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Nucleus and chloroplast: A necessary understanding to overcome heat stress in *Pinus radiata*

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Abstract

The recovery and maintenance of plant homeostasis under stressful environments are complex processes involving organelle crosstalk for a coordinated cellular response. Here, we revealed through nuclear and chloroplast subcellular proteomics, biochemical cell profiles and targeted transcriptomics how chloroplasts and nuclei developed their responses under increased temperatures in a long-lived species (*Pinus radiata*). Parallel to photosynthetic impairment and reactive oxygen species production in the chloroplast, a DNA damage response was triggered in the nucleus followed by an altered chromatin conformation. In addition, in the nuclei, we found several proteins, such as HEMERA or WHIRLY, which change their locations from the chloroplasts to the nuclei carrying the stress message. Additionally, our data showed a deep rearrangement of RNA metabolism in both organelles, revealing microRNAs and AGO1 as potential regulators of the acclimation mechanisms. Altogether, our study highlights the synchronisation among the different stages required for thermotolerance acquisition in *P. radiata*, pointing out the role of chromatin conformation and posttranscriptional gene regulation in overcoming heat stress and assuring plant survival for the following years.

KEYWORDS

abiotic stress, microRNA, omics, stress sensing, stress signalling, subcellular proteomics

1 | INTRODUCTION

Human-induced climate change represents a fundamental challenge for vegetation dynamics, being a concern for crop productivity and the sustainability of some ecosystems. Moreover, climate models predict average temperatures to rise and heat waves to be increasingly frequent (Lesk et al., 2016; O'Neill et al., 2017). Fortunately, as sessile organisms, plants have evolved sophisticated molecular mechanisms to perceive and cope with environmental stresses like high temperatures and even "learn" from them (Bäurle, 2016; Lamelas, Valledor, et al., 2020; Ling et al., 2018). This molecular-based memory represents an essential strategy for plants, especially for those species with long life cycles, such as forest trees; 'with age comes wisdom'. Despite the importance of high temperatures as one of the key stressors of forest ecosystems, the cellular mechanisms leading to its perception, signalling, and molecular memory acquisition are still poorly understood.

Stress acclimation mechanisms rely on the precise coordination between nuclei and endosymbiotic organelles (Pfannschmidt et al., 2020). While the nucleus is the main regulatory hub of the cell; the chloroplast is the plant-cell defining organelle, which houses photosynthesis and the biosynthesis of secondary metabolite precursors (Dobrogojski et al., 2020). Its genome codes around 100 proteins (Daniell et al., 2016). Nevertheless, thousands of genes now encoded in the nuclear genome were transferred from the chloroplast genome during evolution (Dobrogojski et al., 2020). The proteins coded by these genes are still required to be imported into the chloroplast for its proper development in a process called anterograde regulation (Unal et al., 2020).

On the other hand, the chloroplast can regulate nuclear gene expression via organelle-to-nucleus retrograde signalling (Jung & Chory, 2010). Although the coordinated expression of chloroplast and nuclear genes regulated by retrograde signalling is indispensable for plant growth and development, how the organelle-to-nucleus communication takes place is largely unknown (Zhao et al., 2020). Recent studies point out that chloroplasts act as thermosensors or thermal alarms (Dickinson et al., 2018; Zhao et al., 2020). Although photosynthesis is damaged and photorespiration increases (Hu et al., 2020), reactive oxygen species (ROS) and other signals including carotenoid derivatives such as tocopherols, isoprenoid precursors, phospho-nucleotides and heme are released into the cytoplasm (Zhao et al., 2019, 2020). These signals travel to the nucleus where the main cell response can be triggered by, among others, alterations in nuclear gene expression. This coordination is essential to attempt to reach the stress-adapted cell homeostasis required for overcoming the stress.

In recent years, it has been reported that chloroplast retrograde signalling could involve the regulation of microRNA biogenesis in *Arabidopsis thaliana* (Fang et al., 2019; Lin et al., 2018; Ravichandran et al., 2019; Świda-Barteczka et al., 2018). However, the link between both processes is quite little-known, and even less so in long-lived species such as forest trees. MicroRNAs have been also established as key players in vital cellular aspects through microRNA-guided posttranscriptional gene silencing (PTGS); however, it is still unclear, especially for woody plants, how this molecular process reprograms gene expression. Few studies have claimed the relevance of PTGS in the heat stress response in other plant species, mainly through alterations in antioxidant activity and redox homeostasis (Fang et al., 2019; Guerra et al., 2015; Ravichandran et al., 2019), although it remains unknown whether this process is related to damage, signalling, or acclimation.

To get a deeper understanding of these signals and how these processes are synchronised we accomplished two untargeted subcellular proteomics studies (nuclear and chloroplast, independently) in a time-course experiment with high temperature stressed *Pinus radiata* plants. Furthermore, the relative accumulation of microRNAs was analyzed in parallel to targeted transcriptomics analysis to elucidate the role of microRNAs in retrograde signalling.

2 | MATERIAL AND METHODS

2.1 | Plant material and stress treatment

Six-month-old *P. radiata* D. Don seedlings (height 22 ± 4 cm) were kept in 1-dm^3 pots inside a climate chamber (Fitoclima 1200; Aralab) under a 16-h photoperiod (400 μmol m^{-2} s^{-1}) at 25°C and 50% relative humidity (RH) as well as an 8-h night period at 15°C and 60% RH. The plants were watered with nutritive solution (5:8:10 N:P:K).

Heat exposure began each day of the stress treatment with an increasing temperature gradient from 15°C to 45°C for 5 h, and then the temperature was maintained at 45°C for 6 h. During the following 5 h, the temperature was returned to 15°C and maintained for 8 h, thus mimicking a day–night scenario. Control plants (C) were sampled, and the stress exposure began the following day. Plant material was sampled at the end of the 6 h heat exposure on Day 1 (T1), Day 3 (T3) and Day 5 (T5), as indicated in Figure S1. Needles were frozen in liquid nitrogen immediately after sampling and stored at −80°C for further analysis. Plants were divided into four pools, constituting of needles of three plants each. These pools were kept across the experiment and formed the four independent biological replicates that were analyzed.

2.2 | Physiological parameters and biomarkers quantification

The percentage of electrolyte leakage (EL) was used to determine cell membrane damage in the control and the heat-stressed plants (C, T1, T3 and T5). Needles (70 mg) of each biological replicate were collected at each sampling point and processed according to Lamelas, Valledor, et al. (2020). EL was determined as the ratio between the conductivity of water containing needle chunks after an 18 h incubation period and after autoclaving for 20 min at 120°C (sensION + MM150 portable meter, Hach). Chlorophyll fluorescence measurements were taken just before sampling using a pulse-amplitude modulation fluorimeter (OS1-FL; Opti-Sciences, Hudson). Biomarker quantification including chlorophyll a, chlorophyll b, carotenoids, malondialdehyde, free amino acids (FAAs), total soluble sugars, starch, total flavonoids and total phenolic compounds were performed according to López-Hidalgo et al. (2021), starting from 10 mg of lyophilised needles.

