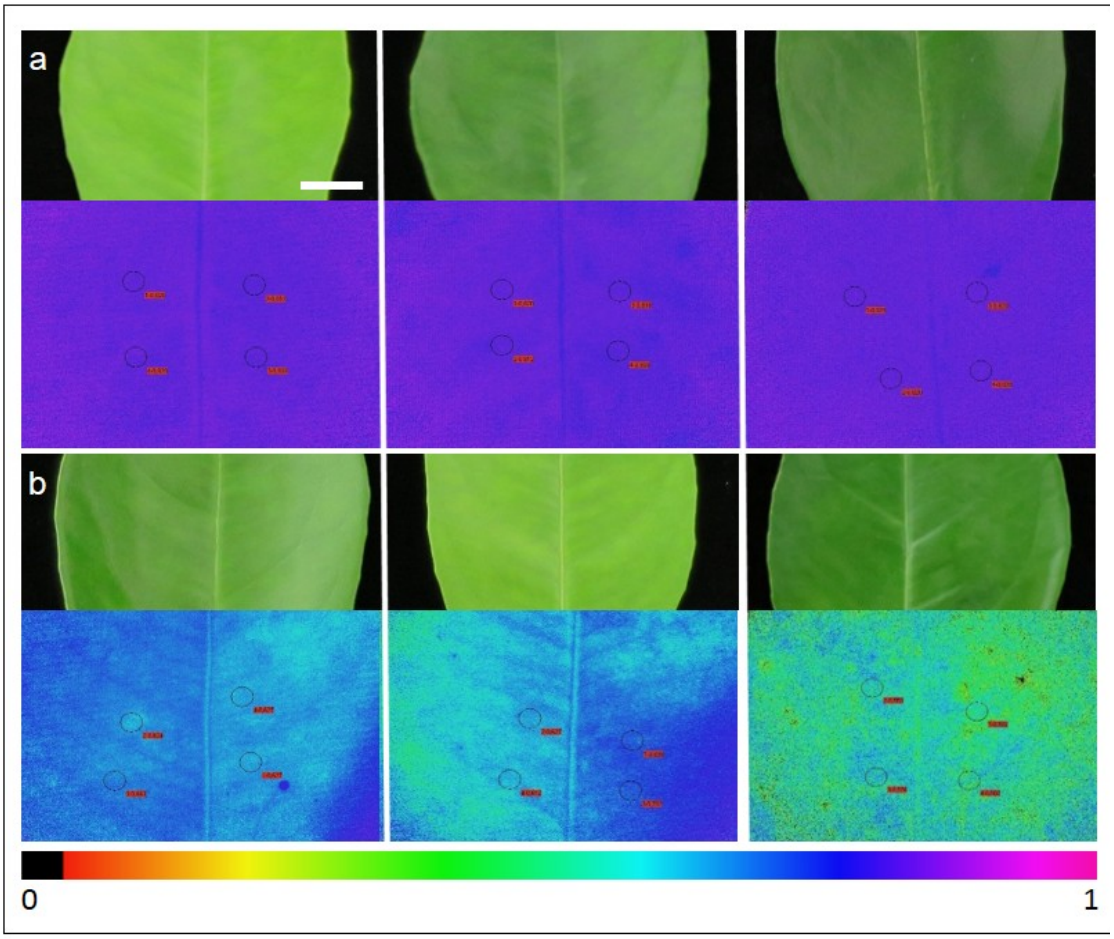


Fig. 2. Fv/Fm image that represents 0 and 6 weeks with cold damage in leaves. Top, a leaf photography; bottom, a Fv/Fm leaf image with values ranging from 0 (black) to 1 (pink). (a) Control plants at 0 weeks of cold, (b) plants grown for 6 weeks in the cold. Image shows three leaves of different plants per treatment week.

Cold conditions also lowered the chlorophyll *a* (Table 3) concentration by about 25%, while the chlorophyll *b* value lowered to a large extent (37%, from 32.25 µg g^{-1} DW to 20.41 µg g^{-1} DW). Thus the chlorophyll total concentration went from 124.06 µg g^{-1} DW to 89.72 µg g^{-1} DW when plants were grown according to the cold treatment.

Table 3**.** Fluorescence parameter (Fv/Fm) and chlorophyll concentration (a, b and Total, in µg g-1 DW) measured in the leaves of the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 6 weeks. The values are the means ± SE of six biological replicates (n=6). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001.

	Control	Cold
Fv/Fm	0.81 ± 0.002	0.55 ± 0.07 ***
Chl *a*	91.81 ± 4.3	69.31 ± 4.6 **
Chl *b*	32.25 ± 2.1	20.41 ± 6.3 **
Chl *Total*	124.06 ± 6.0	89.72 ± 6.2 **

3.4. Phylogenetic analysis

There ~~It~~ were constructed 5 phylogenetic trees (Fig. 3), a tree for each proline route enzyme. All proteins were clustering into an expected group leaving out the second *Arabidopsis* hit (outgroup). P5CS enzyme (Fig. 3a) has a duplicate gene in *Arabidopsis* that was confirmed for *Citrus clementina* too.

