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Chloroplast proteomics reveals transgenerational cross-stress priming in *Pinus radiata*

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ABSTRACT

Plants do have stress memory and chloroplast signaling has been revealed as crucial element to acquire and extend this memory into future generations, allowing plant adaptation to changing environments and providing novel tools in the field of crop improvement. Despite the process is known, how a plant is capable to transfer some aspects of its "life-long learning" to progeny, as well as the role of chloroplast proteome mediating transgenerational cross-stress priming effects, remain unknown. To fill this gap, this study examines the impact of the physiological status of *Pinus radiata* parentals over the capacity of their progeny to acclimate to their first stress period in a common garden experiment. Seedlings were originated in subpopulations with the same genetic background, but grown in two locations with contrasting environments (stressed vs non-stressed plants). Physiological measurements (fluorescence-based and biochemistry) and chloroplast proteomics were employed to study plant stress responses. Results demonstrated a differential seed priming. Those seedlings originated from stressed plants responded quicker and more efficiently than those originated from unstressed counterparts. Unprimed responses showed proteome remodeling driven by lipid peroxidation and photoinhibition, whereas primed subpopulation quickly faced stress rearranging secondary metabolism, replacing damaged lipids, reducing photooxidative damage, and promoting photorespiration and redox homeostasis in order to reduce lipoperoxidation and maintain photosynthesis. These results not only delve into cross-stress memory in long-lived species, but also suggest a new biotechnological potential for current seed orchards if adequate management is performed.

1. Introduction

Across evolution, plants have evolved sophisticated adaptive mechanisms to withstand environmental stress at physiological, cellular, molecular, and biochemical levels, enabling them to survive under a variety of stress conditions. This long-term adaptive mechanism coexists with short-term acclimation responses. Among these, there are several ways that can determine plant capability to respond to reiterated stresses (Bruce et al., 2007). This effect is called priming or stress memory, which often makes a plant more tolerant to future exposures to same -stress tolerance (Lamelas et al., 2020a; Leuendorf et al., 2020)- or even different -cross-stress tolerance (Foyer et al., 2016; Munne-Bosch, 2013; Walter et al., 2013)- stress factors. In this context, the most important and best documented common response of plants to different abiotic and biotic stresses, such as heat, cold, high-light intensities, drought, osmotic shock, wounding, UV-B radiation, ozone, and pathogens is the accelerated production of active oxygen species (Barrios and Brown, 2014; Locato et al., 2018; Pastori and Foyer, 2002).

This ability of plants to store and recall information from previous events and then change their responses to recurring stresses is essential for their adaptation and survival because of their sessile nature. Although an enhanced stress-resistance may compromise plant productivity in the short-term, for example through a reduction of photosynthesis, it represents increased tolerance to subsequent stress. Consequently, plants are able to select, at least to some extent, the most appropriate response to certain changes in the environment, boosting productivity in the long-term (Bruce et al., 2007). This is particularly important for long-lived species such as trees, which may face several

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stresses during their life. Occasionally, stress-memory may also be extended into future generations (Kumar et al., 2015; Molinier et al., 2006; Robinson and Robinson, 2020; Vancostenoble et al., 2022; Wang et al., 2016), process called transgenerational stress-memory, which may be an evolutionary force for plants adapting to rugged environments (Ramírez-Carrasco et al., 2017; Slaughter et al., 2012). Despite the process is known, and epigenetics has been proposed as the main player, providing a mechanistic basis for a stress memory (Grossniklaus et al., 2013; Lämke and Bäurle, 2017; Tricker, 2015), how a plant is capable to transfer some aspects of its "life-long learning" to progeny is still unknown. Explaining the mechanisms behind transgenerational stress memory will provide not only a better understanding of evolutive mechanisms and the behavior of natural populations, but also novel tools in the field of crop improvement (Bilichak and Kovalchuk, 2016; Liu et al., 2021).

Within plant cells, chloroplast is one of the principal drivers of stress response through different organellar signaling pathways (Kmiecik et al., 2016). It acts as a hub in the cellular response to environmental changes optimizing different cell functions for triggering an appropriate response to stressful conditions, and this often involves broad proteome changes (Phee et al., 2004; Taylor et al., 2009; Watson et al., 2018). So, chloroplast proteome is rapidly modified in response to a range of changing environmental conditions or impacts, which is key to plant's stress response, acclimation, and survival (Tamburino et al., 2017; Yang et al., 2019; Lande et al., 2020, 2022). As chloroplast genome represents only a minor proportion of its proteins, roughly counting 120 genes in crop and trees species (Daniell et al., 2016; Dobrogojski et al., 2020), the definition of the chloroplast proteome also involves a fine tuning of nuclei transcription, which is the result of a dynamic bidirectional communication with the nucleus (Crawford et al., 2018; Lamelas et al., 2021; Woodson and Chory, 2012), as well as cytosolic post-translational protein modifications and activated cell trafficking system (Emanuelsson et al., 2000; Jarvis and López-Juez, 2013; Lee et al., 2013; Li and Chiu, 2010). Chloroplast signaling has also been revealed as crucial element to gate stress tolerance (Dickinson et al., 2018) and acquire this memory. In the model species *Arabidopsis thaliana*, chloroplast calcium signaling dependent on CALCIUM SENSING RECEPTOR (CAS) protein regulates thermo-memory (Pollastri et al., 2021), and/or chloroplast reactive oxygen species (ROS) signaling mediated by transcriptional regulation of thylakoid ASCORBATE PEROXIDASE (tAPX) induces cold-priming (van Buer et al., 2019), whereas the crosstalk and synchronization between chloroplast and nucleus has been required for thermotolerance acquisition in *Pinus radiata*, revealing microRNAs and protein ARGONAUTE 1 (AGO1) as potential regulators (Lamelas et al., 2021).

In nature, one of the direct stressors of chloroplast is UV radiation as it quickly induces damage at different levels. Apart from triggering chloroplast movements (Hermanowicz et al., 2019) and altering pigment content and the structure of both grana and stroma thylakoids (Kirchhoff, 2014), UV is directly absorbed by intracellular macromolecules, causing them damage, and ultimately inhibiting photosynthesis and inducing chloroplast turnover (Kataria et al., 2014), which reduces plant development and tree productivity in a meaningful way. In this context and according to current and future UV irradiance prediction models (Bais et al., 2015; Neale et al., 2021), UV has become one of the most important challenges facing forest species, such as the commercially *P. radiata* (Dash et al., 2019; Wei et al., 2019). Although its UV response has been well characterized at different levels in the last years (Pascual et al., 2016, 2017; Valledor et al., 2012), the dynamics of chloroplast proteome triggered by UV stress, as well as its role mediating stress response modulation and memory effects, remain unknown. Interestingly, UV priming has been reported to induce a wide cross-stress tolerance, improving tolerance to herbivory (Dillon et al., 2018), fungal (Xu et al., 2019), salt (Ouhibi et al., 2014; Sen et al., 2021; Thomas and Puthur, 2019) and drought (Saenz-de la O et al., 2021; Sen et al., 2021; Sen and Puthur, 2021; Thomas and Puthur, 2019) stress in many species. In this context, stress tolerance may be also induced in the other direction, meaning other biotic and/or abiotic stresses might toughen plants against UV stress. The elucidation of common stress-response components, such as ROS elements (Foyer et al., 2016; Pastori and Foyer, 2002), has enormous potential and has, therefore, become a priority in research and breeding programs aimed at improving plant stress tolerance (Janni et al., 2020).

To fill this gap, we have employed *P. radiata* seedlings originated from subpopulations with the same genetic background grown in two locations with contrasting environments to conduct a transgenerational cross-stress memory study. Physiological and chloroplast proteome analyses demonstrated that seedlings originated from the seeds of the different subpopulations showed distinctive capabilities to handle UV-B stress, highlighting photorespiration and signaling mediated by redox, lipid, and secondary metabolism as relevant pathways to drive transgenerational cross-stress memory. This knowledge has potential application for understanding tree stress response and adaptation processes, for providing new tolerance markers to the breeders, and for suggesting a new biotechnology based on the management of seed orchards to produce more tolerant trees.

2. Material and methods

2.1. Plant material and experimental design

The assay was conducted in a climate chamber under controlled conditions (Fitoclima 1200, Aralab). Seeds from one clone named 0027 growing in two different Chilean locations (subpopulations) were collected in March 2017: Escuadrón (E) (36°56′49.26″ S; 73°8′49.41′ W) seeds were produced by 93 ramets (fertirrigated), and Tranguilvoro (T) (37°59′35.04″ S; 73°21′41.48′ W), seeds produced by 18 ramets grown under natural conditions (non-fertirrigated). Ramets were established in 1981, by grafting the clone 0027 which at that time had 18–20 years old. The irrigation of E subpopulation during dry months (November to March) was applied once per week using a drip system to recover the 30 % of water loses by evapotranspiration (ETo; estimated after local meteorological information), avoiding drought stress periods during Summer, contrary to T subpopulation (Supplementary Table S1 -the information of precipitations and temperatures for the occurrences of each orchard was obtained from the Chelsa-climate database (Karger et al., 2017) according to Alarcon and Cavieres (2018) using the R libraries: raster (https://CRAN.R-project.org/package=raster) (Hijmans, 2022) and rgdal (https://CRAN.R-project.org/package=rgdal) (Bivand et al., 2022)-). A total of fifteen one-year-old *P. radiata* seedlings of each of these two Chilean E and T subpopulations (plant height 35 ± 5 cm and 34 ± 2 cm, respectively) in 1 dm^3 pots -(peat:perlite) (7:2) supplemented Osmocote- were randomized inside the climate chamber. Afterwards, they were kept over a one-month period under a photoperiod of 16 hours (h) (400 μmol m^{-2} s^{-1}) at 25 °C, and 50 % relative humidity (RH), and 15 °C and 60 % RH during the night period (8 h) to acclimate. Plants were regularly watered at field capacity.