2.3 | Proteomics analysis

Nuclear and chloroplast fractions for proteomics analyses were purified as described in (Lamelas, García, et al., 2020). In brief, samples were homogenised, incubated in their respective cellular lysis buffers and then filtered. Organelle enrichment was achieved with sucrose and sucrose-Percoll discontinuous gradients for intact nucleus and chloroplast isolation. Protein extraction was performed following phenol-sodium dodecyl sulfate (SDS) protocol (Valledor &

Weckwerth, 2014). As protein samples were dissolved with the detergent SDS, in-gel digestion was performed using trypsin (Roche, cat. no. 03 708 969 001) according to the manufacturer's indications. Peptides were extracted and desalted as previously described (Valledor & Weckwerth, 2014). The peptides were analyzed using a 1D nano-flow LC coupled to an MS/MS Orbitrap Fusion spectrometer (ThermoFisher Scientific), using a 60-min gradient starting with 0.1% formic acid and with 80% acetonitrile as the mobile phase.

Protein identification was performed with Proteome Discoverer v2.2 (ThermoFisher). A combined database was compiled with three protein databases and used for protein identification, including the *Pinus taeda* genome v.1.01 (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/), UniProt/SwissProt Viridiplantae and an in-house *P. radiata* transcriptome. The mass spectrometry proteomics data including RAW, msf and pepXML files have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the data set identifier PXD029114.

Identified protein sequences were blasted using the following in silico localization tools: BUSCA (Savojardo et al., 2018), Localizer (Sperschneider et al., 2017), YLoc (Briesemeister et al., 2010), TargetP (v2.0) (Almagro Armenteros et al., 2019), NucPred (Brameier et al., 2007), NLStradamus (Nguyen Ba et al., 2009), NLSdb (Nair et al., 2003) and Plant TFDB (v4.0) (Jin et al., 2017). Then, proteins were annotated with sma3s (Casimiro-Soriguer et al., 2017) and Mercator MapMan (Lohse et al., 2014) tools.

Finally, we addressed contamination issues by dropping for downstream analyses those proteins with less than two matches for their cellular organelle considering in silico localization tools or with no positive subcellular location in the annotation according to Mercator Mapman or sma3s.

2.4 | Statistical analyses

All statistical analyses were performed in R (v 4.0.2) (R Core Team, 2020); proteomics datasets were analyzed using pRocessomics R package, (available at htttps://github.com/Valledor/pRocessomics), to perform data preprocessing, univariate (Venn) and multivariate analyses (principal component analysis [PCA], kmeans, sparse partial least squares [sPLS]). Self-organising maps (SOM) were built using kohonen R package (Wehrens & Buydens, 2007); and T-distributed stochastic neighbour embedding (t-SNE) was calculated with Rtsne package (van der Maaten & Hinton, 2008).

In brief, each proteomics data set was preprocessed independently, keeping those proteins that were present in at least 15% of the samples or in all the replicas that constituted a treatment; missing values were imputed using the Random Forest algorithm. After data preprocessing, univariate analyses were performed and then both datasets were *z* scaled for multivariate analysis.

Correlation-based networks were inferred using sPLS multivariate analysis, independently for each cellular compartment, by splitting each data set in nuclear- or chloroplast-encoded proteins and gathering the correlations between both groups of proteins, using a cut-off value of 0.7, networks were depicted using Cytoscape (v3.7.2) tool (Shannon et al., 2003), following the recommendations of Escandón et al. (2020).

2.5 | (Micro)RNA extraction, quantification, cDNA synthesis and quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

A set of common microRNA sequences previously identified in *Pinus pinaster* (Rodrigues et al., 2019), were blasted in miRbase (Release 22.1) (Kozomara et al., 2019). Those sequences conserved in other tree species were kept and blasted against the transcripts corresponding to the proteins in this study, which were found to be significantly accumulated when comparing control to stress conditions using psRNATarget tool (Dai et al., 2018).

The (micro)RNA extraction was performed according to Valledor, Escandón, et al. (2014), with minor modifications to enrich microRNA concentration: absolute ethanol was added to RNA-containing supernatant and then passed through the silica columns. RNA and microRNA were quantified using Qubit Assay Kits (Thermo Scientific, RNA cat. no. Q33223; miRNA cat. no. Q32880), and a total amount of 500 ng was retro-transcribed using mi-X-RNA kit (Takara, cat. no. 638315).

The qPCR reactions were performed in a CFX Connect Real-Time PCR machine (Bio-Rad) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), using three biological and three analytical replicates. Normalized relative quantities (NRQ) and standard errors of RQ were determined according to Hellemans et al. (2007). Expression levels of ACTIN (ACT) and glyceraldehyde-3-phosphate dehydrogenase were used as endogenous controls for mRNA quantification as described in previous studies with the same experimental system (Lamelas, Valledor, et al., 2020) and U6 snRNA expression levels for microRNA quantification as recommended in mi-X-RNA kit (Takara, cat. no. 638315). Detailed information about the primers used for RT-qPCR experiments is available in Table S1.

3 | RESULTS

3.1 | Impaired photosynthesis and proteomics rearrangement as stress indicators

We mimicked a 5-day heatwave in a time series experiment to elucidate the coordinated response in nuclei and chloroplasts, mainly through their proteomes. With this experimental design, we aimed to track the crosstalk between these two organelles from both 'points of view'. In addition, we characterized the physiological profile according to well-known biomarkers (López-Hidalgo et al., 2021), as well as immediate measurements of photosynthesis and EL.

The applied heat stress had a strong physiological impact on the seedlings as shown in Figure 1a. Most of the measured biomarkers