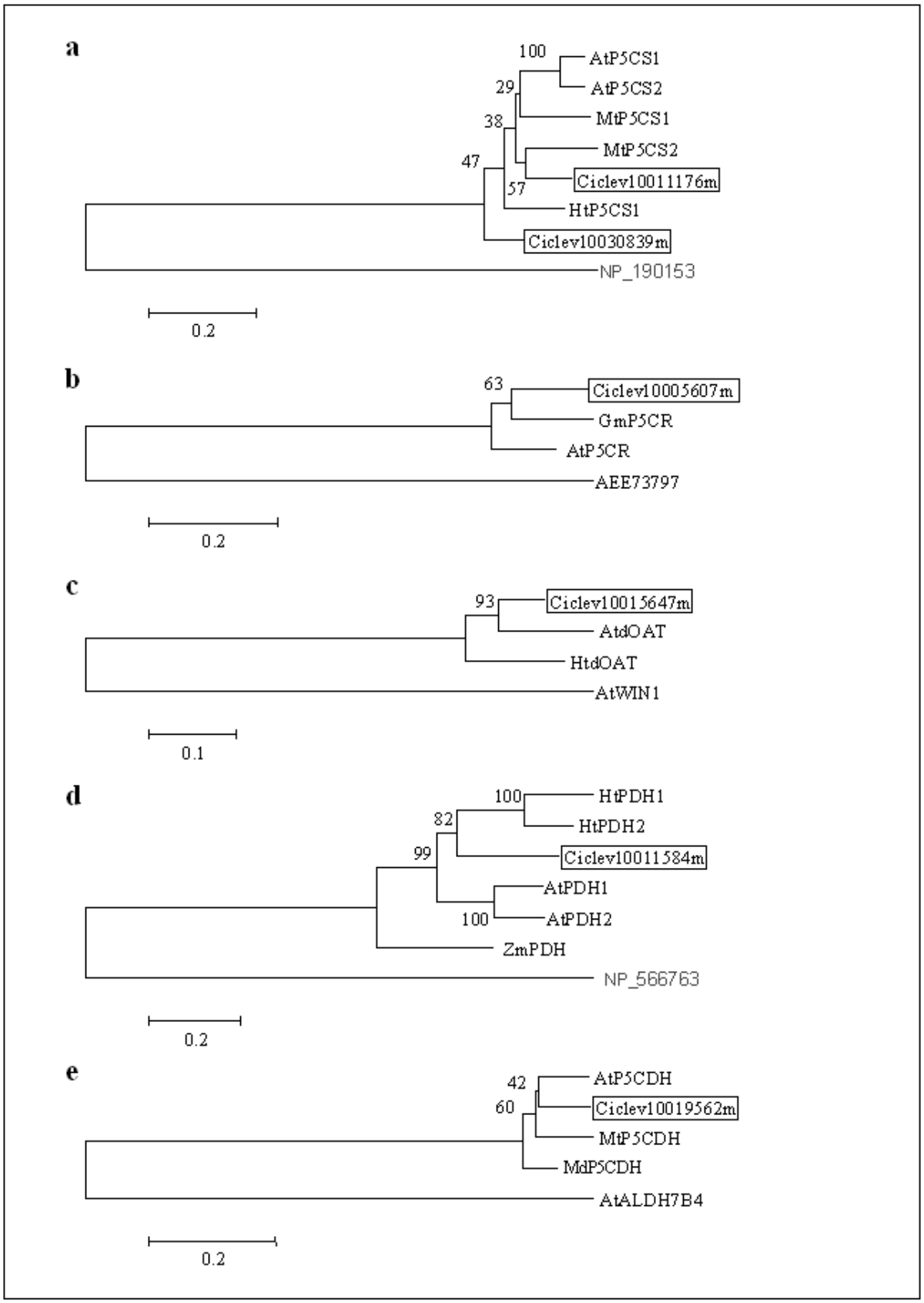

a
100
AtP5CS1
29
AtP5CS2
MtP5CS1
38
MtP5CS2
47
Ciclev10011176m
57
HtP5CS1
Ciclev10030839m
NP_190153
0.2
b
63
Ciclev10005607m
GmP5CR
AtP5CR
AEE73797
0.2
c
93
Ciclev10015647m
AtdOAT
HtdOAT
AtWIN1
0.1
d
100
HtPDH1
82
HtPDH2
99
Ciclev10011584m
AtPDH1
100
AtPDH2
ZmPDH
NP_566763
0.2
e
42
AtP5CDH
60
Ciclev10019562m
MtP5CDH
MdP5CDH
AtALDH7B4
0.2

Fig. 3. Phylogenetic trees of proline synthesis and degradation route proteins. The trees were constructed using the Maximum Likelihood method and bootstrapped with 1000 replicates. *Numbers* next to each node represent the bootstrap values in percentage. The *scale bar* indicates the branch length that corresponds to the number of substitutions per amino acid position. GenBank accession numbers: (a) *Arabidopsis thaliana* AtP5CS1 (OAP10756), *Arabidopsis thaliana* P5CS2 (OAP05097), *Medicago truncatula* MtP5CS1 (CAC82184), *Medicago truncatula* MtP5CS2 (AET87352), *Citrus clementina* CcP5CS2 (Ciclev10011176m), *Helianthus tuberosus* HtP5CS1 (AHJ08569), *Citrus clementina* CcP5CS1 (Ciclev10030839m), *Arabidopsis thaliana* FMN-linked oxidoreductases superfamily protein (NP_190153); (b) *Citrus clementina* CcP5CR (Ciclev10005607m), *Glycine max* GmP5CR (NP_001235914), *Arabidopsis thaliana* AtP5CR (OAO94594), *Arabidopsis thaliana* 6-phosphogluconate dehydrogenase family protein (EE73797); (c) *Citrus clementina* CcdOAT (Ciclev10015647m), *Arabidopsis thaliana* AtdOAT (AED95350), *Helianthus tuberosus* HtdOAT (AHJ08571), *Arabidopsis thaliana* AtWIN1 (OAP16808); (d) *Helianthus tuberosus* HtPDH1 (AHJ08572), *Helianthus tuberosus* HtPDH2 (AHJ08573), *Citrus clementina* CcPDH (Ciclev10011584m), *Arabidopsis thaliana* AtPDH1 (AEE77659), *Arabidopsis thaliana* AtPDH2 (NP_198687), *Zea mays* ZmPDH (NP_001147577), *Arabidopsis thaliana* uxin-responsive family protein (NP_566763); (e) *Arabidopsis thaliana* AtP5CDH (NP_568955), *Citrus clementina* CcP5CDH (Ciclev10019562m), *Medicago truncatula* MtP5CDH (XP_003609016), *Malus domestica* MdP5CDH (ACL13549), *Arabidopsis thaliana* AtALDH7B4 (NP_175812).

3.5. Proline quantification

Cold treatment increased the proline concentration value in leaves over time (Fig. 4) to reach a maximum of 14.6 mg g^{-1} DW 4 weeks after the experiment began (2.9-fold higher than the control leaves). Interestingly, the proline concentration in the cold-treated plants at the end of the experiment (8.9 mg g^{-1} DW) was 31.5% lower than at 4 weeks (14.6 mg g^{-1} DW).

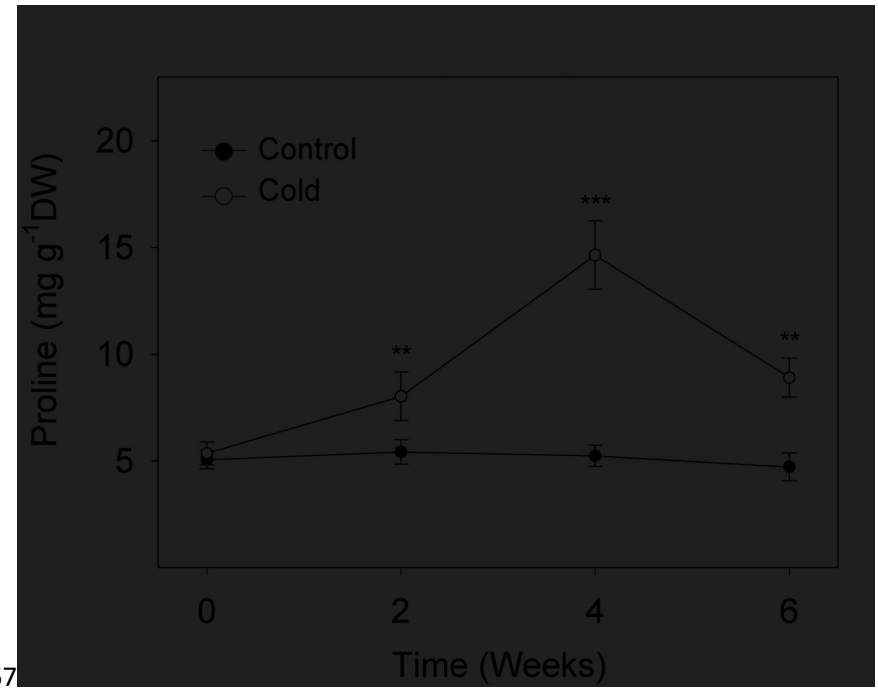


Fig. 4. Proline concentration (mg g^{-1} DW) measured in the leaves of the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 0, 2, 4 and 6 weeks. The values are the means±SE of six biological replicates (n=6). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001.

3.6. Gene expression analysis

Fig. 5 shows the relative gene expression of genes coding for enzymes related with the proline biosynthesis pathway in cell leaves over time. As an early response to low temperatures (2 weeks after treatment), an almost 2-fold increase (maximum value) was recorded for the cold leaves in the expression of the first implicated gene, *P5CS1* (Fig. 5a). From this point, the transcription of this gene slightly reduced in the cold leaves (by 27.7%) 4 weeks after treatment, and also to similar levels to the control ones by the end of the experiment. The expression of the second gene in the pathway, *P5CS2,* progressively increased, which was recorded in the cold-treated leaves (Fig. 5b) compared to the control ones, and reached 370% induction 6 weeks after applying cold stress. The third biosynthetic gene, *P5CR* (Fig. 5c), was not apparently affected by

low temperatures throughout the cold treatment. Finally, the *dOAT* gene expression results reflected the induction of its activity under the cold conditions at all the tested times (Fig. 5d). Two weeks after treatment, it showed a moderate 2.7-fold increase over the controls. At 4 weeks, the *dOAT* transcript level underwent a peaked expression (12.2-fold higher in the cold leaves than in the control ones) and the messenger level remained high (6.5-fold over the control) until the end of the experiment. The relative gene expressions of the proline degradation genes are shown in Figure 6.