Same conditions were maintained during UV stress assay (Supplementary Fig. S1), in which plants were irradiated with 1.0 W/m^2 UV-B light measured in the apical part (TL 40 W/12 RS UV-B Broadband Philips -290 to 315 nm-) 8 h each day (Supplementary Fig. S1_A). Apical needles were sampled and photosynthetic activity was measured at different intervals (Supplementary Fig. S1_B): just before UV-B stress (control, T0), after 2 h of UV-B stress (T0.5), once maximal efficiency of PSII (Fv/Fm) in leaves pre-adapted to darkness for 20 min (see below) was fewer than 0.75 (T2, 2 days) and once Fv/Fm values were fewer than 0.70 (T8, 8 days). Mature needles that were closest to the apex were sampled and chloroplasts were immediately isolated. For biochemical and proteomic analyses, plants were flash-frozen in liquid nitrogen and stored at − 80 °C until use. Five biological replicates for each treatment (T0, T0.5, T2, T8) and subpopulation (E, T) were constituted pooling needles of three different plants. These pools were kept across the

experiment and constitute the biological independent replicates that were analyzed.

2.2. Photosynthesis performance measurements

To evaluate photosynthetic damage, chlorophyll fluorescence was measured immediately after the end of each individual treatment (T0, and UV-B exposed T0.5, T2, T8) employing a fluorometer (OS1p-FL, Opti-Sciences, USA). Maximal efficiency of PSII (Fv/Fm) in leaves pre-adapted to darkness for 20 min were calculated according to Maxwell and Johnson (2000).

2.3. Metabolism markers extraction and quantification

Metabolism markers were extracted and quantified following the protocol described by López-Hidalgo et al. (2021). In brief, 10 mg of lyophilized needles were grounded into a homogeneous and fine powder and homogenized in 1 mL of cold ethanol 80 % (v/v). Samples were then incubated in ultrasonic bath during 10 min with ice, and centrifuged for 10 min, at 10,000 *g*, and 4 °C. Since pellet was used to determine starch (STA) content, aliquots from supernatant were employed to quantify photosynthetic pigments (PHP) (chlorophyll a, chlorophyll b, ratio of chlorophyll a to chlorophyll b (chlorophyll a/b ratio), carotenoids), free amino acids (FAA), malondialdehyde (MDA) -used as a lipid peroxidation marker under several stresses (Jbir-Koubaa et al., 2015; Zhou et al., 2015a)-, total phenolic compounds (TPC), total flavonoids (TFL), and total soluble sugars (TSS). PHP were extracted by ethanol 80% (v/v), measured at 470, 649, and 664 nm, and quantified according to the following equations: chlorophyll a (Chla) (μg/mL) = 13.36 A664 − 5.19 A649, chlorophyll b (Chlb) (μg/mL) = 27.43 A649 − 8.12 A664, carotenoids (μg/mL) = (1000 A470 − 2.13 Chla − 97.63 Chlb)/209 (Lichtenthaler, 1987). MDA was determined using the trichloroacetic acid (TCA) / 2-thiobarbituric acid (TBA) test, employing TCA (20 % (w/v)) / TBA (0.5 % (w/v)) in ddH_2O as positive reaction reagent (+ TBA) and TCA (20 % (w/v)) in ddH_2O as negative reaction reagent (-TBA). Samples were incubated 30 min at 95 °C, centrifuged, and absorbances read at 440, 532, and 600 nm. Following equations were used to quantify MDA content: A = [(A532$_{+TBA}$ − A600$_{+TBA}$) – (A532$_{-TBA}$ − A600$_{-TBA}$)], B = [(A440$_{+TBA}$ − A600$_{+TBA}$) 0.0571], MDA equivalents (nmol/mL) = ((A − B)/157.000) * 10^6 (Hodges et al., 1999; Landi, 2017). FAA content was determined employing Commercial Ninhydrin Reagent 2 % Solution (Sigma-Aldrich) and L-Proline + L-Glycine in 80 % (v/v) ethanol as standard curve (Moore and Stein, 1954). Samples, as well as standard solutions, were incubated at 100 °C for 10 min, reaction was stopped by adding 95 % (v/v) ethanol to each tube, and absorbances were read at 440, 520, and 570 nm (Seracu, 1987). TPC content was measured employing 10 % Folin-Ciocalteu reagent (Merck) (Ainsworth and Gillespie, 2007) and gallic acid standard in 80 % (v/v) ethanol. After 2 min, reaction was neutralized by 700 mM Na_2CO_3 in ddH_2O, and tubes were incubated at room temperature and darkness for 2 h, reading then absorbance at 720 nm. TFL quantification was carried out using the aluminum chloride method (10 % of $AlCl_3$) (Huang et al., 2018) and quercetin standard in 80 % (v/v) ethanol. Tubes were incubated at room temperature for 30 min and absorbance was read at 415 nm. Both TSS and STA were measured using anthrone reagent (Chow and Landhäusser, 2004), as well as D-Glucose standard in 80 % (v/v) ethanol and 30 % (v/v) perchloric acid, respectively, for the standard curve. Although both metabolism biomarkers were determined by incubating samples with anthrone at 100 °C for 10 min and reading absorbance at 625 nm, TSS were measured in supernatant and STA was measured after starch hydrolysis by incubating initial pellet in 1 mL of 30 % (v/v) perchloric acid in ddH_2O at 60 °C during 1 h.

2.4. Chloroplast isolation and purification and protein extraction

Chloroplast isolation and purification, as well as protein extraction, were carried out according to Lamelas et al. (2020b). In summary, sampled needles (approximately one gram) were cut in 2–3 mm pieces and immediately homogenize in 12 mL of precooled chloroplast isolation buffer (CIB) (0.35 mM sorbitol, 50 mM HEPES-KOH pH 7.4, 5 mM EDTA, 5 mM $MgCl_2$, 15 mM β-mercaptoethanol, 0.5 mM PMSF, 1 % (w/v) BSA) using a rotor–stator homogenizer at 6500 rpm for 20 s three times each. Rotor-stator system was cleaned in a fresh tube with 8 mL of CIB, and this CIB was added to the homogenized needles. Both homogenates were mixed and filtered through four layers of cheesecloth/Miracloth. Filtered solution was centrifugated for 3 min at 200 *g* at 4 °C in a swinging rotor. Supernatant was transferred to a new tube and centrifugated 20 min at 3000 *g* and 4 °C, discarding new supernatant and washing the raw chloroplast pellet with 10 mL of CIB. This last step was made twice. 3 mL of discontinuous percoll-sucrose gradient (DPSG) solution A (9 vol 3 M sucrose, 5 mM $Mg(C_2H_3O_2)_2$, 0.1 mM EDTA, 10 mM Tris–HCl pH 8.0, 1 mM DTT, 1 vol CIB) were added to a 15 mL tube and overlaid with 3 mL of DPSG solution B (percoll 70 % (v/v) diluted in CIB). Cleaned chloroplast pellet was resuspended in 3 mL of CIB and the discontinuous gradient was carefully overlaid. Tubes were centrifugated 30 min at 3300 *g* and 4 °C in a swinging rotor with smooth acceleration-deceleration. The lower dark phase of the gradient, where intact chloroplasts were located, was recovered to a 15 mL tube, which was filled with CIB and mixed gently by inversion until homogeneous color was obtained. Tubes were centrifugated 10 min at 3000 *g* and supernatant was discarded. Purity and integrity of chloroplast extracts was tested under bright-light optical microscope.

Once chloroplasts were purified, pellets were resuspended in 300 μL of protein extraction buffer (PEB) (100 mM Tris–HCl pH 8.0, 5 % (w/v) SDS, 10 % (v/v) glycerol, 2 mM PMSF, 10 mM DTT, 1.2 % (v/v) plant protease inhibitor cocktail (Sigma P9599)) and sonicated for 15 s at 60 % amplitude (Hielscher UP200S), and then incubated in a vortex at maximum speed for 15 min at room temperature. 100 μL of 20 % SDS were added to each sample tube and tubes were homogenized by vortex and incubated 2–5 min at 95°. 300 μL of buffer Z (BZ) (1.5 M sucrose, 10 mM DTT, 1 % (v/v) plant protease inhibitor cocktail (Sigma, P9599)) and 300 μL of phenol were added. Tubes were mixed vigorously and centrifugated at room temperature for 5 min at 17,000 *g*. After centrifugation, phenolic phase was saved, and the lower phase was re-extracted by adding 300 μL of phenol. Both phenolic phases were collected, merged, and cleaned with BZ in the same way to conserve the upper phase. Proteins were precipitated by adding two volumes of protein precipitation solution (PPS) (0.1 M ammonium acetate in methanol) and incubating overnight at − 20 °C. Tubes were centrifugated and proteins pellets washed with acetone twice. Dry pellets were dissolved in the minimum amount of protein solubilization solution (PSS) (1.5 % (w/v) SDS, 8 M urea) and protein content was quantified using bicinchoninic acid (BCA) assay (Smith et al., 1985).

2.5. Protein identification and quantitation by nUPLC-Orbitrap/MS analysis

Sixty μg of chloroplast protein were in gel digested as described by Valledor and Weckwerth (2014). In brief, proteins were run in 12 % SDS-PAGEs until the front entered 4 mm into resolving gel. Gel slab was excised, and proteins were digested with trypsin (Roche). Obtained peptides were cleaned, extracted, desalted, and afterwards analyzed in a nUPLC-Orbitrap/MS (Dionex Ultimate Nano - Orbitrap Fusion/MS) employing a 85 min gradient and a 150 mm length RP-C18 column (Zorbax) following a top30 approach.

Four databases were employed for identifying proteins (*Pinus taeda* v.1.0 and *Pinus pinaster* v.1.0 available at Gymnoplaza, Uniprot/SwissProt Viridiplantae (January 2020 version), and an in-house *P. radiata* transcriptome (bioRxiv,https://doi.org/10.1101/2022.07.08.499117)), following the recommendations described by Romero-Rodríguez et al. (2014). Proteome Discoverer 2.3 (Thermo Fisher) and SEQUEST algorithm were employed for the identification and quantitation of proteins

using at least one high-confidence unique peptide for identification (see Lamelas et al., 2020a for a detailed description of parameters).

Identified proteins sequences were analyzed with four independent plant-specific subcellular location tools (Localizer v1.0.4 (Sperschneider et al., 2017), Bologna Unified Subcellular Component Annotator (BUSCA) (Savojardo et al., 2018), TargetP v.2.0 (Emanuelsson et al., 2007), YLoc (Briesemeister et al., 2010a; Briesemeister et al., 2010b)) and two functional annotation tools (Mercator Mapman v3.6 (Lohse et al., 2014; Thimm et al., 2004) and sma3s (Casimiro-Soriguer et al., 2017)) to verify protein location. Proteins not considered chloroplastic by at least two different tools or with no subcellular annotation found by Mercator Mapman or sma3s were dropped from the analysis.