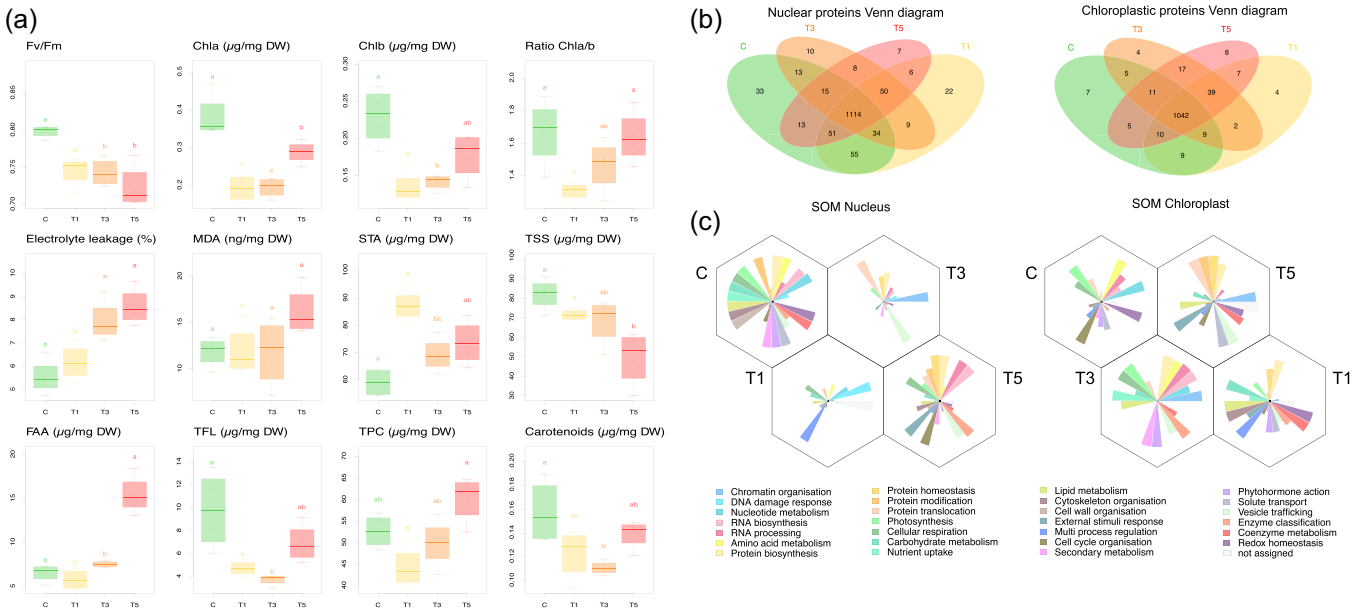

FIGURE 1 Physiological profile and proteomics exploratory analysis. (a) F_v/F_m, chlorophyll a, chlorophyll b, ratio Chla/Chlb, electrolyte leakage, malondialdehyde (MDA), starch (STA), total soluble sugars (TSS), Free amino acids (FAA), total flavonoids (TFL), total phenolic compounds (TPC), and carotenoids content in control conditions (C), after 1 day of stress (T1), 3 days of stress (T3) and 5 days of stress (T5). Different letters indicate significant differences according to HSD test ($p < 0.05$, dots represent mean values of four independent biological replicas and three technical replicas each). (b) Venn diagram representing the overlap of nuclear and chloroplast proteomes across the experiment. (c) Self-organizing map (SOM) of nuclear and chloroplast proteomes. Pie charts inside the cells indicate the relevance of protein functional categories according to Mercator MapMan. Letters close to each cell indicate the experimental conditions of the samples inside that cell. (a–c) All procedures were done with four biologically independent replicates

changed among the sampling points ($p < 0.05$, honestly significant difference [HSD] test). F_v/F_m decreased since the first stress treatment, and the different values of carotenoids, chlorophyll a, b, and their ratio demonstrated an initial heat-induced impairment of photosynthetic function, which started to recover after 5 days of stress (T5). Heat stress did not significantly affect membrane lipid peroxidation (MDA content), although we observed an increased abundance in T5. Membrane permeability, evaluated by E. L., was increased under heat stress.

Regarding the carbohydrate metabolism, an accumulation of starch was observed in the first shock along with a sugar content stabilization followed by their consumption during the stress. The secondary metabolism was characterized by phenolic compound (TPC) and flavonoid (TFL) contents, both well-known antioxidants. The TPC and TFL diminished under the first stress shock and then underwent a basal status at T5. In the case of phenolics, higher levels were found when compared to the control. FAA content drastically increased at T5, revealing deep changes in protein metabolism dynamics after several days of stress.

To characterize the molecular mechanisms driving this response, we accomplished a systems biology analysis combining two subcellular bottom-up proteomics assays. Spectra were identified employing a new *P. radiata* database built after heat-stress RNA-Seq reads (Escandón et al., 2021, under review) allowing a deep characterization of chloroplast proteome with 1182 quantifiable proteins. This database was also employed for reanalysing available nuclei spectra (Lamelas, Valledor, et al., 2020), enhancing previous protein identification in nucleus proteome, obtaining 1451 quantifiable proteins (Table S2). Qualitative proteome distributions across the time points are shown in the Venn diagram (Figure 1b), showing subtle but consistent differences across both organelles.

The biological meaning of proteome quantitative distribution was further studied with SOM. The SOMs of both organelles clustered the biological replicates according to sampling points in the same cells (Figure 1c). The nuclear proteome was remodelled after the first stress exposure, with an increased DNA damage response and multiprocess regulation MapMan categories. Following the bibliography, as the stress time increased, the nuclear proteome dynamics turned to increased chromatin organisation (Lamelas, Valledor, et al., 2020), protein translocation (Krause et al., 2012; Li et al., 2017) and vesicle trafficking (Wang et al., 2020) categories. Finally, after 5 days under stress, more biological processes became relevant, especially those related to RNA and proteins. Meanwhile, in the chloroplast, SOM clusters corresponding to Control and T1 were in nonadjacent cells, indicating a drastic initial response, mainly described by a photosynthesis-related proteins depletion in T1. The multiprocess regulation category was firstly triggered by the stress along with coenzyme and lipid metabolism, which was maintained in T3 when secondary metabolism and RNA clusters became more abundant until T5, where protein transport and trafficking classifications were the most relevant.

3.2 | Chloroplast proteome response stopped evolving at midterm heat exposure, while nuclear proteome kept changing

After exploring the main mechanisms leading the heat stress response in both organelles, we were interested in identifying those concrete protein candidates that were the most representative of each stress stage. To this end, we performed multivariate analysis over the proteome datasets, including PCA and t-SNE.