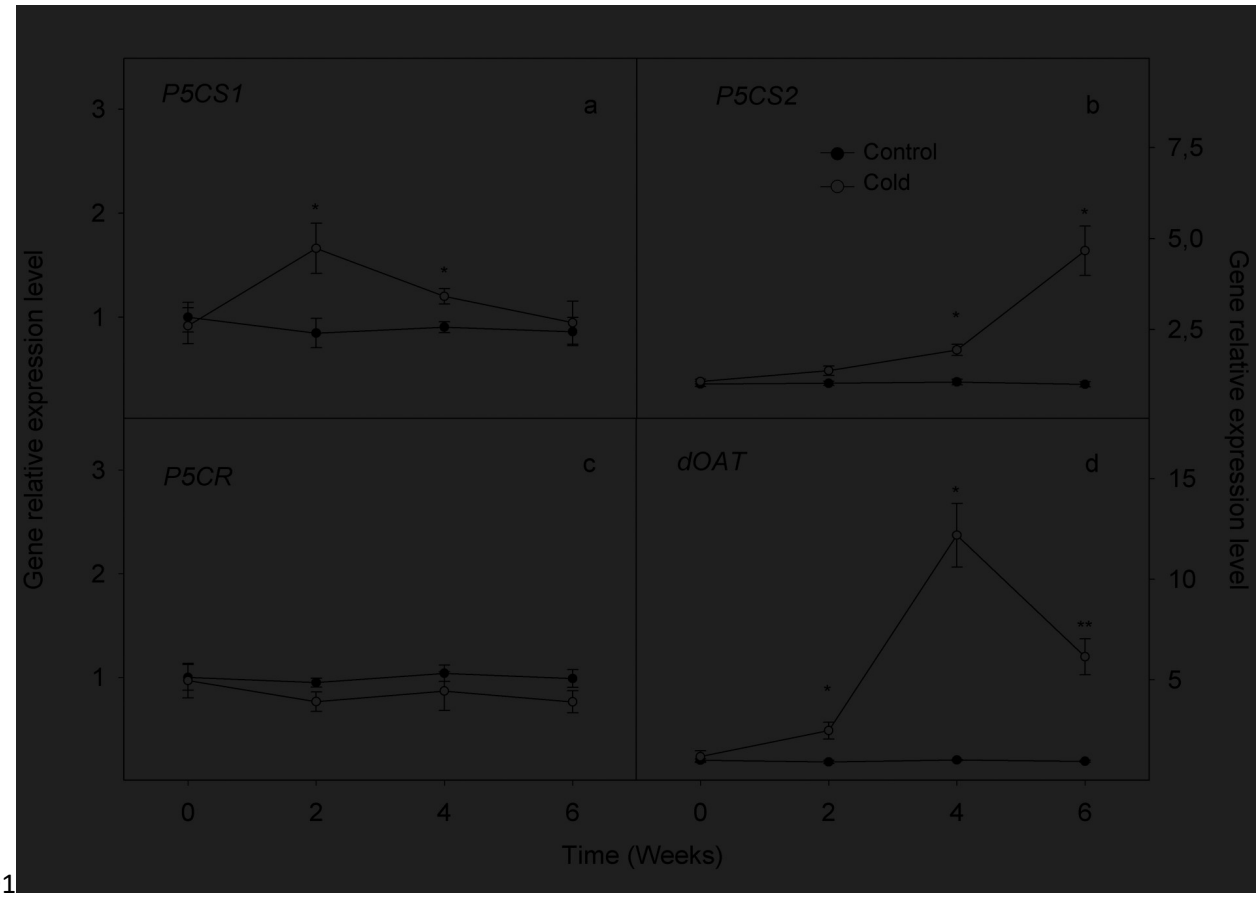


Fig 5. The relative gene expression level of the (a) *P5CS1*, (b) *P5CS2*, (c) *P5CR* and (d) *dOAT* genes related with the proline biosynthesis pathway. Samples were measured in the leaves of the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 0, 2, 4 and 6 weeks. The values are the means±SE of three biological replicates (n=3) and three technical replicates per biological sample. The treatment effect tested by a one-way ANOVA is indicated as follows*P< 0.05; **P< 0.01; ***P< 0.001.

The *PDH* gene (Fig. 6a) surprisingly increased by more than 25-fold (maximum expression level) after 2 weeks of cold treatment compared to the controls. Although the *PDH* messenger level considerably fell until the end of the experiment, the expression remained significantly higher in the cold leaves than in the controls (5.0-fold and 2.7-fold over the controls at 4 and 6 weeks, respectively). Finally, the *P5CDH* transcript level (Fig. 6b) was always higher in the cold-treated leaves than in the control ones, and at all the tested points. Initially, the cold treatment gradually increased the gene expression level of *P5CDH*, which displayed maximum activity 4 weeks after treatment (15.5-fold higher than the controls). At the end of the experiment, the *P5CDH* expression in the cold leaves lowered to similar values to those recorded at 2 weeks (around 7.5-fold higher than the controls).

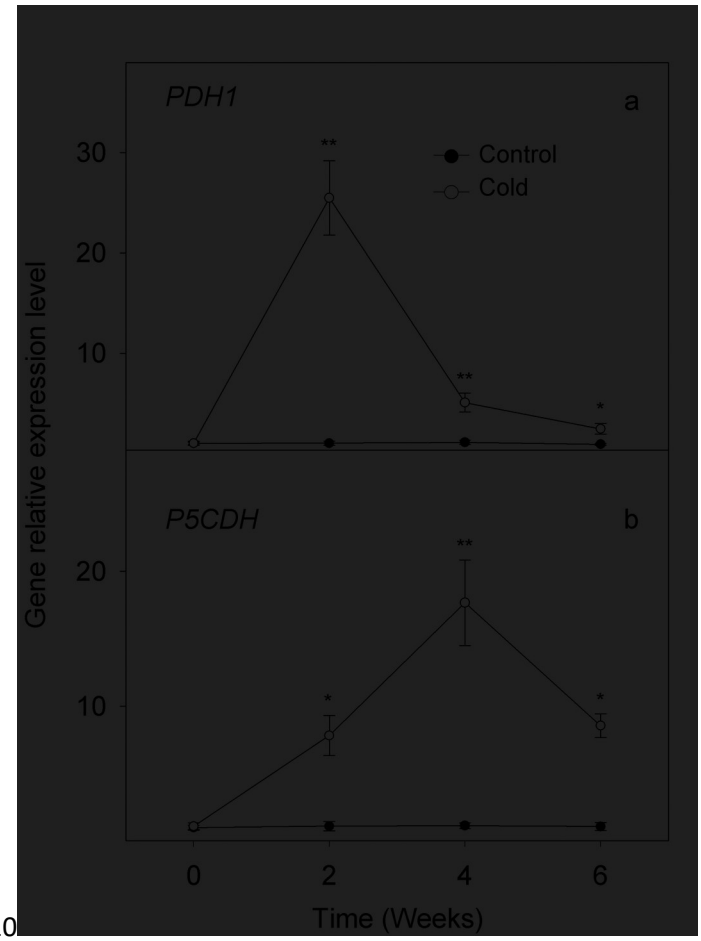


Fig. 6. Relative gene expression level of the (a) *PDH1* and (b) *P5CDH* genes related with the proline degradation pathway. Samples were measured in the leaves of the Carrizo citrange plants grafted with the Valencia delta seedless grown under the cold (1°C) and control conditions for 0, 2, 4 and 6 weeks. The values are the means±SE of three biological replicates and three technical replicates (n=3). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001.