2.6. Statistical and bioinformatics analyses

Per subpopulation (E and T) and treatment (T0, T0.5, T2, T8), fifteen biological replicates were used for photosynthetic measurements, and four biological replicates were used for metabolism markers quantification and proteome analyses. All statistical procedures were conducted with the R programming language (https://www.rproject.org/) (R Core Team, 2020) running under RStudio v.4.0.2 (http://www.rstudio.org/) (RStudio Team, 2020) employing the agricolae package (http://CRAN.R-project.org/package=agricolae) for performing ANOVAs of the physiological measurements and metabolism markers datasets (p-value < 0.05), and pRocessomics (https://github.com/Valledor/pRocessomics) for analyzing omic dataset and integrating data following the recommendations of Valledor and Jorrín (2011) and Valledor et al. (2014). In brief, missed values were imputed using a sequential Random Forest approach (Stekhoven and Buhlmann, 2012), and then variables not present in at least 45 % of samples or in all replicates of a sampling time were filtered out to reduce noise. Protein abundances within samples were normalized employing a treatment-centric approach. Data were subjected to univariate analyses (one-way ANOVA + Tukey HSD post-hoc test, p-value < 0.05), as well as Venn diagrams and multivariate analyses: heatmap clustering, K-means, principal component analysis (PCA), and sparse partial squares regression analysis (sPLS). sPLS-based multivariate models were performed employing mixOmics package (Rohart et al., 2017), providing networks using proteins as predictors and physiological measurements as response. Networks were pruned and represented employing Cytoscape v.3.7.2 (Shannon et al., 2003).

3. Results and discussion

3.1. Growing conditions of parental plants determined the physiological responses to UV-B stress of their progeny

In this work, we have studied the response of pine seedlings to moderate dosages of UV-B, which can be considered environmentally realistic, in a time-course experiment. We focused on analyzing physiological and chloroplast proteome responses, as well as exploring transgenerational cross-stress memory by using seedlings originated from plants grown in two different locations with different fertirrigation regimes (E subpopulation, seeds from non-stressed plants, and T subpopulation, seeds from stressed plants). These differences related to precipitations are available in Supplementary Table S1: while E subpopulation was irrigated from November to March, critical months in Chile to establish epigenetic memory associated to seed development in pine, T subpopulation was grown under natural conditions, facing with drought stress periods along these months. Furthermore, drought events in Tranguilvoro were promoted by more extreme temperatures (maximum and minimum) than Escuadrón, considering an additive stressful effect.

Applied UV-B dosage did not cause any accountable macromorphological damage to the plants, but stress affected photosynthetic activity as Fv/Fm decreased significantly along experiment (Fig. 1A; Supplementary Table S2_a). Photosynthetic pigment content was not significantly affected by this stress (Fig. 1B–D; Supplementary Table S2_b) and exhibited similar abundances than previously described in this species (Pascual et al., 2017; Valledor et al., 2012), suggesting the activation of photoprotective mechanisms leading to the decrease of Fv/Fm. Interestingly, chlorophyll a/b ratios (Fig. 1E; Supplementary Table S2_b) showed lower values at short-term (T2) than after 8 irradiation periods (T8), which was unexpected as chlorophyll b has been reported to be selectively reduced under UV stress (Marwood and Greenberg, 1996). The physiological role of the initial slight decrease of chlorophyll b may be signaling a stressful situation since chlorophylls can be seen as major components of stress biology (hormesis) (Agathokleous et al., 2020), whereas plants seem to achieve acclimation at long-term probably due to the fact that other stress defense mechanisms have been activated.

Despite the absence of significant changes in carotenoid content, pigments also involved in plant UV-B photoprotection (Emiliani et al., 2018; Middleton and Teramura, 1993), total phenolic compounds were progressively accumulated in needles in both subpopulations, showing faster significant changes in T subpopulation (T0 vs T2) than E (T0 vs T8) (Fig. 1F; Supplementary Table S2_b). Furthermore, T subpopulation also showed significant differences in flavonoids (Fig. 1G; Supplementary Table S2_b), which suggests a quicker adaptive capacity of subpopulation originated from stressed plants. The different types of accumulated phenolics (Agati et al., 2012; Schulz et al., 2021; Xu et al., 2021) have two physiological roles, acting as sunscreens for preventing photosynthetic damage (Barnes et al., 2008; Jansen et al., 1998) and scavenging ROS species to reduce oxidative damage, having flavonoids a main role under this stress (Landry et al., 1995; Li et al., 1993). As phenolics presented a similar tendency at short-term in both subpopulations, stressed-parents subpopulation might have another ROS-preventing mechanism to face stress, maybe acquired by transgenerational cross-stress memory (Barrios and Brown, 2014; Foyer et al., 2016; Locato et al., 2018; Pastori and Foyer, 2002), as increased lipid peroxidation was only observed at this time in E subpopulation (Fig. 1H; Supplementary Table S2_b), emphasizing the role of redox signals as key elements in cross-stress tolerance (Foyer et al., 2016). Conversely, free amino acids increased in T subpopulation at long-term (T8) (Fig. 1I; Supplementary Table S2_b), maybe as they are required for many biosynthetic pathways involved in stress response as phenylpropanoid metabolism required for increasing phenolic accumulation (Fu et al., 2021; Martínez-Luscher et al., 2014).

Primary metabolism is also highly sensitive to environmental stresses (Couee et al., 2006; Ho et al., 2001; Sami et al., 2016; Zhou et al., 2015b), releasing energy or sugars to reduce oxidative damage (Van den Ende and Valluru, 2009) and help mitigate the stress (Hilal et al., 2004; Thalmann and Santelia, 2017). Although non-significant differences were observed on starch determination (Fig. 1J; Supplementary Table S2_b), total soluble sugars were significantly accumulated at the beginning of the stress (T0.5) in E subpopulation (Fig. 1K; Supplementary Table S2_b), which may be linked to the oxidative stress observed at the same experimental point in this subpopulation (Fig. 1H; Supplementary Table S2_b). This result points out towards the enhanced production of sugars involved in cell homeostasis rather than sugars linked to the production of phenolics (Colina et al., 2020), also suggesting lower cell damage in T subpopulation as plants do not need to produce/release sugars to counter the stress.

Accordingly, this multiple physiological correlation through different cellular pathways suggests, a priori, a stronger, faster, and more coordinated response to diminish UV-B stress in T subpopulation (originated from stressed plants). In this context, these results showed not only the triggering of changes affecting the physiology of plants under UV-B stress but also differences between subpopulations probably related to priming effects. To go in depth in the comparison between subpopulations and connect physiological changes to potential molecular biomarkers used for a description of genotype's level of stress

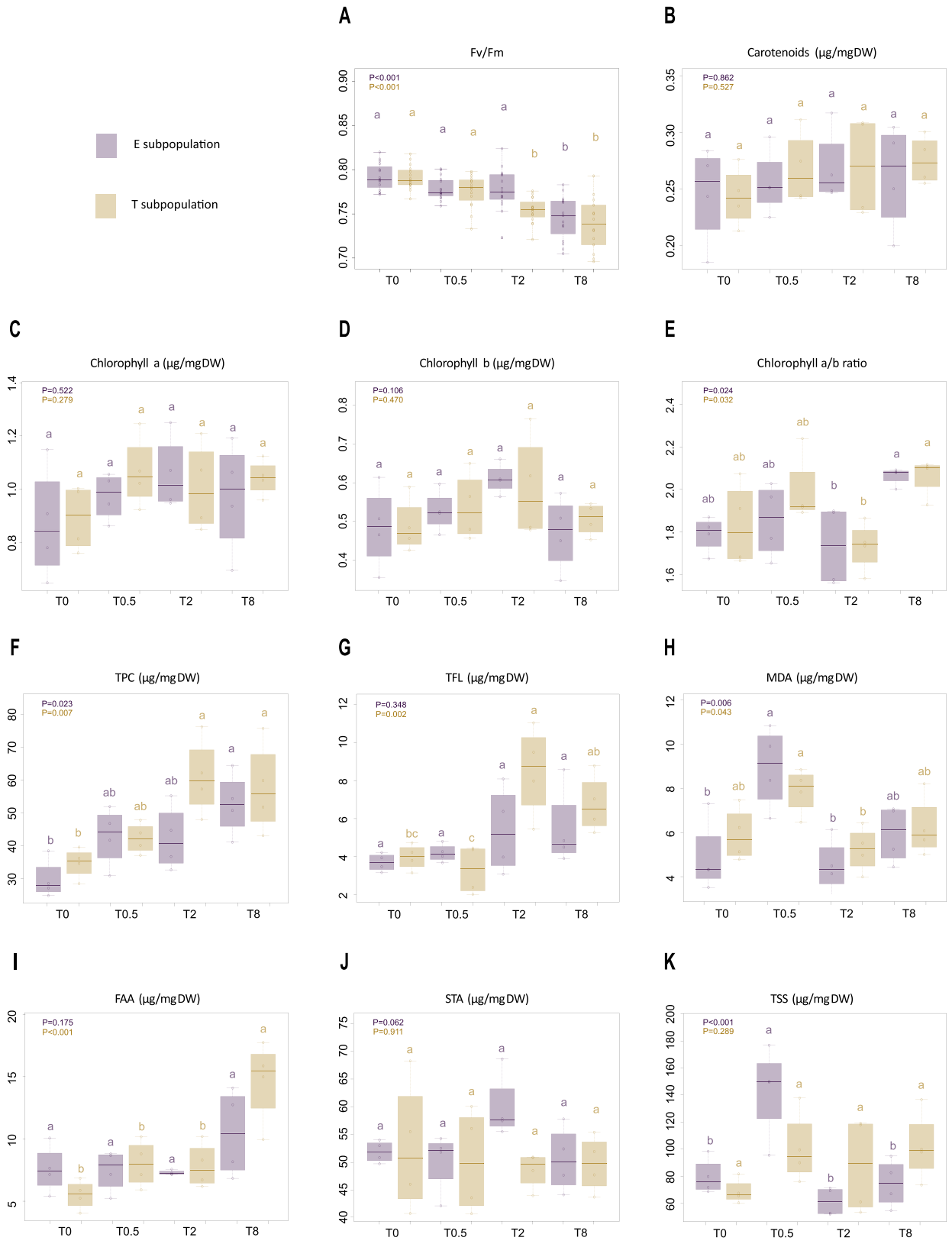

Fig. 1. Physiological measurements in needles of *P. radiata* seedlings subjected to UV-B stress. A) Maximal efficiency of photosystem II (Fv/Fm); **B)** Carotenoids (µg/mg DW); **C)** Chlorophyll a (µg/mg DW); **D)** Chlorophyll b (µg/mg DW); **E)** Chlorophyll a/b ratio; **F)** Total phenolic compounds (µg/mg DW); **G)** Total flavonoids (µg/mg DW); **H)** Malondialdehyde (µg/mg DW); **I)** Free amino acids (µg/mg DW); **J)** Starch (µg/mg DW); **K)** Total soluble sugars (µg/mg DW). Different letters indicate significant differences for each subpopulation across stress (purple and yellow for E and T subpopulations, respectively) as determined by ANOVA followed by Tukey HSD test (p-value < 0.05).

tolerance, chloroplast proteome has been explored, showing the dynamic behavior between both levels as well as connections between them.