PCA allowed the determination of the main sources of variation among our sampling points in both proteomic datasets and their combination. In each case (Figure 2, Table S3), the sum of Principal Components 1 and 2 (PC1, PC2) explained more than 45% of the variance. In addition, in all analyses, PC1 separated control from stressed plants, while PC2 highlighted different processes depending on the organelle. Nuclear proteome PC2 gathered the variance related to stress exposure (Figure 2a), whereas chloroplast and combined proteomes separated the first stress shock (Figure 2b,c) from

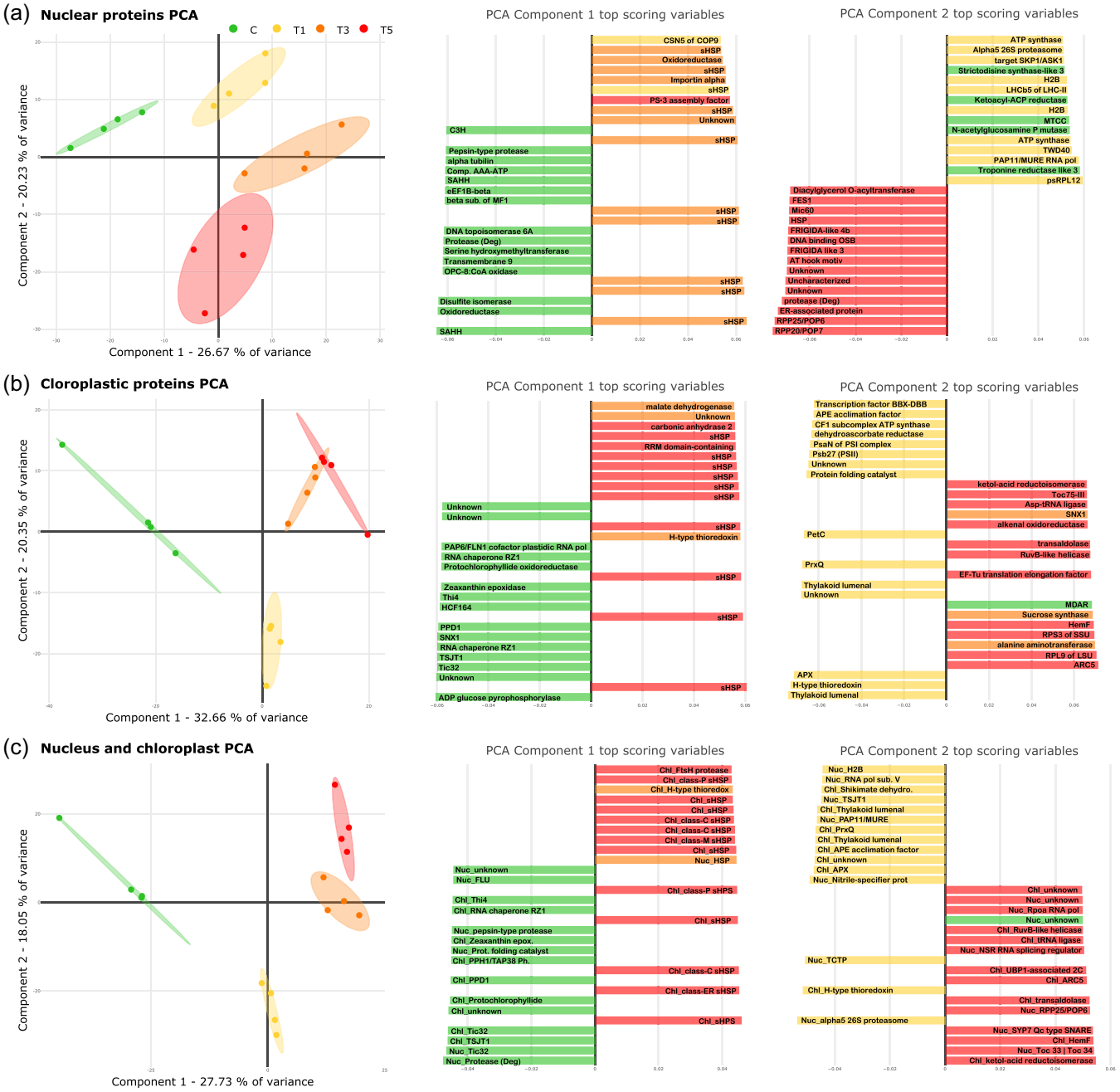

FIGURE 2 PCA Score plot and PC1 and PC2 top-ranked proteins. (a) Nuclear proteome, (b) chloroplast proteome and (c) their combination. (a–c) Top 30 scoring loadings (15 highest and 15 lowest) of PC1 and PC2 are shown by row for each PCA, bar colours indicate the experimental condition in which each top-scoring protein is more accumulated. Ellipses show a 75% confidence interval. Different colours indicate different experimental conditions ($n = 4$ biologically independent replicates) [Color figure can be viewed at wileyonlinelibrary.com]

the other two sampling points. In addition, chloroplast proteome PCA (Figure 2b) was unable to differentiate between T3 and T5 samples. This indicated that, in terms of proteome variation, chloroplasts reached in T3 a stationary state, which was maintained in T5. To further check sample distribution, we performed a t-SNE analysis (Figure S2), known to be insensitive to data collinearity and overfitting. t-SNE corroborated chloroplast PCA clustering showing no differences between T3 and T5 and revealed similarities (by cluster overlap) between C and T5 in the nuclear data set.

Once PCA sample distribution was validated by t-SNE, we turned to top-scoring loadings of PC1 and 2. The proteins showing top positive loadings in PC1 (overaccumulated along with the stress) showed, as expected, the relevance of heat shock proteins (HSP) as a common heat stress response in both organelles. However, a closer look into the proteins, which were more abundant under control conditions, revealed relevant organelle-specific processes. These included S-adenosylhomocysteine hydrolase (SAHH), related to the methyl cycle in the nucleus (Figure 2a). As well as redox, RNA binding and photosynthesis-related proteins in the chloroplast (Figure 2b) and in the combined datasets (Figure 2c), such as PPD1, protochlorophyllide oxidase, zeaxanthin epoxidase, FTSH protease 2 and HCF164 proteins, all of them linked to D1 chloroplast protein synthesis and assembly (Schult et al., 2007) and to the photosystem II (PSII) repair cycle (Kato & Sakamoto, 2018), known to be sensitive to stress.

Regarding PC2, in the chloroplast (Figure 2b) and combined datasets (Figure 2c), samples were clustered distinguishing first versus subsequent stress exposure days. Acclimation of photosynthesis to the environment (APE) acclimation factor and other proteins related to photosynthesis, such as PSB27, previously linked to PSII-independent adaptation to light stress (Hou et al., 2015), showed high negative loadings and an overaccumulation in T1 samples. Contrastingly, carbon metabolism proteins (such as sucrose synthase or transaldolase), EF-TU translation elongation factor, related to heat tolerance (Ristic et al., 2007), ARC5 essential for chloroplast division and biogenesis (Gao et al., 2003) and RUVB-LIKE HELICASE, also known as heat-responsive and related to thermotolerance in rice (Saifi et al., 2018), presented their highest positive values and peaked in longer exposure times (T3 and T5).

The combined analysis of both datasets besides revealed a cluster of proteins with high scoring values related to RNA metabolism, such as NSR RNA splicing regulator, RPOA RNA polymerase or RNA polymerase V. The latter is a multisubunit plant-specific nuclear RNA polymerase required for the normal function and biogenesis of small interfering RNA (siRNA) and is involved in the regulation of gene expression by siRNA-directed DNA methylation (Xie & Yu, 2015). Dual-located proteins as key players in the coordination of stress response mechanisms.