3.7. Nitrogen quantification

The amount of total nitrogen in leaves (Table 4) increased by 25.8% in the cold-treated plants at the end of the experiment compared with the control ones. In contrast, the cold conditions did not significantly affect the percentage of proteic nitrogen (about 70% of the total N in both treatments). However, the inorganic nitrogen percentage was much higher (1.56-fold) in the cold-treated leaves than in the control ones.

Table 4. Nitrogen (N) concentration (total, proteic and inorganic, in %N in dry weight) measured in the leaves of the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 6 weeks. The values are the means ± SE of six biological replicates (n=6). ~~and SD~~

	Control	Cold
Total N	2.29 ± 0.03	2.88 ± 0.16 *
Proteic N	1.69 ± 0.09	1.94 ± 0.13 ns
Inorganic N	0.60 ± 0.08	0.94 ± 0.04 **

3.8. Water potential and osmotic potential

The leaf water potential (Ψ_W) increased in the cold-treated plants compared to the control ones from the very beginning of the experiment (Fig. 7a). Six weeks after the low temperature conditions were applied, Ψ_W was 12-fold higher in the cold-treated leaves than in the controls (from -0.08 to -0.96 MPa, respectively). In general terms, cold stress did not significantly change the leaf osmotic potential (Ψ_π) compared with the control conditions (Fig. 7b), with a value that came close to -1.55 MPa throughout the experiment. Interestingly, a slightly significant decrease in the Ψ_π value was recorded only after a short time (one experiment week) in the cold-treated leaves (with a value that came close to -1.7 MPa).

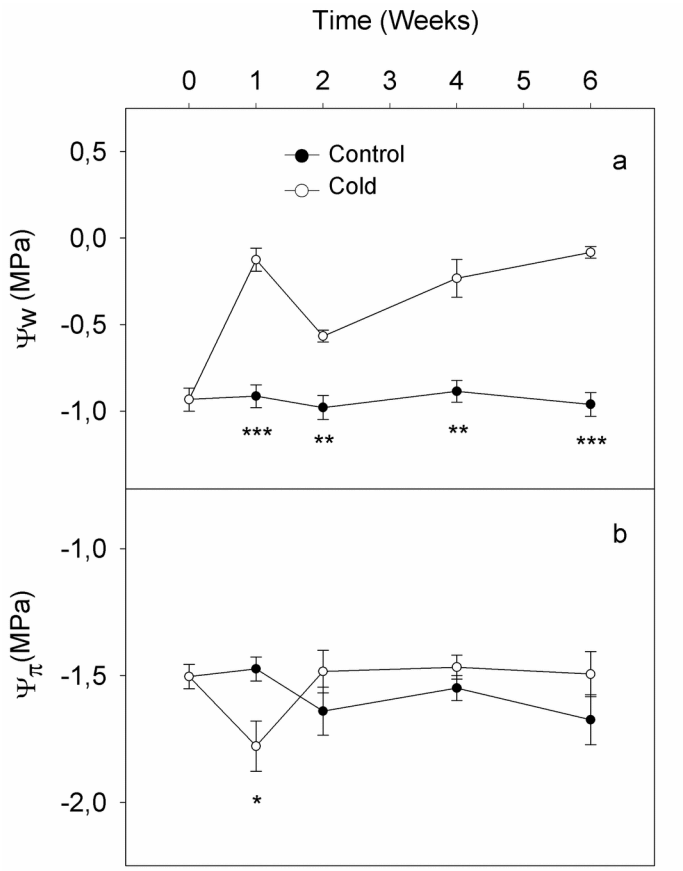


Fig. 7. (a) The leaf water (ψW) and (b) osmotic (ψπ) potentials (in MPa) measured in the leaves of the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 0, 2, 4 and 6 weeks. The values are the means±SE of six biological replicates (n=6). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001.

3.9. Soluble sugars and starch

Fig. 8 depicts the starch (ST) and total sugar (SU) concentrations in leaves at the end of the experiment. As a result of the cold treatment, the ST concentration (Fig. 8a) lowered by 27%, from 48.59 mg g^{-1} DW in the control plants to 35.57 mg g^{-1} DW in the cold-treated ones. The total SU concentration (Fig. 8b) also dropped by 31.7% as a result of low temperatures, and went from 10.9 mg g^{-1} DW in the control plants to 7.5 mg g^{-1} DW in the cold-treated ones.

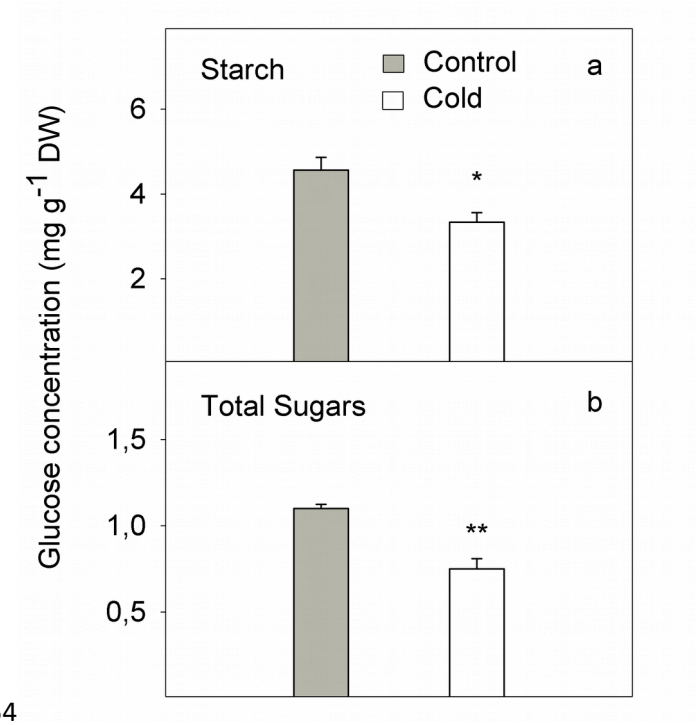


Fig. 8. (a) Starch and (b) total soluble sugars measured as the glucose concentration (in mg g-1 DW) in the leaves of the Carrizo citrange grafted with the Valencia delta plants grown under the cold (1°C) and control conditions for 6 weeks. The values are the means of six biological replicates (n=6). The treatment effect tested by a one-way ANOVA is indicated as follows: *P< 0.05; **P< 0.01; ***P< 0.001. DW: Dry Weight.

4. DISCUSSION

Cold stress causes several physiological responses in citrus plants that affect their development, including photosynthesis inhibition, stomatal closure at 50% and, therefore, diminished plant evapotranspiration (Fig. 1a, b and c). Impaired plant growth is the first symptom of cold stress (Adam and Murthy, 2014; Megha et al., 2014), as seen in Table 2. One of the most important physiological responses to cold is photosynthesis inhibition (Allen and Ort, 2001; Ribeiro et al., 2009; Batista-Santos et al., 2011; Santos et al., 2011; Ribeiro et al., 2014). Stomatal closure is caused early by photosynthesis falling due to cold, which produces membrane rigidity and decreases

cellular activities (Paul et al., 1992; Allen and Ort, 2001). For long cold times, plants synthesize proline and ABA, and these ABA accumulation is known produce a stomatal closure (P M Chandler and Robertson, 1994; Roelfsema and Prins, 1995). In addition, the response is accompanied by a 2-fold increase in the internal CO_2 concentration (Ci) (Fig. 1d), which occurs in citrus and in other subtropical species like coffea (Ramalho et al., 2003; Partelli et al., 2009; Batista-Santos et al., 2011; Ramalho et al., 2014; Machado et al., 2013). Consequently, carbon assimilation is seriously limited, as reflected by the C_i levels, which suggests that an increased proportion of electrons is driven to alternative electron sinks, e.g., photorespiration, Mehler reaction or water-water cycle (Allen and Ort, 2001; Long and Bernacchi, 2003; Ribeiro et al., 2009; Santos et al., 2011). So under these conditions, excess energy must be dissipated both successfully and photochemically, as listed above, or in other non-photochemical ways as the assimilation of the CO_2 generated in the photochemical phase of photosynthesis, photorespiration, nitrate reduction and ammonium assimilation consumes ATP and NADH/NADPH, and contributes to energy dissipation.