3.2. Analyzed subpopulations showed differential dynamics of chloroplast proteome in response to UV-stress

Environment modulates chloroplast proteome through complex mechanisms (Dinh et al., 2019; Goulas et al., 2006; Schulz et al., 2021; Tamburino et al., 2017; Taylor et al., 2009). In this work, we employed untargeted proteomics to deepen our insights into how chloroplasts respond to UV stress and analyze the potential effects of transgenerational stress memory by comparing proteomes of E and T subpopulations.

A total of 3225 proteins (Supplementary Table S3) were initially identified employing public and in-house protein databases. Following a conservative approach, it was decided to filter out all proteins which were inconsistent and not predicted to be chloroplast located. To this end, six independent plant-specific subcellular location and annotation tools (Localizer v1.0.4, BUSCA, TargetP v2.0, YLoc, Mercator v3.6, sma3s) were employed, considering 1848 proteins as chloroplast proteins (Supplementary Fig. S2) (many of the lost proteins (around 800) would not exceed quality/reproducibility threshold). After data preprocessing and statistical analysis (ANOVA + TukeyHSD, $p < 0.05$), 1552 and 1515 unique chloroplast proteins in E (Supplementary Table S4_a) and T (Supplementary Table S4_b) subpopulation, respectively, exceeded criteria for their use in quantitative analyses, as well as 503 and 981 were defined differentially accumulated. Despite both subpopulations presented a similar number of chloroplast proteins, T subpopulation showed a stronger response than E subpopulation since it almost doubled the number of differentially accumulated proteins throughout stress (Supplementary Table S4).

Venn analyses revealed qualitative differences between subpopulations. Analyzing both subpopulation datasets independently

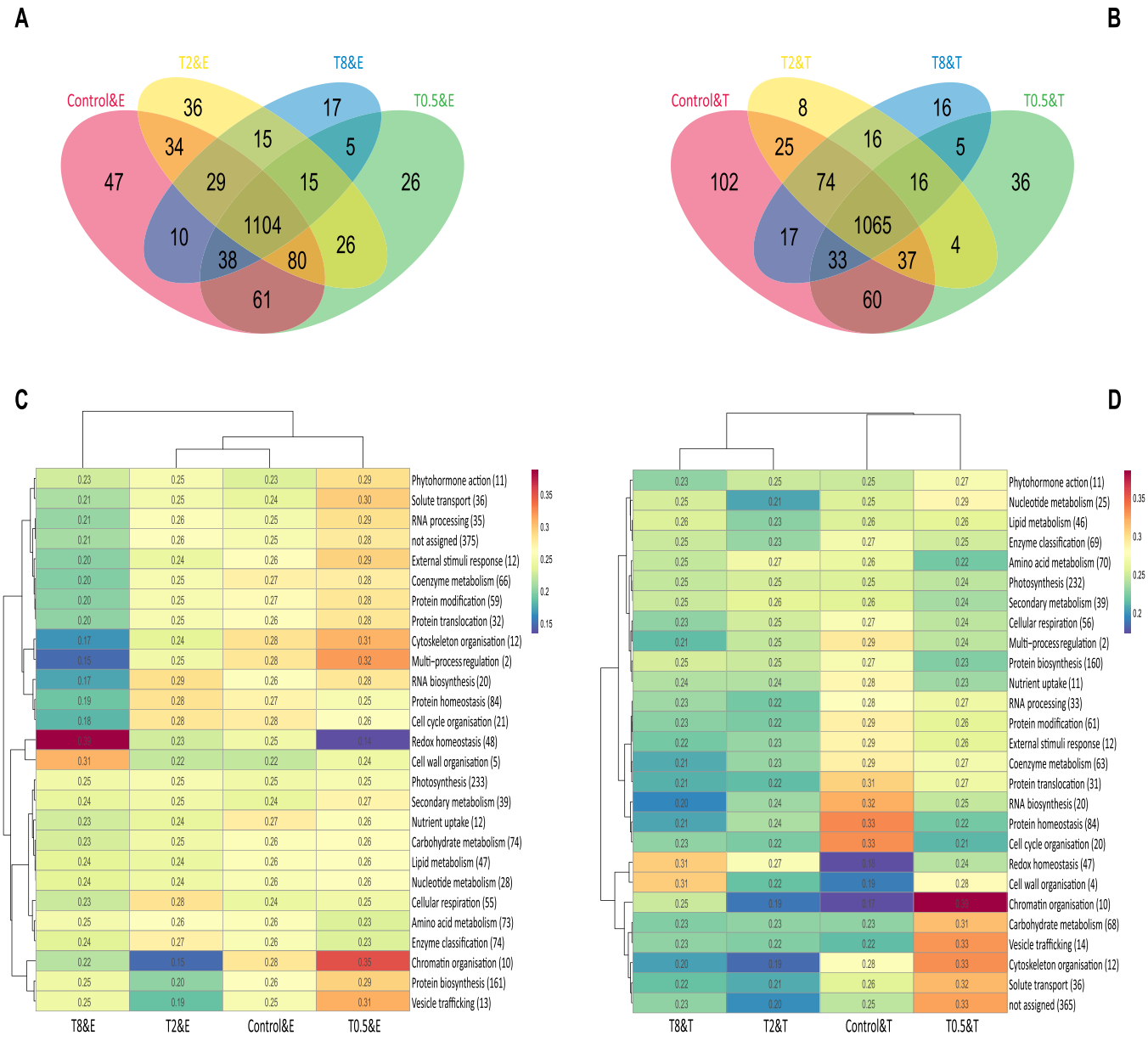

Fig. 2. Qualitative differences in chloroplast proteins among UV-B stress for E and T subpopulations (A and B, respectively) presented by Venn analyses, as well as heatmap clustering (C and D, respectively) using MapMan categorization pathways. The numbers indicate the scaled abundance according to the MapMan functional bin. Distances were established employing Manhattan distance and aggregated according to Ward`s method. The sampling times correspond to the experimental set-up shown in Supplementary Fig. S1.

(Fig. 2A, B; Supplementary Table S5_a, b), T subpopulation presented more characteristic proteins at control conditions than E (102 and 47, respectively), which may be related to priming effects. Contrary to E, proteome remodeling and lipid metabolism seems to play a key role in T subpopulation control state. Furthermore, the higher number of shared proteins in E subpopulation than T considering T0, T0.5, and T2 (80 and 37, respectively), as well as the number of shared proteins between short-term points-experiment (T0.5 vs T2) (26 and 4, respectively), pointed at a faster proteome remodeling related to UV-B stress in subpopulation originated from stressed plants, although both subpopulations seem to achieve acclimation at the end of the stress (17 and 16 characteristic proteins, respectively). All these hypotheses were supported by the analysis of both datasets comparing jointly shared proteins among experimental-points and subpopulations. Despite both populations shared 27 proteins before the stress (T0), T subpopulation presented 21 unique characteristic proteins, some of them related to redox (GLUTAREDOXIN, FERREDOXIN-NADP REDUCTASE), stress signaling (MLP-LIKE PROTEIN 423, CHAPERONE DNAJ C76, DNA-DAMAGE-REPAIR/TOLERATION DRT102), and lipid metabolism (GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE), contrary to E subpopulation (Supplementary Fig. S3; Supplementary Table S5_c). In this context, subpopulation originated from stressed plants (T) seems to be stress-prepared even at control conditions. For example, MLP423 protein positively regulates drought tolerance in *Nicotiana tabacum* (Liu et al., 2020), and T subpopulation comes from non-fertirrigated plants (Supplementary Table S1), so results suggest that its parents could have been capable to transfer this learning to the progeny, preparing T plants to rugged environments. Moreover, the abundance and location of FERREDOXIN-NADP REDUCTASE is involved in redox poise and stress tolerance (Kozuleva et al., 2016) and some stresses, such as freezing, induce a decline in phosphatidylglycerol in *A. thaliana* (Welti et al., 2002), also supporting previous hypothesis. Same dynamic was observed at short-term (T0.5), highlighting at this point the absence of 36 proteins in T subpopulation that appeared in the rest of treatments, which suggest its great short-term proteome specialization (Supplementary Fig. S3; Supplementary Table S5_c). HISTIDINOL DEHYDROGENASE, up-regulated in response to UV-B stress (Zimmermann et al., 1999), was also found at T0 in T subpopulation but not until T2 in E subpopulation. At this point (T2), E subpopulation also presented 23 unique characteristic proteins related to pathways which had been already found earlier in T subpopulation, such as Fe SUPEROXIDE DISMUTASE or heat shock proteins, suggesting a slower response to the stress. Additionally, 19 proteins related to all treatments were not found during T subpopulation acclimation (T8&T), including proteins linked to accelerate cell death (PAO), refolding aggregates (ClpB1) or DNA repair (DEAD/DEAH box helicase) (Supplementary Fig. S3; Supplementary Table S5_c). A more accurate and faster acclimation state in subpopulation originated from stressed plants (T) could dispense with these proteins, what along with previous results suggests that suffering cross-stress memory events could be not only linked to a faster but also a greater specialization.