An increasing number of proteins are found dually localized in the plastids and the nucleus (Krupinska et al., 2020). Many nuclear transcription factors were shown to be controlled by signals generated in the organelles. In addition to the metabolites involved in retrograde signalling (Zhao et al., 2020), there is accumulating evidence suggesting a role for proteins in plastid-to-nucleus communication. Indeed, several proteins exhibiting a dual localization in the plastids and the nucleus are promising candidates for direct signal transduction involving regulatory protein storage in the plastids (Krause et al., 2012). In this study, 340 proteins were detected in both organelles (Table S4).

An overview of the changes in these double agents across the stress in both organelles was performed using the *k*-means algorithm, obtaining 25 different clusters according to their abundance profile (Figure 3a). Several clusters showed a similar trend in both organelles. An increasing accumulation pattern during stress conditions was shown in Clusters 2 and 22. Low levels in T1 and high levels in T3–T5 for Clusters 1 and 19 and a decreasing accumulation in Clusters 18 and 23, where we found HEMERA protein (also known as pTAC12 and PAP5). This protein exhibits different functions depending on its cellular location; in the nucleus, it is related to phytochrome signalling (Chen et al., 2010), and in the chloroplasts, it is required for gene expression (Steiner et al., 2011).

The most recurring trend was an opposite profile (Clusters 5, 7, 8, 11, 12 and 17). These organelle-specific accumulated clusters included proteins related to carbohydrate metabolism, cell cycle organisation, chromatin organisation, photosynthesis and cellular respiration (Figure 3b), all of these essential processes in plant metabolism. Within these groups, DNA/RNA binding protein WHIRLY1 was noteworthy (Cluster 8, Table S4), since this protein has been proposed to move from the chloroplast to the nucleus in response to environmental cues such as high light intensity, in which a WHIRLY1-dependent increase of nuclear microRNAs was reported (Świda-Barteczka et al., 2018). Now, through this study, WHIRLY1 was found to follow the same pattern in high-temperature response as well.

Strictly increasing and decreasing clusters were mainly composed of redox homeostasis and photosynthesis proteins, respectively (Figure 3b); while the profile of Clusters 1 and 19, with low levels in T1 and high levels in T3–T5, which could be connected to acclimation processes and possibly to signalling or memory acquisition. These clusters essentially collected proteins from RNA metabolism and protein biosynthesis (Figure 3b), as RNA splicing regulator, RH3 plastid RNA basal splicing factor and ribosomal proteins (Table S4).

3.3 | Nuclear-chloroplast crosstalk, a two-way road

Both nuclei and chloroplasts contain proteins whose DNA is encoded in the genomes of other organelles. These 'nonnative' proteins are known to play a role in organellar communication along with the dually located proteins already identified. To further explore this crosstalk, we performed an integrative analysis by evaluating the correlations among the proteins quantified. To do so, each data set was independently divided into 'native' (encoded in this organelle genome) and 'nonnative' proteins, and the correlations among them were evaluated using sPLS algorithm and depicted as two protein–protein networks (Figure 4, Table S5). The relations between

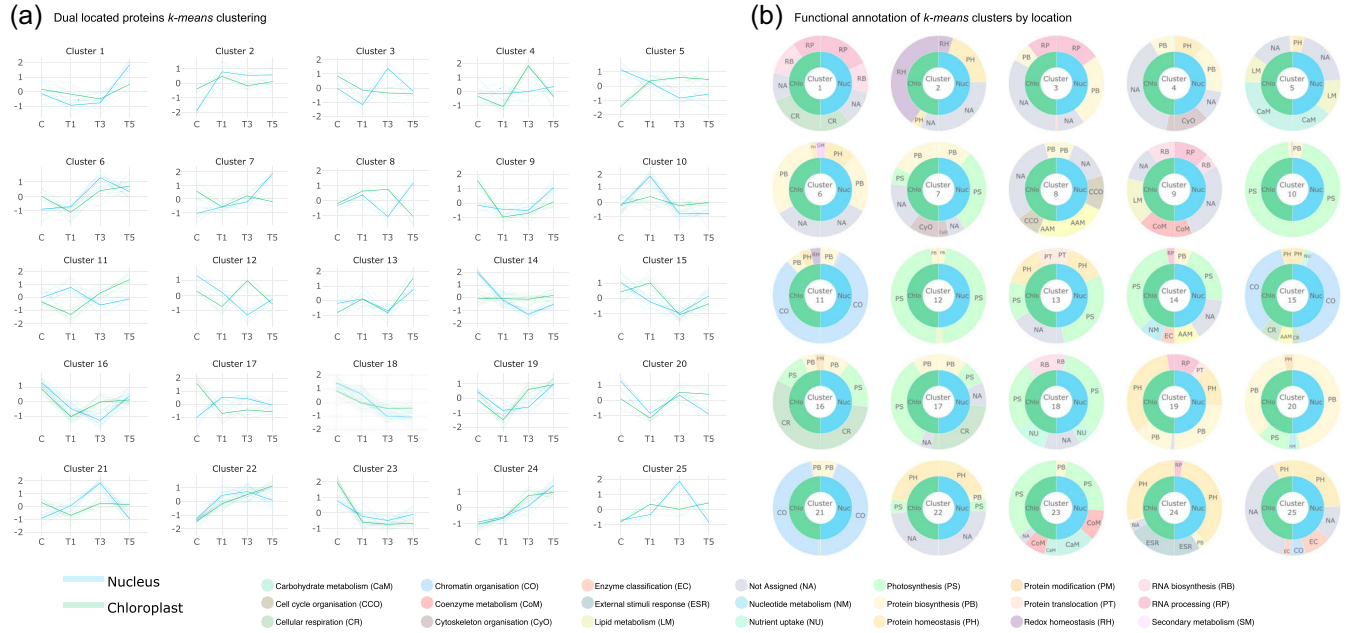

FIGURE 3 Pattern-clustering of dual localized proteins. (a) *k*-means analysis of the abundance of dual located proteins across the stress. Three hundred forty proteins showing dual localization were identified, scaled in each data set, and *k*-means clustered yielding 25 groups. Colours indicate the cellular location; nuclear in light blue and chloroplast in green. Continuous lines indicate mean values for each protein at each experimental condition ($n = 4$ biologically independent replicates) and bold lines indicate mean values for each cluster at each experimental condition. (b) Pie charts of most abundant function annotation classifications for each cluster and cellular location. Different colours indicate different protein functional annotation classification according to Mercator Mapman [Color figure can be viewed at wileyonlinelibrary.com]