The chloroplast is usually the only organelle to be rapidly and profoundly affected during cold stress (Adam and Murthy, 2014). Therefore, chlorophyll (Chl) biosynthesis is affected in situations of stress and excess energy (Table 3). Chl a and Chl b are degraded by about 25% and 37%, respectively, which causes an imbalance in PSII through exposure to low temperatures, as well as a consequent alteration in Chl antenna complexes (Ensminger et al., 2006; Adam and Murthy, 2014). The Fv/Fm value lowered in the cold-treated plants at 6 weeks from 0.8 to 0.55 (Table 3 and Fig. 2). However, Fig. 2 shows how cold affected PSII in the cold-treated plants with no

apparent visual leaf damage. This is very interesting because CFI (Chlorophyll Fluorescence Image) technique permits visualize leaf damage to be evaluated before plants display evident cold symptoms.

Before genes quantifications, sequence from Phytozome was supported with a phylogeny analysis. Tree results (Fig. 3) could confirm that all proteins described for *Citrus clementina* were clustering into an expected group. For P5CS1 our phylogenetic analysis coincides with the Phytozome annotation too, Ciclev10011176m was clustered with MtP5CS2 and probably is P5CS2 enzyme and Ciclev10030839 is likely P5CS1. Furthermore, metabolic enzyme families are considered to be highly conserved and have been used to reconstruct the deep branching patterns of the tree of life (Doolittle et al., 1996).

Proline accumulation (Fig. 4), which has been subjected to cold, can increase by synthesis (Fig. 5 and Fig. 6) from glutamate, which is carried out by the *P5CS1*, *P5CS2* and *P5CR* synthesis genes. Synthesis proline genes from glutamate can explain proline accumulation, *P5CS1* shows a slight induction ~~of~~ at ~~2~~ week two~~s~~ and *P5CS2* in week 6. *P5CS1* gene transcription slightly increased by 1.5-fold at 2 weeks, but no increased transcription was observed in the *P5CS2* gene at 2 weeks (Fig. 5a and 5b). It is known that in *Arabidopsis thaliana,* genes *P5CS1* and *P5CS2* perform different functions in spite of these two genes being a gene duplication. The *P5CS1* gene was induced by salt, drought, glucose and sucrose when ABA and signal derived from H_2O_2 are present (Székely et al., 2008).The *P5CS2* gene is considered a housekeeping gene in *Arabidopsis thaliana* seedlings as *p5cs2* mutants show~~s~~ aberrant development and infertility (Székely et al., 2008). In citrus the function of *P5CS1* and *P5CS2* is still

unknown. Instead the proline biosynthetic gene from the ornithine pathway has a *dOAT* gene. The expression of *dOAT* gene increases 12-fold (Fig. 5d) at 4 weeks, which coincides with the maximum proline concentration (Fig. 3) in leaves at 4 weeks. On the one hand, we can speculate that proline could have been synthesized mainly by the ornithine route, but more experiments are needed to confirm this idea. On the other hand, further research exists where the ornithine pathway, together with the glutamate pathway, plays an important role in proline accumulation during osmotic stress. An example is found in (Roosens et al., 1998) young plantlets of *Arabidopsis thaliana,* and seems to be related to the need to dispose of an easy recycling product, glutamate. As in *Arabidopsis*, salt-stress treatment in *Medicago* plants induced the accumulation of *MtOAT* transcripts in the whole plant regardless of the developmental stage, and a positive correlation was found between proline and *MtOAT* transcript accumulation (Armengaud et al., 2004). However, proline levels were not affected in *Arabidopsis oat knockout* mutants, and it has been suggested that OAT facilitates nitrogen recycling from arginine through P5C, which is converted into glutamate by P5CDH (Funck et al., 2008).

It is not surprising that, despite the induction of 12-fold *dOAT*, the proline concentration increased only 3-fold, and one of the reasons for this is the high induction of degradative proline genes. *PDH* expression at 2 weeks of cold treatment increased by more than 20-fold (Fig. 6a) and *P5CDH* increased by 15-fold at 4 weeks of cold treatment (Fig. 6b). Activation of the *PDH* genes occurs during plant rehydration in drought stress and in the presence of proline. Instead, the *P5CDH* gene is activated with a high proline concentration in leaves (Hayat et al., 2012). Thus it seems that the

high expression of degradative genes *PDH* and *P5CDH* could be induced by proline concentration.

The concentration of total and inorganic nitrogen increased significantly (Table 4), from 2.29% to 2.88% and from inorganic 0.60% to 0.94%, respectively. Although the bibliography does not currently provide a consensus as to whether a moderate to high tissue nitrogen concentration has been shown to increase, reduce or have no effect on frost resistance, it is generally agreed that a low tissue nitrogen concentration usually hinders cold acclimation (Harvey et al. 1997, Thomas et al. 1999, Andivia et al. 2011, Taulavuori et al. 2014, Villar-Salvador et al. 2015). In this way, the high nitrogen concentration reached in citrus leaves under cold stress possibly indicates that the plant might be attempting to resist low temperatures. High total and inorganic nitrogen could connect the idea about *dOAT* gene activation. The *dOAT* gene seems to predominate in mitochondria with a high nitrogen supply, whereas the glutamate pathway acts during osmotic stress and nitrogen limitation in green bean plants that have been submitted to high nitrogen doses (Sánchez et al., 2001).

In our case, the water potential (Ψh) (Fig. 7a) increased from -1 MPa to -0.5 MPa after 6 weeks of treatment. This increased water potential may be due to halted photosynthesis, stomatal closure and evapotranspiration reduction, for which plants do not suffer from water requirements. Furthermore, both cold and freezing reduce water availability. Some authors have reported an increased proline concentration in experiments where cold hardening caused no changes in water content. Osmotic potential (Ψo) (Fig. 7b) did not significantly change at the end of the experiment. Other authors have observed in maize roots grown at low water potentials that proline

accumulation may account for approximately 45% of the total osmotic adjustment in the root apex (Voetberg et al. 1991). However, others have been able to induce stresses and proline accumulation in plants, and report no change in osmotic potential (e.g. heavy metal toxicity, anaerobiosis, nutrient deficiency, atmospheric pollution and photooxidative stress) (Kavi Kishor 2004, Verbruggen et al. 2008, Szabados et al. 2010, Verslues et al. 2010, Anwar Hossain et al. 2014, Kaur et al. 2015, Saibi et al. 2015). This idea could support our hypothesis, in that the biosynthetic pathway from glutamate in citrus would not be of much importance in this experiment because an osmotic component is important in the synthesis proline pathway from glutamate (Hare and Cress 1997, Kaur et al. 2015). However, more experiments are necessary.