Proteins were annotated according to Mapman in 26 functional bins, and quantitative pathway analyses showed subpopulation-specific proteome dynamics in response to UV stress (Fig. 2C, D). RNA biosynthesis and processing, protein homeostasis, modification and translocation, external stimuli response, coenzyme metabolism, multi-process regulation, and cell cycle organization showed a progressive reduction in T subpopulation along the stress, but only clear changes at long-term (T8) (acclimation) in E subpopulation. These results are in concordance with previous studies carried out using UV-B stressed and UV-B stress-recovered *P. radiata* seedlings (Pascual et al., 2017), and suggest faster and ongoing changes in subpopulation originated from stressed plants than non-stressed. Redox homeostasis category showed a divergent pattern between subpopulations. T subpopulation presented a quick increase in proteins related to redox homeostasis from the beginning of UV-B stress, which might be linked to the non-significant differences observed in MDA content in this subpopulation (Fig. 1H), whereas this increase was only present at the end of the stress in E subpopulation. ROS are key elements in plant stress signaling (Baxter et al., 2014; Oelze et al., 2008) and induce programmed cell death in plants (Petrov et al., 2015), which acts controlling the number of cells by eliminating damaged, old or unnecessary cells to maintain cellular homeostasis (Valandro et al., 2020). In this regard, results involve a better acclimation in T subpopulation, connecting this remark to the continuously decrease of proteins grouped in multi-process regulation category in this subpopulation and supporting ROS signals as common elements involved in cross-stress tolerance and memory (Barrios and Brown, 2014; Foyer et al., 2016; Locato et al., 2018; Pastori and Foyer, 2002). Surprisingly, heatmap clustering analysis did not show changes in secondary metabolism pathway, but many proteins related to this category were differentially accumulated (Supplementary Table S4) along the stress and between subpopulations (Fig. 1F, G): VIOLAXANTIN DE-EPOXIDASE presented its higher abundance at control conditions in T subpopulation and at short-term in E subpopulation; FARNESYL PYROPHOSPHATE SYNTHETASE increase its abundance since the beginning of the stress in T subpopulation, contrary to no changes observed in E; DIHYDROFLAVONOL-4-REDUCTASE presented a high increase during T subpopulation acclimation, whereas its abundance decrease significantly with the first impact of stress (T0.5) in subpopulation E. All these results suggest that subpopulation originated from stressed plants could be able to prevent oxidative stress damage in a more effective way (Fig. 1H).

Carbohydrate metabolism pathway did not present changes in E subpopulation, but an important increase at short-term (T0.5) followed by a high reduction (T2) was showed in T subpopulation. This result could be linked to two differentes responses. On the one hand, the secondary role of some sugars such as sucrose and glucose as osmolytes to maintain cell homeostasis (Gupta and Kaur, 2005), although this hypothesis would not be consistent with TSS content (Fig. 1K) since statistical significant changes were only observed in E subpopulation. On the other hand, the involvement of pentose phosphate pathway in oxidative stress control by producing NADPH (de Freitas-Silva et al., 2017), which could be related to MDA measurements (Fig. 1H).

3.3. Chloroplast proteomes revealed subpopulation specific responses to UV-stress

Proteins were optimally classified in 20 clusters according to their accumulation patterns employing a K-means approach (Fig. 3; Supplementary Table S6). This approach allowed to distinguish overall and inter-subpopulation protein dynamics. Results supported previous observations, as the number of proteins overaccumulated in T was higher, and its accumulation range faster and greater (clusters 9, 13, 17, and 19) than those proteins overaccumulated in E (clusters 10, 14, and 20). ROS-scavenging enzymes (ASCORBATE PEROXIDASE, DEHYDROASCORBATE REDUCTASE, X-TYPE THIOREDOXIN, GLUTATHIONE PEROXIDASE, several PEROXIREDOXINS) (Barrios and Brown, 2014; Dumanovic et al., 2021; Herbette et al., 2011; Locato et al., 2018; Pastori and Foyer, 2002), photosystems components, specially PsbD and PsbS proteins, and signaling and regulator-related proteins (SUPPRESSOR OF QUENCHING 1 (Bru et al., 2020), PsbP-like PROTEIN 1 (Ishihara et al., 2007), ACCLIMATION OF PHOTOSYNTHESIS TO THE ENVIRONMENT protein (Walters et al., 2003)), as well as lipid (Hou et al., 2016) (CYTOSOLIC NADP-DEPENDENT MALIC ENZYME, KETOACYL-ACP SYNTHASE II, ENOYL-ACP REDUCTASE) and photorespiration related proteins (Voss et al., 2013) (GLYCOLATE OXIDASE, SERINE HIDROXYMETHYL TRANSFERASE) were quickly overaccumulated in T subpopulation (clusters 9, 13, 17, and 19), pointing the importance of these mechanisms to overcome UV-B stress, also acting coordinately at nuclei, chloroplast, peroxisome, and mitochondria levels.

E subpopulation was, on the other hand, characterized by an increase

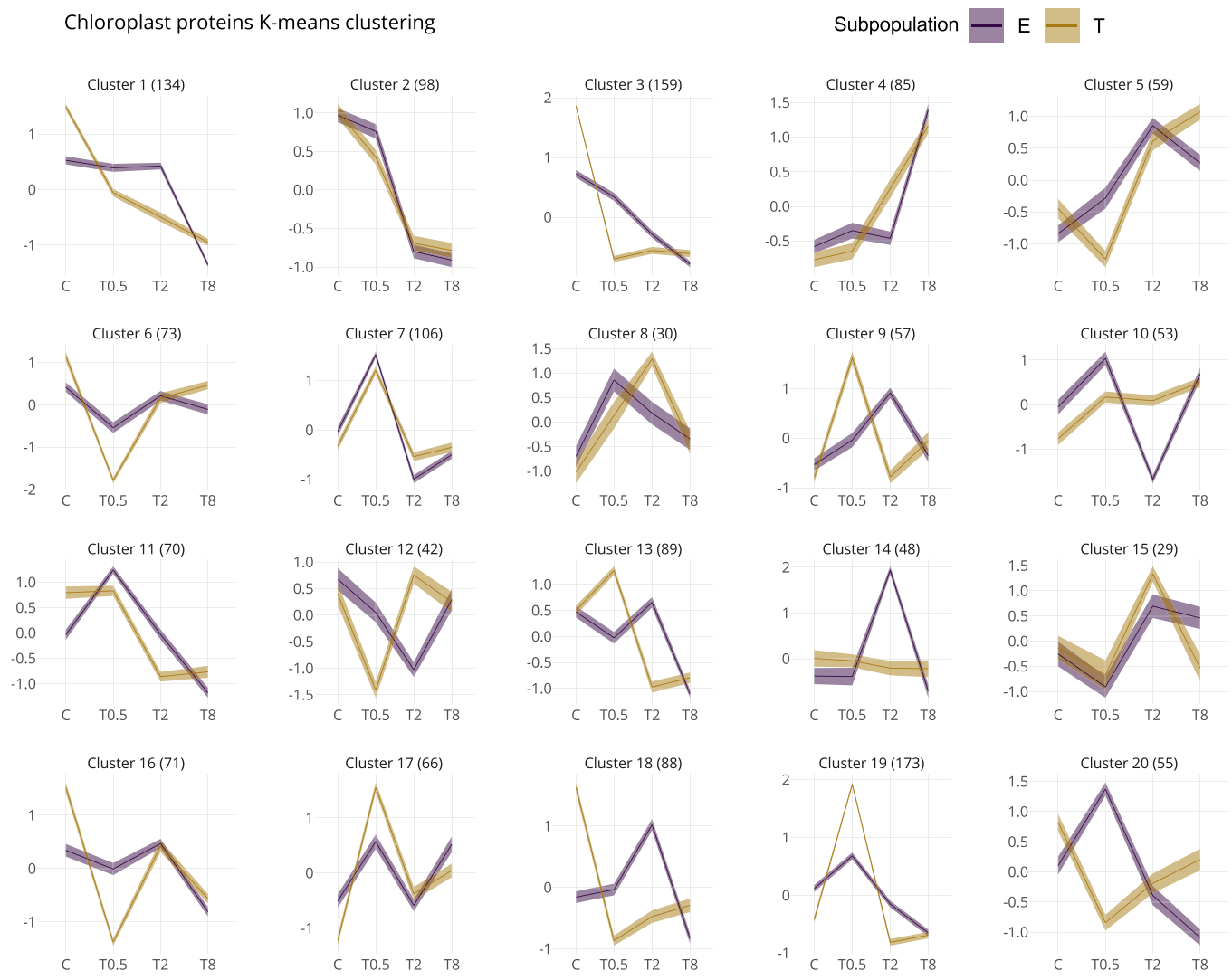

Fig. 3. K-means clustering comparing both subpopulations under UV stress. Graphic representation of the variables grouped in 20 clusters that were determined based on the different accumulation patterns showed during the UV-B experiment. The different clusters correspond to different quantitative trends showed in the study. Dashed lines show individual patterns and bold lines the mean for each cluster. The number of proteins included in each cluster are indicated in brackets. The sampling times correspond to the experimental set-up shown in Supplementary Fig. S1.

in ribosomal subunits, chloroplast and mitochondrial proteome-related remodeling proteins (CHAPERONES, PROTEIN FOLDING CATALYST, EF-G TRANSLATION ELONGATION FACTOR, PROTEIN DCL, PENTATRICOPEPTIDE REPEAT PROTEINS), and the activation of photooxidative stress responses (PROTEIN KINASE ABC1 (Jasinski et al., 2008; Yang et al., 2012), BETAINE ALDEHYDE DEHYDROGENASE 1 (Tang et al., 2014; Yang et al., 2015), CATALASE (Mishra et al., 1993), DEGRADATION OF PERIPLASMIC PROTEINS (Kapri-Pardes et al., 2007; Lucinski et al., 2011)) (Clusters 14, 18 and 20), being this behavior characteristic of a first stress exposure (Kosova et al., 2018; Lv et al., 2014; Pascual et al., 2017; Ytterberg et al., 2006). The expression of *A. thaliana DCL1* gene decreases extensively after drought treatment, and rice *DCL3* and *DCL4* reduce their expression significantly under drought or salt stresses (Liu et al., 2009), as well as *BETAINE ALDEHYDE DEHYDROGENASE* mRNA levels decrease when different abiotic stresses are attenuated in barley (Ishitani et al., 1995), indicating a faster response in T subpopulation one more time. Subpopulation T comes from non-fertirrigated plants (drought stress periods), supporting the hypothesis of transgenerational cross-stress memory. This faster response may be also appreciated in clusters 1, 3, and 11 grouping many proteins involved in the necessary proteome remodeling to achieve plant resilience such as LON protease (Li et al., 2010; Ostersetzer et al., 2007). Otherwise, finding proteins which have an essential function on modulating redox homeostasis and stress responsiveness suggest more oxidative damage in E subpopulation once again.