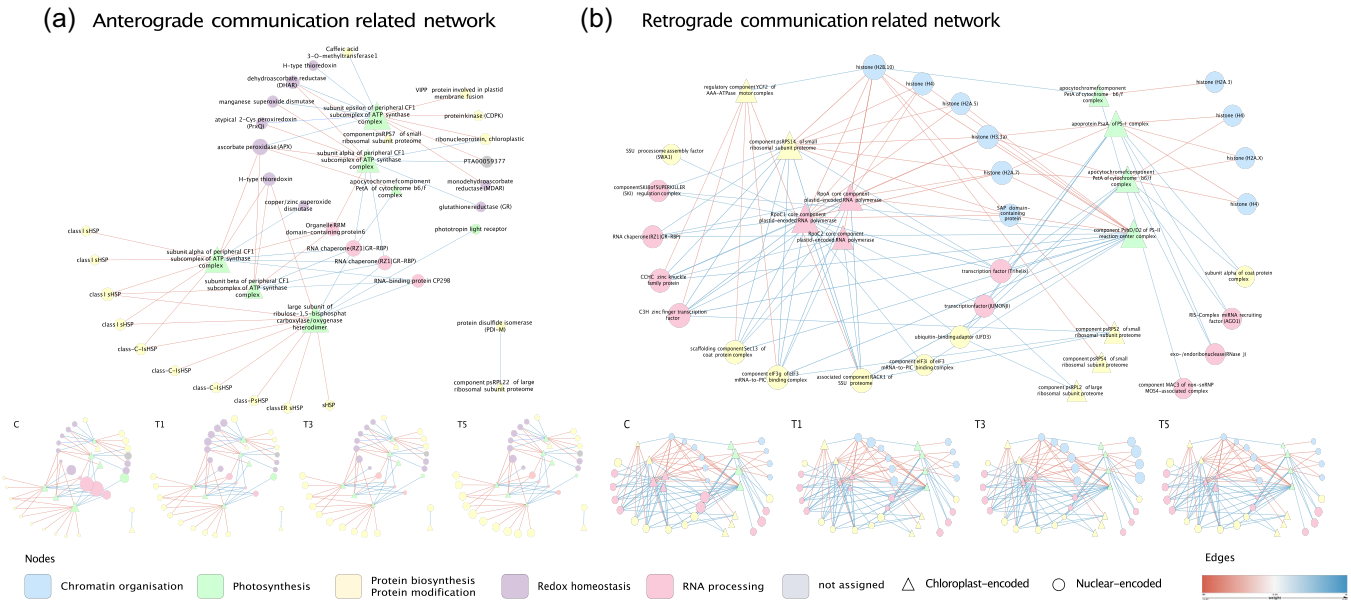

FIGURE 4 Reconstruction of nuclei-chloroplasts communication by sPLS networks. (a) Anterograde communication-related network, was built using chloroplast targeted proteins, which were divided according to their gene localization (nuclear or chloroplast genome), network links depict the correlations found among nuclear-encoded proteins and chloroplast-encoded proteins. (b) Retrograde communication-related network, was built following the same schema, being this time nucleus targeted proteins divided according to their gene localization. Triangles indicate chloroplast-encoded proteins and circles nuclear-encoded proteins. Node colours indicate Mercator MapMan classification according to the legend. Edge colour indicated inversely proportional (negative) relations in red and proportional (positive) relations in blue. Edges below a 0.75 cutoff were removed. C, T1, T3 and T5 subnetworks in the bottom represent the proteins' abundance distribution across the control and heat-stress treatments. All models were built using four biologically independent replicates [Color figure can be viewed at wileyonlinelibrary.com]

nuclear-encoded proteins found in the chloroplast proteome might give insights related to anterograde communication, and the relations between chloroplast-encoded proteins found in the cell nucleus might be related to retrograde communication.

Anterograde communication traces (Figure 4a) were found in the chloroplasts, where the ubiquitous HSPs and RNA chaperones were transcribed in the cell nuclei and sent to the chloroplasts, probably as the desirable consequence of the gene expression alterations at cell nuclei as a defence shield against stress. These proteins are known to be essential to maintain chloroplast functionality, along with redox enzymes (APX, thioredoxins, SOD) also highlighted in chloroplast PCA loadings (Figure 2b, PC2), which have a role in counteracting the electron transfer flux disruption and redox imbalance triggered by the hyperthermal stress (Fang et al., 2019). Furthermore, this network provided potential candidates and indicated that RNA metabolism rearrangement is also required in the chloroplasts and was at least partially driven from the cell nucleus, with the opposite abundance changes of organelle RRM domain-containing protein 6 (Fu et al., 2007) and RNA binding protein CP29B linked to photosynthesis and RNA metabolism (Hackett et al., 2017).

On the other hand, retrograde communication (Figure 4b) seems to be linked to chloroplast-encoded RNA polymerases, whose expression were altered concomitantly with histones and RNA splicing factor MAC3, transcription factors (C3H Zinc Finger, Triple Helix and jumonji) and protein metabolism (processome components and ribosomal subunits such as SWA1 [Shi et al., 2005] and RACK1 [Guo et al., 2011]).

Taken together, both networks covered potential players of the nucleus-chloroplast communication, involving chromatin organisation, photosynthesis, redox, RNA and protein metabolisms.

3.4 | MicroRNA contents raised at long-term heat stress exposure

To further investigate the role of RNA modulation in the stress response we quantified microRNA abundances (Figure 5a). Our results showed an increasing trend along with stress and a significant change in T5.

In addition, we aimed to find concrete microRNAs and mRNA pairs relevant to heat stress acclimation mechanisms. To do so, transcripts from nuclear-encoded proteins, which exhibited significant variations in nuclear or chloroplast proteomes, were blasted as targets against conserved microRNA (Table S6). The in silico identified target-microRNA pairs were filtered to keep the most probable pairs. Among them, we selected biological relevant targets covering translation (eIF3G; miR160), chromatin conformation (H2A; miR947), genome methylation (SAHH; miR482), photosynthesis (CP29B; miR396_1), retrograde communication (HEMERA/PAP5; miR394 and GUN4; miR1131), redox homeostasis (APX; miR396) and RNA metabolism (CP29B; miR396_1, RNA helicase; miR162). miR160 has been previously linked to acquired thermotolerance (Lin et al., 2018) and miR162, miR394, miR396 and miR482 have been linked to heat stress, with no common pattern among different species or experimental designs, and none of them has been monitored for more than 24 h under stress (reviewed by Liu et al., 2015). Proteins, mRNAs and their potential microRNAs change folds are shown in Figure 5b. As expected, the discrepancy between protein and mRNA abundances can be explained with microRNA expression changes, especially in long-term heat-treated samples. eIF3G, H2A, SAHH, CP29B, HEMERA transcripts were upregulated along with their proposed microRNA pairs, and the protein abundance decreased, while for the other pairs, their relation is not clear. These results indicate possible additional regulatory layers that complicate the interpretation of the results and reveal a complex network involved in their regulation.