Finally, in the presence of cold stress, major plant metabolism-related functions are impaired (photosynthesis, stomatal closure, growth, etc.). So we hypothesize that citrus plants were forced to meet their requirements by using their reserves. In our study, citrus plants stopped growing completely, and their weight remained almost constant throughout the experiment, as reflected by the RGR value (nearly 0 g per week in the cold treatment) (Table 2). Hence this behavior suggests that plants prioritise the mobilization of their reserves to cushion any damage caused by cold stress rather than attempt to keep growing. As the proline route is central in metabolism and is connected with many other routes through ornithine and glutamate (Fig. 9) (Kaur et al. 2015), its accumulation appears an excellent means for storing energy since the oxidation of one proline molecule can yield 30 ATP equivalents (Hu et al. 1992, Szabados et al. 2010). The localization of proline degradation and the presence of the glutamate dehydrogenase enzyme in the mitochondrial matrix indicate that this process can

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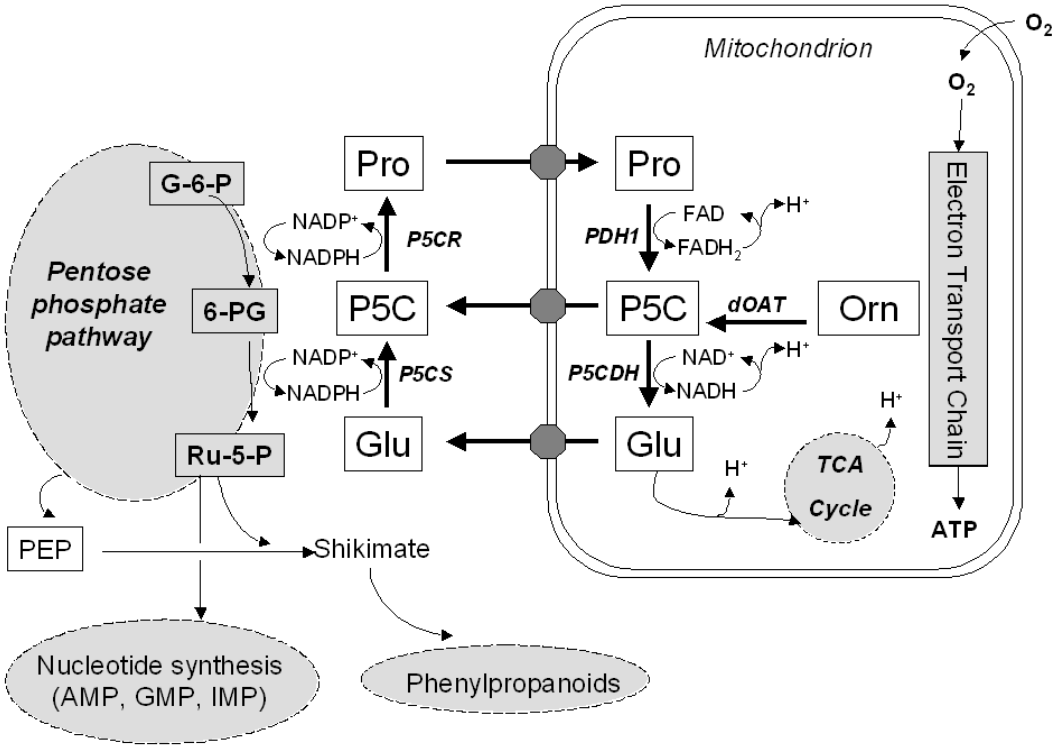
585contribute carbon to the TCA cycle (Hare et al. 1997, Szabados et al. 2010, Kaur et al
5862015). Apart from contributing carbon, the mitochondrial degradation of proline to 2-
587oxoglutarate can also help reduce the equivalents needed for the electron transport
588chain, and can generate ATP to recover from stress and to repair cold stress-induced
589damage (Araújo et al., 2012). A similar effect has been observed in barley, which
590recovered from drought through proline degradation as it contributed 20% of total
591respiration to the TCA cycle (Guo et al., 2009).



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593Fig. 9. Schematic drawing of the proline biosynthesis pathway (modified from (Hare and Cress, 1997). 594AMP, adenosine monophosphate; G-6-P, glucose-6-phosphate; Glu, glutamate; GMP, guanosine 595monophosphate; IMP, inosine monophosphate; dOAT, ornithine aminotransferase; Orn, ornithine; 6-PG, 5966-phosphogluconate; PEP, phosphoenolpyruvate; Pro, proline; PDH1, proline oxidase; P5C, 1 -pyrroline-5975-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthetase; Ru-5-P, 598ribulose-5-phosphate; TCA, tricarboxylic acid cycle.
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Besides considering proline synthesis an important osmoprotectant, ST and SU can also act as osmoprotectants in citrus plants, which also occurs with other abiotic stresses like salinity, waterlogging or drought (Yelenosky, 1985). However the final ST and SU contents did not appear to accumulate as an osmoprotector mechanism under the long-term cold conditions in Carrizo citrange plants. ST content (**Figure 8a**) lowered in the citrus trees that had suffered cold stress, and went from 48.6 mg g DW to 35.6 mg g DW. In our case, and unlike other stresses, ST hydrolysis provided a significant part of soluble SU during cold hardening, which would be required to stabil cell membranes against cell dehydration, which is caused by the freezing of extracellular water, and accumulation would avoid ice nucleation (Levitt 1980, Uemura et al. 2003). Glucose also lowered by 31.7% (**Figure 8b**). Glucose units, which hydrolyse from starch, can be diverted to maintain metabolism to the pentose phosphate route because photosynthesis, the main source of carbohydrates, is locked. Similarly to our results, premature reduction in cold hardiness due to higher non-structural carbohydrate respiratory consumption has been reported during spells with mild winter temperatures (Ögren et al. 1997, Ruelland et al. 2009, Villar-Salvador et al. 2015).

5. Conclusion

Our results showed that long-term cold stress ~~in Carrizo citrange plants cold stress at long-term i~~induces an increase of proline concentration in Carrizo citrange plants mainly through activation of the ornithine aminotransferase~~y by dOAT synthesis~~ route. Further, ~~degradation~~ proline degradation ~~route took place~~was favored by ~~with an~~ increases ~~of~~in *PDH1* and *P5CDH* gene expression. The decrease of S~~s~~tarch and soluble sugars

suggest ~~decrease consuming p~~plant reserves are being consumed~~in order~~ to resist the stress due to the photosynthesis arrest by cold stress.

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REFERENCE

Adam, S., Murthy, S.D.S., 2014. Effect of Cold Stress on Photosynthesis of Plants and Possible Protection Mechanisms, in: Gaur, R.K., Sharma, P. (Eds.), Approaches to Plant Stress and Their Management. Springer India, pp. 219–226. https://doi.org/10.1007/978-81-322-1620-9_12

Agüero, J., Vives, M. del C., Velázquez, K., Pina, J.A., Navarro, L., Moreno, P., Guerri, J., 2014. Effectiveness of gene silencing induced by viral vectors based on Citrus leaf blotch virus is different in Nicotiana benthamiana and citrus plants. Virology 460–461, 154–164. https://doi.org/10.1016/j.virol.2014.04.017

Allen, D.J., Ort, D.R., 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci. 6, 36–42. https://doi.org/10.1016/S1360-1385(00)01808-2

Andivia, E., Márquez-García, B., Vázquez-Piqué, J., Córdoba, F., Fernández, M., 2011. Autumn fertilization with nitrogen improves nutritional status, cold hardiness and the oxidative stress response of Holm oak (Quercus ilex ssp. ballota [Desf.] Samp) nursery seedlings. Trees 26, 311–320. https://doi.org/10.1007/s00468-011-0593-3

Anwar Hossain, M., Hoque, Md.A., Burritt, D.J., Fujita, M., 2014. Chapter 16 - Proline Protects Plants Against Abiotic Oxidative Stress: Biochemical and Molecular Mechanisms A2 - Ahmad, Parvaiz, in: Oxidative Damage to Plants. Academic Press, San Diego, pp. 477–522.