Otherwise, clusters 6 and 16 grouped those proteins quickly degraded only in T subpopulation, probably related to priming effects. These were mainly linked to lipid metabolism (PHOSPHOLIPASES A1 and D, UDP-SULFOQUINOVOSE SYNTHASE, STEAROYL-ACP DESATURASE) and secondary metabolism (ZETA-CAROTENE DESATURASE (ZDS) or 4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE REDUCTASE), highlighting their essential role in stress response modulation (Kato et al., 2019; Zhang et al., 2021), compared to cluster 10, which only presented changes in E subpopulation. Many ribosomal subunits proteins were grouped in this cluster, showing a similar pattern to the aforementioned cluster 14.

3.4. While subpopulation E noticed the stress, subpopulation T quickly activated stress-response mechanisms, demonstrating differential priming status of the seedlings

Principal component analysis (PCA) was employed to reduce the dimensionalities of the datasets, pointing to the most important proteins within the studied experimental system. For each subpopulation,

Principal Components 1 and 2 (PC1, PC2) explained more than 50 % of the total variance (Fig. 4; Supplementary Table S7_a, b). However, sample distribution was different comparing subpopulations.

E subpopulation was characterized by overlapped sample distribution considering the different treatments, suggesting progressive but slow changes in the tree's consequence of UV-B stress (Fig. 4A, Supplementary Table S7_a). PC1 (Fig. 4B) seems to be related to acclimation status, as distinguished T8 from other treatments. The proteins showing higher correlations to this PC were classified in many different pathways, pointing to a initial non-specialized response compared to T subpopulation, as discussed below. SHIKIMATE KINASE was one of the proteins most correlated to PC1, demonstrating the importance of phenolics in the protection against UV-B, according to physiological results (Fig. 1F, G). Photoprotection and redox related machinery was also gathered in this component, such as VIOLAXANTHIN DE-EPOXIDASE (Demmigadams and Adams, 1992), as well as other proteins involved in isoprenoid biosynthesis, photosystems related, and THIOREDOXINS. Interestingly, FARNESYL DIPHOSPHATE SYNTHASE showed negative correlation to this component despite its role in terpenoids biosynthesis, as other proteins associated with photosynthetic light and carbon reactions, sugars, and key enzymes of nitrogen metabolism, pointing the importance of these pathways for stress acclimation (T8 samples). PC2 (Fig. 4C; Supplementary Table S7_a) only seemed to distinguish the damage of T2 samples, highlighting proteins linked to photosynthesis (STN protein kinase (Bellafiore et al., 2005; Tikkanen and Aro, 2012)), redox (CATALASE (Mishra et al., 1993), NADPH-DEPENDENT ALKENAL/ONE OXIDOREDUCTASE (Yamauchi et al., 2012)) and protein folding (Hsc70, Hsp70, PROTEIN FOLDING CATALYST).

On the other hand, PCA showed different dynamics in T subpopulation (Fig. 4D; Supplementary Table S7_b). The clear separation between control (T0) and short-term response (T0.5), as well as the joining of T2 and T8 experimental points hinted at a faster response in this subpopulation. PC1 (Fig. 4E; Supplementary Table S7_b) gathered the variation related to initial stress response, distinguishing between control and T0.5. Proteins that showed high positive correlation to PC1, which were accumulated in T0.5, were classified mainly in three major activities: 1) reducing power and energy production (light reaction of photosynthesis (SUBUNIT GAMMA OF PERIPHERAL CF1 SUBCOMPLEX OF ATP SYNTHASE COMPLEX, COMPONENT LHCb4 OF LHC-II COMPLEX (de Bianchi et al., 2011), CHLOROPHYLL DEPHYTYLASE (Lin et al., 2016)) and sugar metabolism (GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE, FRUCTOSE-BISPHOSPATE ALDOLASE, CARBONIC ANHYDRASE)), 2) ROS scavenging (redox-dependent regulator (BolA4) of iron-sulfur cluster assembly machinery (Talib and Outten, 2021)), and 3) genetic reprogramming (Hsp20, Hsp60, ribosomal proteins, histone H4-like, CHAPERONE AKR2), suggesting that plants of T subpopulation, already primed by stressed parents, have a quicker and more focused and prepared response than E plants. PC2 (Fig. 4F; Supplementary Table S7_b) distinguished acclimated (T2, T8) and non-acclimated plants (T0, T0.5). Proteins related not only to RNA binding and active proteome remodeling, such as COMPONENT RPL7a OF LARGE RIBOSOMAL SUBUNIT PROTEOME and PAP6/FLN1 COFACTOR OF PLASTID-ENCODED RNA POLYMERASE, but also to photorespiration (CYTOSOLIC GLUTAMINE SYNTHETASE (Cai et al., 2009), METHYL-TETRAHYDROFOLATE-DEPENDENT METHIONINE SYNTHASE) and phenolic and lipid metabolism (5-ENOLPYRUVYL--SHIKIMATE 3-PHOSPHATE (EPSP) SYNTHASE, ACETYL-CoA C-ACYLTRANSFERASE) showed high loadings in this component.

It is known that stress affects cell available energy, which forces a remodeling of the central metabolic pathway to allow the survival with a minimum support to growth. The precise control of FRUCTOSE-BISPHOSPATE ALDOLASE plastid isoforms depends on the environmental and physiological circumstances (Pascual et al., 2017), including the redox state of cells, as well as the iron-sulfur cluster (and associated proteins such as BolA4) has been proposed as key element in fast environmental stimuli response (Talib and Outten, 2021), so having found them as relevant loadings at T0.5 in primed subpopulation (T) was not surprising. CARBONIC ANHYDRASE (CA) is important not only for photosynthesis and for several metabolic pathways, but also it is necessary under certain stress conditions (Polishchuk, 2021). The expression of a *CARBONIC ANHYDRASE* gene was induced by environmental stresses, for example, in *Oryza sativa* (Yu et al., 2007). Li et al. (2020) have also shown a slight increase in the total content of βCA1 with its relocation from chloroplasts to cytoplasm under drought stress in *Nicotiana benthamiana*, probably indicating its involvement in stress retrograde signaling. So, T subpopulation may have learnt this knowledge from previous exposures to stress (Supplementary Table S1) in order to avoid energy-limitations in the future that could compromise plant development, and maybe to control energy supply. Thus, photorespiration mechanism could be also essential in this subpopulation as key proteins of this process were found (CYTOSOLIC GLUTAMINE SYNTHETASE, METHYL-TETRAHYDROFOLATE-DEPENDENT METHIONINE SYNTHASE) (Fig. 4F; Supplementary Table S7_b) and overacummulated (Fig. 3, cluster 20; Supplementary Table S6). In this context, RuBisCO oxygenase activity seems to be favored, as previously reported (Pascual et al., 2017), because of glycolate accumulation during the early stages of UV stress. On the other hand, the xanthophyll cycle represents an essential photoprotection mechanism against oxidative stress generated by high-light intensity (Latowski et al., 2011), and the concentrations of the xanthophyll cycle pigments have been found to increase by some environmental stresses because of higher need of energy dissipation (Demmigadams and Adams, 1992). Since light-driven proton pumping at the thylakoid membrane (ATPase) was severe damage due to UV-B in non-primed subpopulation (Supplementary Table S4), E plants may have presented lumen acidification, which assists VIOLAXANTHIN DE-EPOXIDASE accumulation (Demmigadams and Adams, 1992) (Fig. 4B; Supplementary Table S7_b) to prevent damage along with ROS-scavenging enzymes. Dehydroascorbate has reported to be overaccumulated under UV-B stress in pine (Pascual et al., 2017).

3.5. *Interaction network revealed that unprimed responses to UV-stress observed in subpopulation E require protein biosynthesis and were possibly driven by lipid peroxidation and photoinhibition*

sPLS network was constructed considering proteomics as predictor matrix for physiology, revealing a small network directed towards preventing phototoxicity in E subpopulation (Fig. 5A; Supplementary Table S8_a). Increased oxidative damage, supporting the main role of ROS stress responses to acclimate for general abiotic stress (Barrios and Brown, 2014; Foyer et al., 2016; Locato et al., 2018; Pastori and Foyer, 2002) and monitored as lipid peroxidation, was correlated to an active chloroplast proteome remodeling, as induced the accumulation of a large number of ribosomal proteins, and also to increased vesicle traffic for importing nuclear proteins (PIP, VAMP7) (Fig. 5A). Changes were especially notable at short-term (Fig. 5B), supporting physiological results (Fig. 1H). Photoprotective changes at electron transport chain through STN protein kinases (Bellafiore et al., 2005; Tikkanen and Aro, 2012), reducing PSII activity by uncoupling antennas from PSII, as well as flavodiiron protein (FLV) (Wada et al., 2018), which protects PSI from photodamage efficiently sinking electrons through the photoreduction of O_2 to H_2O, were also positively correlated to MDA node (Fig. 5A). Reversibly phosphorylation of several PSII-LHCII proteins (Tikkanen and Aro, 2012) and PsbS- and xanthophyll cycle- dependent thermal dissipation of excitation energy (NPQ) (Steen et al., 2020) are the two major distinct mechanisms related to photosystem photoprotection. They are involved in light excitation energy redistribution leading migrations and reorganizations of the PSII-LHCII complexes along the thylakoid membrane, and their regulation has a redox-dependent component (Aro and Ohad, 2003; Chen et al., 2017; Hall et al., 2010). Thus, it is highly conceivable that both mechanisms function in great synchrony in order to together maintain the energetic balance of the