4 | DISCUSSION

An important unanswered question in stress plant biology is how signalling coordination between organelles takes place and what experimental approach can be used to address these molecular mechanisms. Here, we show that subcellular untargeted proteomics is a powerful strategy to provide insights into how plants orchestrate physiological responses.

4.1 | The acclimation stage seems to be reached firstly in the chloroplast and then in the nucleus

Our experimental design allowed us to monitor organelle and whole-cell responses after one, three and five consecutive days of heat stress. The differences found after the first and subsequent stress exposures revealed a better physiological performance in T5, such as increased phenolics abundance, recovery of pigment contents and flavonoids and restored primary metabolism (Figure 1a); indicating that a whole-cell acclimation process took place.

Regarding organelles, chloroplasts exhibited a more drastic proteomic variation after the first day of stress (Figure 1c), while their proteomic profiles varied slightly from T3 to T5 (Figures 2b and S2), suggesting a new homeostatic state was reached at midterm stress exposure. Taken globally, our results showed various pieces of evidence indicating an acclimation process at midterm stress exposure. The APE acclimation factor, triggered in T1, may be key to survive to the following days, given that *Arabidopsis thaliana ape* mutants showed a defective acclimation response (Walters et al., 2003). Additionally, the chloroplast protein synthesis elongation factor EF-TU raised in T5 was found to be implicated in heat tolerance in maize (Ristic et al., 2007) revealing a possible long-term thermotolerance acquisition process placed in the chloroplast. In addition, the overaccumulation of ARC5 in T5, which is essential for chloroplast division (Gao et al., 2003) gave insights into chloroplast heat-stress-overcoming mechanisms.

Meanwhile, the nucleus triggered after the first heat shock a DNA damage response (Figure 1c). The third stress exposure caused

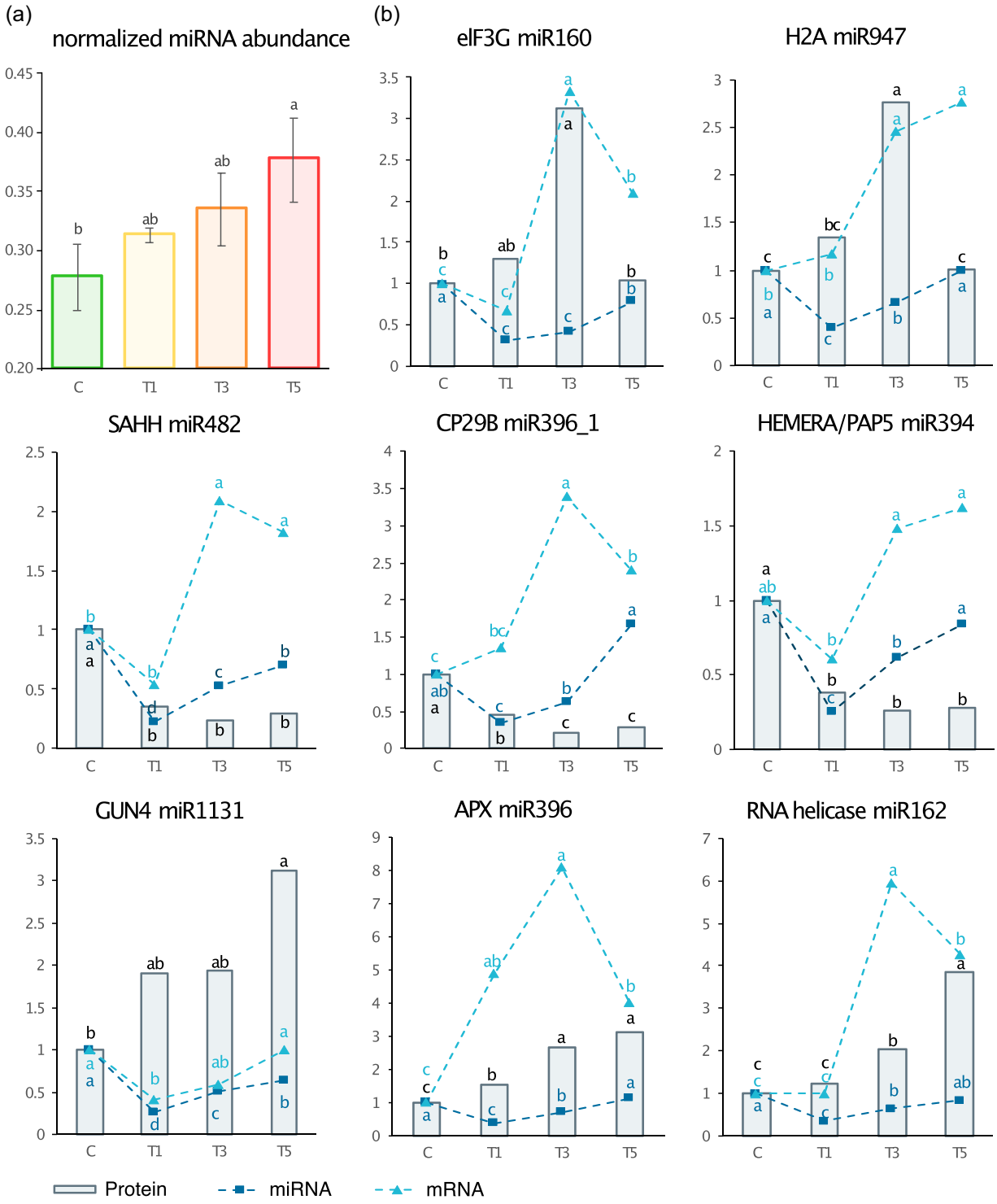

FIGURE 5 microRNAs biogenesis across heat stress and correlation to candidate target mRNA and relative protein levels. (a) Normalized microRNA abundance, (b) candidate microRNAs levels, fold change of their targeted mRNA expression and correlative protein abundance. Proteins are represented in bars (grey) and mRNA (light blue) and microRNA (dark blue) in lines. (a,b) Different letters indicate significant changes according to HSD test ($p < 0.05$) four biologically independent replicates were used for proteomics analysis and, three independent biological replicates were used for gene expression measurements [Color figure can be viewed at wileyonlinelibrary.com]

an accumulation of chromatin organising proteins as histone isoforms (Figures 1c, 4b), and after five days transcription and translation metabolism-related proteins were overaccumulated (Figures 2a,c), coupled to a whole-cell FAA increase, suggesting a deep proteomic rearrangement towards thermotolerance acquisition. These processes were coupled to an upregulation of PTGS complexes through microRNA and AGO1 overaccumulation in the fifth consecutive exposure day (Figures 4b and 5a), when plants finally seemed to be

acclimated to the applied stress. This co-occurrence might imply that the upregulation of microRNAs is a result of an acclimated state; PTGS would function as a protection mechanism against an exaggerated response, which is no longer needed in the new homeostatic state.