Araújo, W.L., Trofimova, L., Mkrtchyan, G., Steinhauser, D., Krall, L., Graf, A., Fernie, A.R., Bunik, V.I., 2012. On the role of the mitochondrial 2-oxoglutarate dehydrogenase complex in amino acid metabolism. Amino Acids 44, 683–700. https://doi.org/10.1007/s00726-012-1392-x

Armengaud, P., Thiery, L., Buhot, N., Grenier-de March, G., Savouré, A., 2004. Transcriptional regulation of proline biosynthesis in Medicago truncatula reveals developmental and environmental specific features. Physiol. Plant. 120, 442–450. https://doi.org/10.1111/j.0031-9317.2004.00251.x

Attaway, J.A., 1997. A History of Florida Citrus Freezes. Florida Science Source.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207. https://doi.org/10.1007/BF00018060

Batista-Santos, P., Lidon, F.C., Fortunato, A., Leitão, A.E., Lopes, E., Partelli, F., Ribeiro, A.I., Ramalho, J.C., 2011. The impact of cold on photosynthesis in genotypes of Coffea spp.—Photosystem sensitivity, photoprotective mechanisms and gene expression. J. Plant Physiol. 168, 792–806. https://doi.org/10.1016/j.jplph.2010.11.013

Bremner, J.M., 1965. Total Nitrogen. Methods Soil Anal. Part 2 Chem. Microbiol. Prop. agronomymonogra, 1149–1178. https://doi.org/10.2134/agronmonogr9.2.c32

Brunner, A.M., Yakovlev, I.A., Strauss, S.H., 2004. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol. 4, 14. https://doi.org/10.1186/1471-2229-4-14

663Calatayud, Á., San Bautista, A., Pascual, B., Maroto, J.V., López-Galarza, S., 2013. Use of chlorophyll fluorescence
664 imaging as diagnostic technique to predict compatibility in melon graft. Sci. Hortic., Advances in Vegetable
665 Grafting Research 149, 13–18. https://doi.org/10.1016/j.scienta.2012.04.019
666Callister, A.N., Arndt, S.K., Adams, M.A., 2006. Comparison of four methods for measuring osmotic potential of tree
667 leaves. Physiol. Plant. 127, 383–392. https://doi.org/10.1111/j.1399-3054.2006.00652.x
668Doolittle, R.F., Feng, D.-F., Tsang, S., Cho, G., Little, E., 1996. Determining Divergence Times of the Major Kingdoms
669 of Living Organisms with a Protein Clock. Science 271, 470–477.
670 https://doi.org/10.1126/science.271.5248.470
671Ensminger, I., Busch, F., Huner, N.P.A., 2006. Photostasis and cold acclimation: sensing low temperature through
672 photosynthesis. Physiol. Plant. 126, 28–44. https://doi.org/10.1111/j.1399-3054.2006.00627.x
673Estornell, L.H., Gómez, M.D., Pérez-Amador, M.A., Talón, M., Tadeo, F.R., 2016. Secondary abscission zones:
674 understanding the molecular mechanisms triggering stylar abscission in citrus. Acta Hortic. 65–72. https://
675 doi.org/10.17660/ActaHortic.2016.1119.9
676Funck, D., Stadelhofer, B., Koch, W., 2008. Ornithine-δ-aminotransferase is essential for Arginine Catabolism but
677 not for Proline Biosynthesis. BMC Plant Biol. 8, 40. https://doi.org/10.1186/1471-2229-8-40
678Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam,
679 N., Rokhsar, D.S., 2011. Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res.
680 gkr944. https://doi.org/10.1093/nar/gkr944
681Guo, P., Baum, M., Grando, S., Ceccarelli, S., Bai, G., Li, R., Korff, M. von, Varshney, R.K., Graner, A., Valkoun, J.,
682 2009. Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in
683 response to drought stress during the reproductive stage. J. Exp. Bot. 60, 3531–3544.
684 https://doi.org/10.1093/jxb/erp194
685Hare, P.D., Cress, W.A., 1997. Metabolic implications of stress-induced proline accumulation in plants. Plant
686 Growth Regul. 21, 79–102. https://doi.org/10.1023/A:1005703923347
687Harvey, H.P., Driessche, R. van den, 1997. Nutrition, xylem cavitation and drought resistance in hybrid poplar. Tree
688 Physiol. 17, 647–654. https://doi.org/10.1093/treephys/17.10.647
689Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., Ahmad, A., 2012. Role of proline under changing
690 environments. Plant Signal. Behav. 7, 1456–1466. https://doi.org/10.4161/psb.21949
691Hu, C.A., Delauney, A.J., Verma, D.P., 1992. A bifunctional enzyme (delta 1-pyrroline-5-carboxylate synthetase)
692 catalyzes the first two steps in proline biosynthesis in plants. Proc. Natl. Acad. Sci. 89, 9354–9358. https://
693 doi.org/10.1073/pnas.89.19.9354
694Kaur, G., Asthir, B., 2015. Proline: a key player in plant abiotic stress tolerance. Biol. Plant. 59, 609–619.
695 https://doi.org/10.1007/s10535-015-0549-3
696Kushad, M.M., Yelenosky, G., 1987. Evaluation of Polyamine and Proline Levels during Low Temperature
697 Acclimation of Citrus 1. Plant Physiol. 84, 692–695.
698Levitt, J., 1980. Responses of Plants to Environmental Stresses: Chilling, freezing, and high temperature stresses.
699 Academic Press.
700Long, S.P., Bernacchi, C.J., 2003. Gas exchange measurements, what can they tell us about the underlying
701 limitations to photosynthesis? Procedures and sources of error. J. Exp. Bot. 54, 2393–2401.
702 https://doi.org/10.1093/jxb/erg262
703Machado, D.F.S.P., Ribeiro, R.V., Silveira, J.A.G. da, Filho, M., Rodrigues, J., Machado, E.C., 2013. Rootstocks induce
704 contrasting photosynthetic responses of orange plants to low night temperature without affecting the
705 antioxidant metabolism. Theor. Exp. Plant Physiol. 25, 26–35. https://doi.org/10.1590/S2197-
706 00252013000100004
707McCready, R.M., Guggolz, Jack., Silviera, Vernon., Owens, H.S., 1950. Determination of Starch and Amylose in
708 Vegetables. Anal. Chem. 22, 1156–1158. https://doi.org/10.1021/ac60045a016
709Megha, S., Basu, U., Kav, N.N.V., 2014. Metabolic engineering of cold tolerance in plants. Biocatal. Agric.
710 Biotechnol., Trait Introduction Methods and Innovation Platforms in Plant Biotechnology 3, 88–95.
711 https://doi.org/10.1016/j.bcab.2013.11.007
712Moran, R., 1982. Formulae for Determination of Chlorophyllous Pigments Extracted with N,N-Dimethylformamide.
713 Plant Physiol. 69, 1376–1381. https://doi.org/10.1104/pp.69.6.1376
714Moran, R., Porath, D., 1980. Chlorophyll Determination in Intact Tissues Using N,N-Dimethylformamide. Plant
715 Physiol. 65, 478–479. https://doi.org/10.1104/pp.65.3.478
10435

Nesbitt, M.L., Ebel, R.C., Findley, D., Wilkins, B., Woods, F., Himelrick, D., 2002. Assays to Assess Freeze Injury of Satsuma Mandarin. HortScience 37, 871–877.