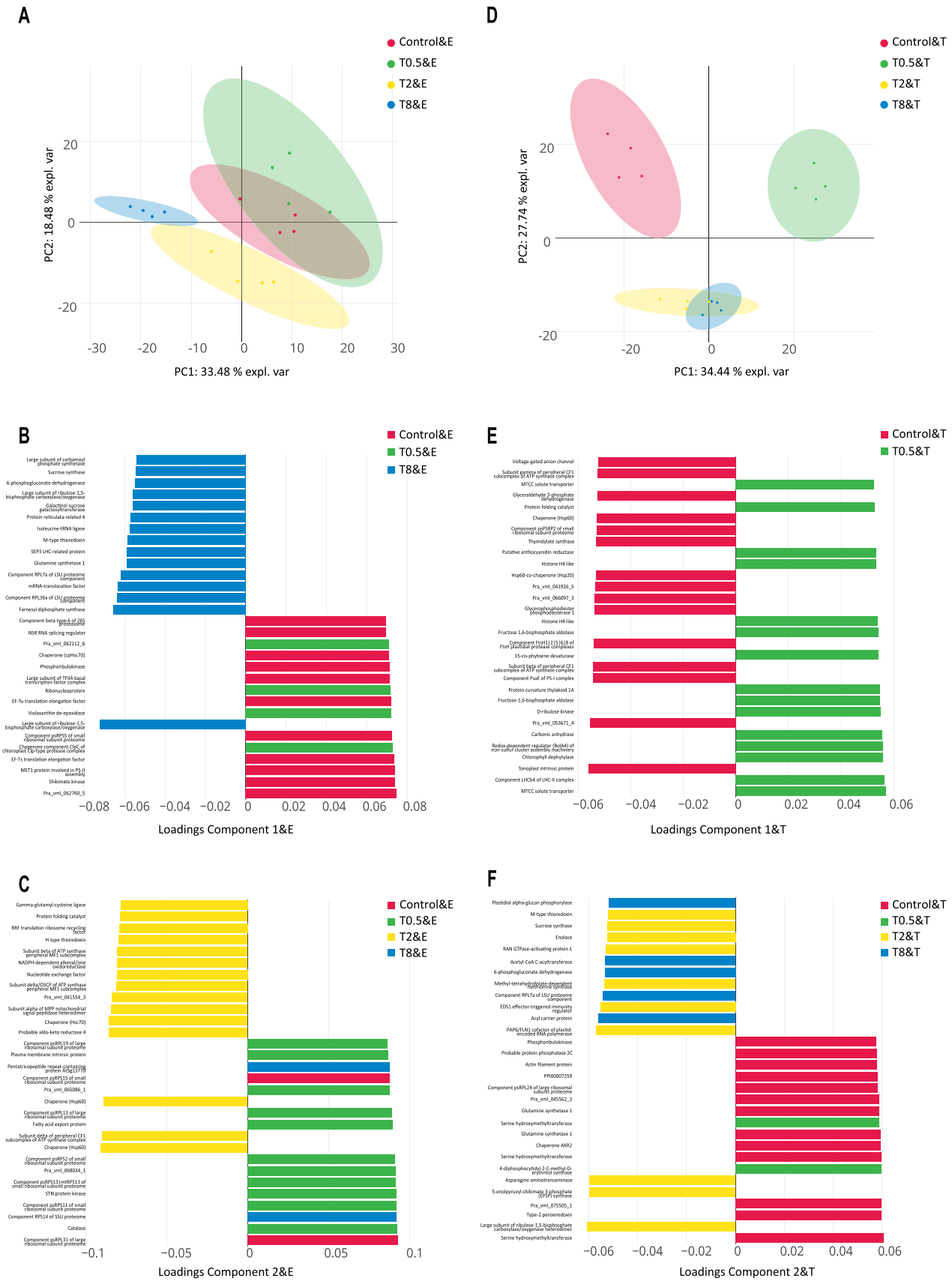

Fig. 4. Principal component analysis of chloroplast proteins of subpopulation E (A) and T (D), showing components 1 and 2 in horizontal and vertical axes, respectively. Loadings plots show the proteins with greatest correlation to components 1 (**B** and **E**) and 2 (**C** and **F**) for E and T subpopulation, respectively. The color of the protein loading bars represents the treatment with higher protein abundance. Less variable proteins were filtered out before PCA (ANOVA p-value < 0.01, 50 % above average IQR).

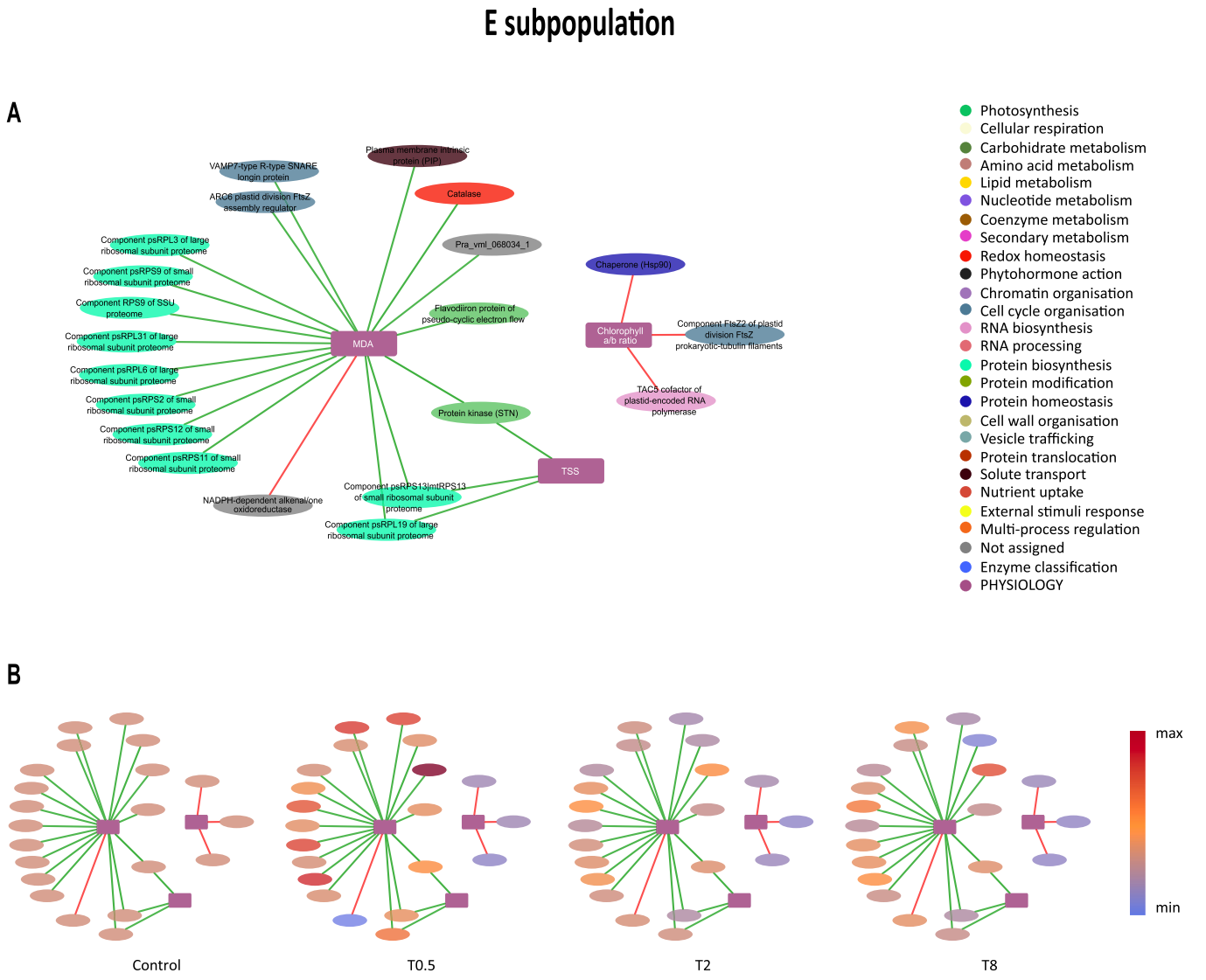

Fig. 5. sPLS-network using proteomics as predictive matrix for the changes observed in physiological measurements for E subpopulation. A) Node color indicates Mapman functional bin and shape indicate proteins (circle) or physiological measurements (square). Positive correlations are denoted in green, whereas red edges represent negative correlation values. Only those correlations equal or higher, in absolute value, than 0.7 are shown. **B)** Same representation in which node color indicates each protein abundance along four time-points experiment expressed by fold-change.

electron transfer reactions and prevent excess photodamage of PSII upon changing environmental cues, connecting to previous results (Fig. 4B). Interestingly, NADPH-DEPENDANT ALKENAL OXIDOREDUCTASE was negatively correlated to MDA, showing the lowest abundance at short-term (Fig. 5B), and thus explaining its role in preventing lipid peroxidation (Fig. 1H).

Chlorophyll a/b ratio was linked to chaperones and plastid RNA polymerase cofactors, also promoting proteome remodeling, and to CELL DIVISION PROTEIN FtsZ2, which is not only involved in chloroplast fission assembled and/or stabilized by ACCUMULATION AND REPLICATION OF CHLOROPLASTS PROTEIN 6 (ARC6) (Irieda and Shiomi, 2017; Sung et al., 2018) (positively correlated to oxidative stress, Fig. 5A), but also shows transcriptional changes due to different abiotic stresses (Li et al., 2021). Statistical differences between chlorophyll a/b ratios were observed in *ftsZ2* and *arc6 A. thaliana* mutants under light stress (Dutta et al., 2017), supporting the connection between both proteins and physiological nodes (MDA, chlorophyll a/b ratio).