4.2 | Heat and light stresses seem to reprogram nucleus-chloroplast crosstalk through pigments in a similar way

In this study, we observed that heat stress provoked a moderate and maintained decrease in PSII maximum efficiency. Interestingly, the decreased abundance of photosynthetic pigments, such as chlorophyll and carotenoids, matched the decrease in F_v/F_m only in short and midterm heat exposures (Figure 1a). The mismatch found after five days between these measurements lead us to the chloroplast proteome, where it was found that D1 protein was depleted along with the enzymes related to the PSII repair cycle. Hence, PSII impairment likely explains the F_v/F_m drop. Additionally, since photosynthetic machinery is in part controlled by nuclear genome gene expression (Unal et al., 2020), these may indicate a reprogramming of basal communications between the two organelles during the stress.

The chlorophyll depletion in short- and midterm stress can be due to different phenomena mainly including chlorophyll being used as a source for other metabolites, chlorophyll biosynthesis pathway downregulation or a combination of both processes. This latter option was likely to occur in different phases of each day stress exposure. As chlorophyll has been proposed to be a source for the biosynthesis of tocopherol through the phytol recycling pathway (Muñoz & Munné-Bosch, 2019), which is a signalling metabolite (Fang et al., 2019; Jung & Chory, 2010; Serrano et al., 2019), the pigment decrease could be linked to a heat-warning signal. In addition, tocopherol has an essential role in avoiding the propagation of lipid peroxidation (Muñoz & Munné-Bosch, 2019), so the MDA content maintenance along with the stress further supports a phytol recycling pathway-based-signalling towards stress. In addition, carotenoids serve as precursors of a wide variety of signalling molecules (Moreno et al., 2021); their depletion after 6 h of stress on the first and third days seems to indicate that they acted as a source for other metabolites.

On the other hand, in the proteome, we found some clues about pigment changes, such as the underaccumulation of protochlorophyllide oxidoreductase (Figure 2b, PC1), an enzyme implicated in chlorophyll biosynthesis. In any case, free chlorophyll usually generates phototoxic catabolites; thus, the decrease in this pigment content may prevent a further toxic effect. Simultaneously, zeaxanthin epoxidase was also depleted under heat stress (Figure 2b, PC1), which may imply a xanthophyll shift towards an increased zeaxanthin amount, one well-known photoprotective pigment under high-light conditions (Jahns et al., 2009).

Interestingly, we have found several candidates involved in chloroplast heat reprogramming, such as the APE acclimation factor, PSB27, HEMERA and WHIRLY, which were previously linked to light adaptation (Chen et al., 2010; Foyer et al., 2014; Hou et al., 2015; Krause et al., 2012; Krupinska et al., 2020; Walters et al., 2003), but so far had not been related to heat stress or heat tolerance. This could be explained by the assumption that in natural conditions light and heat stress are often simultaneous, and that these candidates are related to the signalling of photosynthetic damage through ROS, which is a shared signalling pathway for several abiotic stressors. Among them, WHIRLY and HEMERA showed dual localization, probably acting as messengers between the two organelles. Additionally, our results showed HEMERA as a potential candidate to be regulated by miR394, as its protein abundance decreased along with an increase in its transcript and miR394 in mid and long-term stress exposures evidencing the complexity of organelle signalling systems.

4.3 | RNA metabolisms seem to be involved in organelle communication

As depicted in the anterograde network (Figure 4a), plastid-encoded RNA polymerases seem to be actively involved in stress response. Unexpectedly, we found plastid-encoded RNA polymerases along with other 10 chloroplast-encoded proteins in the cell nucleus (Figure 4b, Table S5). This finding should be taken cautiously as to our knowledge there is no precedent record of chloroplast-encoded proteins targeted to the cell nucleus. Despite this fact, the presence of nuclear localization signals in these protein sequences may suggest them as potential dual-targeted proteins, which may enter the cell nucleus after chloroplast membrane disruption or fluidization, which is known to happen in heat stress conditions (Hu et al., 2020). Another option is the direct protein export from the chloroplasts to the nucleus, which has been proven to be possible through stromules, which are tubular channels that allow metabolite and protein exchange (Hanson & Sattarzadeh, 2013; Köhler et al., 1997). Moreover, ROS production in the chloroplasts triggers plastid movement towards the nucleus and stimulates stromule formation (Brunkard et al., 2015; Hu et al., 2020; Kwok & Hanson, 2004; Mullineaux et al., 2020), which may be intended to communicate the stress signal avoiding cytoplasmic diffusion. However, further studies are required to test the extent and functionality of this physical communication.

In the nucleus, RNA metabolism also exhibited a characteristic profile in the last stress exposure (Figures 1c and 2). The main role of PTGS and microRNA previously linked to organelle crosstalk under stress conditions (Fang et al., 2019; Zhao et al., 2019, 2020), as well as our results related to dual located proteins (Figure 3a,b), support the idea of a central role of RNA metabolism in heat stress response. Besides this, apocytochrome proteins were found to be correlated to RNA metabolism through microRNA biogenesis, exoribonuclease and AGO1 proteins, which are shown to be the main players in stress signalling by directing gene repression (Fang et al., 2019) from the central regulatory hub of the cell.

In addition, the nucleosome stoichiometric changes that were tracked in the retrograde communication network through two

groups of different histone isoforms (Figure 4b) may lead to thermomemory acquisition, as stated in other works (Bäurle, 2016; Lamelas, Valledor, et al., 2020; Lämke et al., 2016), driving the cell to a new primed status. This epigenetic memory based on methylation changes (SAHH) and H2A histone variant could be regulated by microRNA (Figure 5b) since their profile of mRNA expression is not sufficient to explain their protein abundance. Those mismatches provide a proof of concept to the wide variety of functions that microRNAs can regulate. In this study, we provided several mRNA-microRNA potential pairs and a strong evidence of the relevance of this posttranscriptional gene modulation mechanism, which seems to be relevant to come back to 'the new normal' after the first days of stress and to acquire heat stress tolerance and long-term memory. We delved into microRNA-mRNA balance and provided for the first time in this species a set of validated microRNA sequences, a new family of heat-sensitive microRNAs, including miR947 (H2A) and the time-series pattern that these heat-sensitive miRNAs followed during the stress stages.

To sum up, during this study, we combined targeted and untargeted -omics approaches to decipher the biochemical signals relative to stress acclimation, revealing how finely-tuned these sequential mechanisms are, covering ROS detoxifying, chromatin remodelling and their downstream consequences as RNA and protein metabolism reprogramming, which lead to stable changes that allow plants to survive to heat stress. These results increase our understanding of how plants adapt to challenging environments in long-lived species, such as *P. radiata*, which need to acclimate and survive for years to endure as species.

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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, msf and pepXML files have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the data set identifier PXD029114.

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