Obenland, D.M., Aung, L.H., Bridges, D.L., Mackey, B.E., 2003. Volatile Emissions of Navel Oranges as Predictors of Freeze Damage. J. Agric. Food Chem. 51, 3367–3371. https://doi.org/10.1021/jf021109o

Ögren, E., Nilsson, T., Sundblad, L.-G., 1997. Relationship between respiratory depletion of sugars and loss of cold hardiness in coniferous seedlings over-wintering at raised temperatures: indications of different sensitivities of spruce and pine. Plant Cell Environ. 20, 247–253. https://doi.org/10.1046/j.1365-3040.1997.d01-56.x

P. B. Kavi Kishor, S.S., 2004. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr Sci India. Curr Sci 88.

P M Chandler, Robertson, and M., 1994. Gene Expression Regulated by Abscisic Acid and its Relation to Stress Tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 113–141. https://doi.org/10.1146/annurev.pp.45.060194.000553

Partelli, F.L., Vieira, H.D., Viana, A.P., Batista-Santos, P., Rodrigues, A.P., Leitão, A.E., Ramalho, J.C., 2009. Low temperature impact on photosynthetic parameters of coffee genotypes. Pesqui. Agropecuária Bras. 44, 1404–1415. https://doi.org/10.1590/S0100-204X2009001100006

Paul, M.J., Driscoll, S.P., Lawlor, D.W., 1992. Sink-Regulation of Photosynthesis in Relation to Temperature in Sunflower and Rape. J. Exp. Bot. 43, 147–153. https://doi.org/10.1093/jxb/43.2.147

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45–e45. https://doi.org/10.1093/nar/29.9.e45

Ramalho, J.C., DaMatta, F.M., Rodrigues, A.P., Scotti-Campos, P., Pais, I., Batista-Santos, P., Partelli, F.L., Ribeiro, A., Lidon, F.C., Leitão, A.E., 2014. Cold impact and acclimation response of Coffea. Theor. Exp. Plant Physiol. 26, 5–18. https://doi.org/10.1007/s40626-014-0001-7

Ramalho, J.C., Quartin, V.L., Leitão, E., Campos, P.S., Carelli, M.L.C., Fahl, J.I., Nunes, M.A., 2003. Cold Acclimation Ability and Photosynthesis among Species of the Tropical Coffea Genus. Plant Biol. 5, 631–641. https://doi.org/10.1055/s-2003-44688

Ribeiro, R.V., Espinoza-Núñez, E., Junior, J.P., Filho, F.A.A.M., Machado, E.C., 2014. Citrus Rootstocks for Improving the Horticultural Performance and Physiological Responses Under Constraining Environments, in: Ahmad, P., Wani, M.R., Azooz, M.M., Tran, L.-S.P. (Eds.), Improvement of Crops in the Era of Climatic Changes. Springer New York, pp. 1–37. https://doi.org/10.1007/978-1-4614-8830-9_1

Ribeiro, R.V., Machado, E.C., Santos, M.G., Oliveira, R.F., 2009. Seasonal and diurnal changes in photosynthetic limitation of young sweet orange trees. Environ. Exp. Bot. 66, 203–211. https://doi.org/10.1016/j.envexpbot.2009.03.011

Roelfsema, M.R.G., Prins, H.B.A., 1995. Effect of abscisic acid on stomatal opening in isolated epidermal strips of abi mutants of Arabidopsis thaliana. Physiol. Plant. 95, 373–378. https://doi.org/10.1111/j.1399-3054.1995.tb00851.x

Roosens, N.H.C.J., Thu, T.T., Iskandar, H.M., Jacobs, M., 1998. Isolation of the Ornithine-δ-Aminotransferase cDNA and Effect of Salt Stress on Its Expression in Arabidopsis thaliana. Plant Physiol. 117, 263–271.

Ruelland, E., 2009. Chapter 2 Cold Signalling and Cold Acclimation in Plants. Adv. Bot. Res. 35–150. https://doi.org/10.1016/S0065-2296(08)00602-2

Saibi, W., Feki, K., Yacoubi, I., Brini, F., 2015. Bridging Between Proline Structure, Functions, Metabolism, and Involvement in Organism Physiology. Appl. Biochem. Biotechnol. 176, 2107–2119. https://doi.org/10.1007/s12010-015-1713-0

Sakai, A., Larcher, W., 2012. Frost Survival of Plants: Responses and Adaptation to Freezing Stress. Springer Science & Business Media.

Sánchez, E., López-Lefebre, L.R., García, P.C., Rivero, R.M., Ruiz, J.M., Romero, L., 2001. Proline metabolism in response to highest nitrogen dosages in green bean plants (Phaseolus vulgaris L. cv. Strike). J. Plant Physiol. 158, 593–598. https://doi.org/10.1078/0176-1617-00268

Santos, C.M.A., Ribeiro, R.V., Magalhães Filho, J.R., Machado, D.F.S.P., Machado, E.C., 2011. Low substrate temperature imposes higher limitation to photosynthesis of orange plants as compared to atmospheric chilling. Photosynthetica 49, 546–554. https://doi.org/10.1007/s11099-011-0071-6

Szabados, L., Savouré, A., 2010. Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

10736

Székely, G., Ábrahám, E., Cséplő, Á., Rigó, G., Zsigmond, L., Csiszár, J., Ayaydin, F., Strizhov, N., Jásik, J., Schmelzer, E., Koncz, C., Szabados, L., 2008. Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant J. 53, 11–28. https://doi.org/10.1111/j.1365-313X.2007.03318.x

Taiz, L., Zeiger, E., 2010. Plant Physiology. Sinauer Associates.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197

Taulavuori, K., Taulavuori, E., Sheppard, L.J., 2014. Truths or myths, fact or fiction, setting the record straight concerning nitrogen effects on levels of frost hardiness. Environ. Exp. Bot., The Biology of Plant Cold Hardiness: Adaptive Strategies 106, 132–137. https://doi.org/10.1016/j.envexpbot.2013.12.022

Thomas, F.M., Ahlers, U., 1999. Effects of excess nitrogen on frost hardiness and freezing injury of above-ground tissue in young oaks (Quercus petraea and Q. robur). New Phytol. 144, 73–83. https://doi.org/10.1046/j.1469-8137.1999.00501.x

Uemura, M., Steponkus, P.L., 2003. Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from Arabidopsis thaliana leaves. Plant Cell Environ. 26, 1083–1096. https://doi.org/10.1046/j.1365-3040.2003.01033.x

Verbruggen, N., Hermans, C., 2008. Proline accumulation in plants: a review. Amino Acids 35, 753–759. https://doi.org/10.1007/s00726-008-0061-6

Verslues, P.E., Sharma, S., 2010. Proline Metabolism and Its Implications for Plant-Environment Interaction. Arab. Book Am. Soc. Plant Biol. 8. https://doi.org/10.1199/tab.0140

Villar-Salvador, P., Uscola, M., Jacobs, D.F., 2015. The role of stored carbohydrates and nitrogen in the growth and stress tolerance of planted forest trees. New For. 46, 813–839. https://doi.org/10.1007/s11056-015-9499-z

Voetberg, G.S., Sharp, R.E., 1991. Growth of the Maize Primary Root at Low Water Potentials III. Role of Increased Proline Deposition in Osmotic Adjustment. Plant Physiol. 96, 1125–1130. https://doi.org/10.1104/pp.96.4.1125

Vu, J.C.V., Yelenosky, G., 1992. Photosnythetic responses of rough lemon and sour orange to soil flooding, chilling, and short-term temperature fluctuations during growth. Environ. Exp. Bot. 32, 471–477. https://doi.org/10.1016/0098-8472(92)90060-F

Yelenosky, G., 1991. Apparent Nucleation and Freezing in Various Parts of Young Citrus Trees during Controlled Freezes. HortScience 26, 576–579.

Yelenosky, G., 1985. Cold Hardiness in Citrus, in: Janick, J. (Ed.), Horticultural Reviews. John Wiley & Sons, Inc., pp. 201–238.