3.6. Primed responses to UV-stress involved a quick rearrangement of secondary metabolism, apparently producing phenolics in order to reduce lipid peroxidation and maintain photosynthesis

Primed subpopulation showed a sPLS network that presented more functional categories than the previous one, suggesting a more organized and complex response to the stress (Fig. 6A; Supplementary Table S8_b). Furthermore, T subpopulation interaction network lacks the high number of ribosomal proteins exhibited by E subpopulation, maybe explaining why the former exhibited quicker physiological and proteome remodeling responses, also supporting the transgenerational memory effects from previous stresses (Molinier et al., 2006). Most relevant physiological parameters for explaining UV response were total phenolic compounds, total flavonoids, photosynthesis (Fv/Fm), and MDA. MDA was positively correlated to carbon fixation enzymes (FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE, RBCS), which increased their abundance at short-term (Fig. 6B), contrary to E subpopulation, in which MDA was correlated to electron flow. Moreover, the lower number of proteins related to redox activity in this network must be emphasized, which goes along with physiological results (Fig. 1H) and

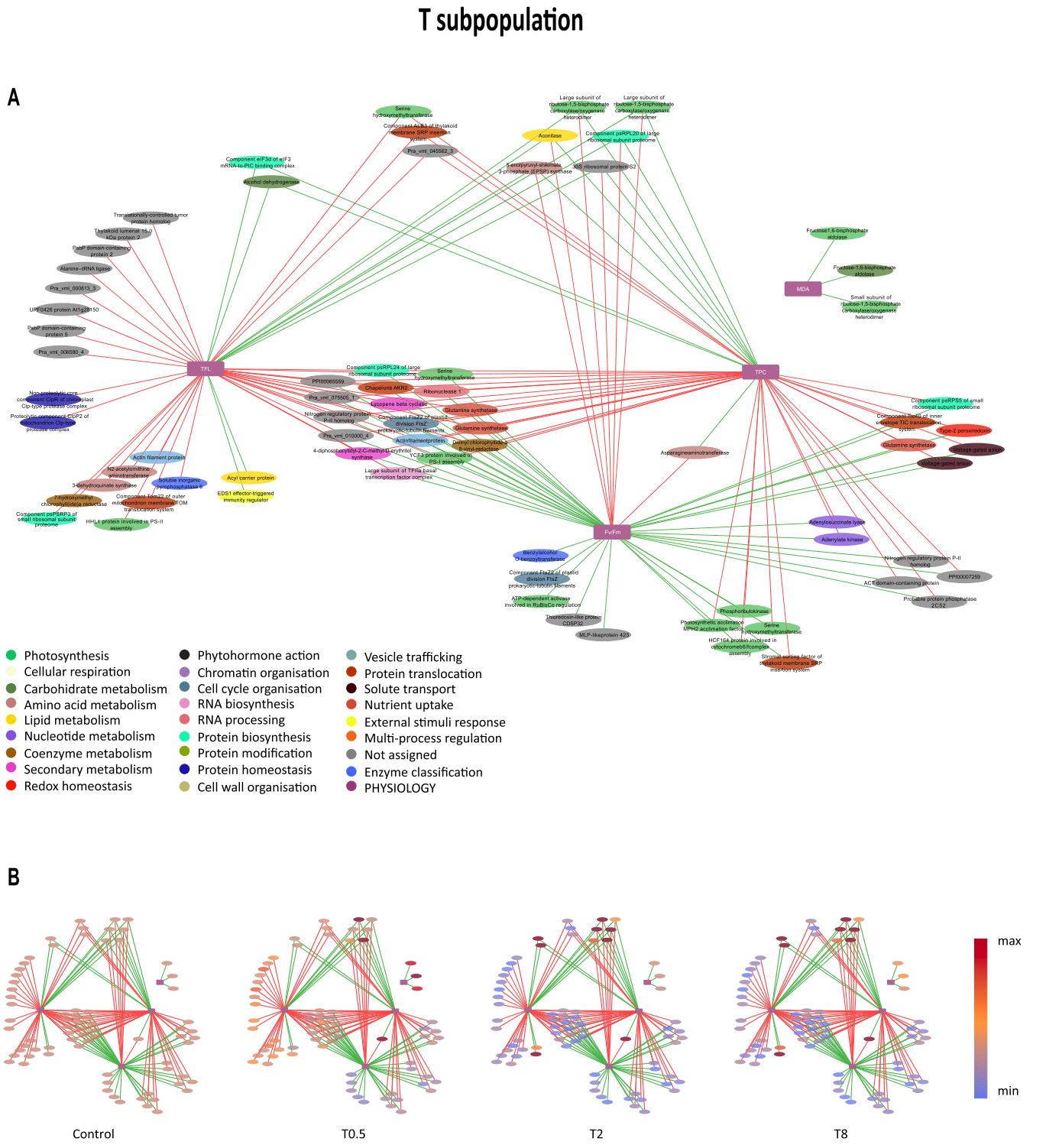

Fig. 6. sPLS-network using proteomics as predictive matrix for the changes observed in physiological measurements for T subpopulation. A) Node color indicates Mapman functional bin and shape indicate proteins (circle) or physiological measurements (square). Positive correlations are denoted in green, whereas red edges represent negative correlation values. Only those correlations equal or higher, in absolute value, than 0.7 are shown. **B)** Same representation in which node color indicates each protein abundance along four time-points experiment expressed by fold-change.

hints at a better plant status. Under UV stress, photosynthesis was negatively correlated to carbon fixation (RBCL), shikimate pathway (EPSP), and nitrogen transport (ASPARAGINE AMINOTRANSFERASE) (Fig. 6A). Prior studies in tobacco (Havir and McHale, 1988), barley (Murray et al., 1987), and *A. thaliana* (Zhang et al., 2013) have associated ASPARAGINE AMINOTRANSFERASE activity, increased since stress began (Fig. 6B), with the photorespiratory enzyme SERINE: GLYOXYLATE AMINOTRANSFERASE. In this context, and as K-means (Fig. 3; Supplementary Table S6) and PCA (Fig. 4; Supplementary Table S7) results showed previously, photorespiration seems to be essential for photosynthesis under these stress circumstances as many elements of this pathway were positively correlated (GLUTAMINE SYNTHETASE, SERINE HYDROXYMETHYLTRANSFERASE) (Fang et al., 2020; Moreno et al., 2005; Voss et al., 2013). This may also explain the

negative correlation between photosynthesis node and ACONITASE, allocating glyoxylate towards photorespiration rather than glyoxylate cycle, boosting the adjustment of redox homeostasis.

Moreover, photorespiration related proteins were negatively correlated to TPC and TFL (Fig. 6A), indicating that the accumulation of this compounds effectively reduce photorespiration, which also connect to the negative correlation between photosynthesis node and EPSP. Since both secondary metabolism (Nakabayashi et al., 2014) and photorespiration (Voss et al., 2013) are involved in preventing oxidative damage, a necessary understanding between them is essential. An increase in the level of expression of several genes of the isoflavonoid pathway was observed in response to active photorespiration to control it (García-Calderón et al., 2020), which was more noticeable in mutants lacking the plastid isoform of GLUTAMINE SYNTHETASE (GS2) (García-Calderón et al., 2015). The presence or absence of GS2 produce important differences in the different pathways for phenolics biosynthesis in *L. japonicus* in response to stress, supporting also the positive correlation shown between ASPARAGINE AMINOTRANSFERASE and TPC.

The relevant importance of phenolic compounds rather than other secondary metabolites such as terpenoids during stress signaling in primed-subpopulation was also emphasized by the negative correlation among secondary metabolism (TFL and TPC) nodes and LYCOPENE BETA CYCLASE and 4-DIPHOSPHOCYTIDYL-2-C-METHYL-D-ERYTHRITOL SYNTHASE proteins (Fig. 6A), both decreased at long-term (Fig. 6B). Interestingly, EDS1 EFFECTOR-TRIGGERED IMMUNITY REGULATOR (EDS1), which is required for rutin-primed plant defense (Yang et al., 2016) and tolerance (Szechynska-Hebda et al., 2016) and enhanced during acclimation response (Fig. 6B), was positively correlated to TFL, pointing the role of these molecules in channeling and transducing redox signals. Due to the similarity of its N-terminal portion to the catalytic site of lipases, EDS1 has also been involved in the release of polyunsaturated fatty acids (Ochsenbein et al., 2006) during oxidative stress in *A. thaliana*, emphasizing a lipid-mediated signaling during abiotic stress that support the positive correlation also presented by ACYL CARRIER PROTEIN and ACONITASE, both overaccumulated at long-term (Fig. 6B). Furthermore, combined loss of EDS1 and actin cytoskeletal activity severely compromise resistance against fungal stress in wheat (Yun et al., 2003), result that may be extended to abiotic stress since ACTIN FILAMENT protein also correlated to physiological nodes.

4. Conclusion

In summary, we employed physiological measurements and proteomics to study the behavior under UV-stress of seedlings produced by water and nutrient stressed or non-stressed plants in order to elucidate transgenerational cross-stress priming effects. Interestingly, apart from demonstrating that the growing conditions of the parental plants have a transgenerational priming effect against stress, we found noticeable differences in response to UV-B stress between seedlings, finding that subpopulation originated from stressed plants (T) responded quicker and more efficiently than those originated from unstressed (E). While E subpopulation needed to build stress response mechanisms almost from scratch (interaction networks showed the link between lipid peroxidation and ribosomal proteins), T subpopulation seemed to start to fight against stress synthetizing quickly phenolic compounds, replacing damaged lipids, reducing photooxidative damage, and protecting the cell against free radicals increasing redox related machinery. These results not only provided a significant advance in the knowledge of the UV-B biological response in conifers, but also demonstrate that the management of seed orchards is a relevant biotechnological aspect that must be considered by breeders in order to produce more tolerant plants to future stresses in only one generation and keeping existing genotypes.

Data availability statement

The data that supports the findings of this study are available in the Supplementary material of this article. The mass spectrometry proteomics data including RAW and MSF files have been deposited to the ProteomeXchange Consortium via the PRIDE (Pérez-Riverol et al., 2022) partner repository with the data set identifier PXD031383.

CRediT authorship contribution statement

Mónica Meijón (M.M.), María Jesús Cañal (M.J.C.), and Luis Valledor (L.V.) conceived and designed the research. Lara García-Campa (L.G-C.), Sara Guerrero (S.G.), and Laura Lamelas (L.L.) performed the experiments. L.G-C., L.L., and L.V. analyzed the data and performed statistical analysis. Rodrigo Hasbún (R.H.) contributed field materials. L.G-C. and L.V. wrote the manuscript draft. All authors corrected the initial draft and participated in the redaction of the final version of this article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

We uploaded our dataset as e-component. RAW and MSF files are available in public repositories (see details at Data Availability Statement).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.105009.

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