PhD Thesis resume

Introduction

Chloroplasts are semi-autonomous cellular organelles responsible for the photosynthesis and autotrophic growth of algae and land plants. Originated by an ancient endosymbiosis with cyanobacteria ancestors (Howe et al., 2008), chloroplasts feature their own genome (plastome) encoding about 5% of plastid proteins in higher plants. The plastid-encoded proteins are assembled in multi-subunit complexes, in precise stoichiometric ratios, with nuclear-encoded proteins imported from the cytoplasm. The physical separation of nuclear and plastid genomes drove the evolution of reciprocal signalling pathways that ensure coordinated gene expression and the maintenance of the plastid protein homeostasis (proteostasis) throughout plastid development and in response to the environment (Jarvis and López-Juez, 2013). Chaperones and proteases, by aiding protein folding and by partially or completely degrading polypeptide chains, are central components in the maintenance of plastid proteostasis (Kim et al., 2013b; van Wijk, 2015; Nishimura et al., 2016). Defects in these factors, as well as abiotic stresses such as heat, lead to altered proteostasis due to the accumulation of unfolded proteins (folding-stress) triggering the upregulation of chaperones and proteases, a process known as chloroplast unfolded protein response (cpUPR) (Sharma et al., 2009; Ramundo and Rochaix, 2014). The UPR was first characterized in the endoplasmic reticulum, one of the main sites of protein synthesis in cells (Hetz et al., 2015). However, it is clear today that also mitochondria and chloroplasts activate comparable pathways when their proteostasis is perturbed (Haynes et al., 2007; Haynes et al., 2010; Pellegrino, 2014; Ramundo et al., 2014; Dogra et al., 2019). In chloroplasts of *Arabidopsis thaliana* and *Chlamydomonas reinhardtii,* the removal of core subunits of the main plastid soluble protease complex CLP or of transmembrane proteases FTSH causes the strong upregulation of chloroplast chaperones and proteases as a compensatory response (Kim et al., 2013a; Ramundo et al., 2014; van Wijk, 2015; Adam et al., 2019; Dogra et al., 2019). Similarly, treatment of wild-type Arabidopsis plants with the chloroplast translation inhibitor lincomycin results in the transcription of nuclear genes usually associated with the heat-shock response and involved in proteostasis (Llamas et al., 2017). Albeit the signalling molecules behind the chloroplast-to-nucleus communication are still unknown, pieces of evidence suggest that peptides derived from proteolysis could act as signalling molecules. Studies conducted in *Caenorhabditis elegans* have revealed that extruded proteolytic products, *i.e.* peptides, from mitochondria are the signalling molecules that mediate the mtUPR in response to heat (Haynes et al., 2010). The mitochondrial peptides are exported in the cytoplasm by the ABC transporter HAF-1, embedded in the inner membrane of the organelle. In particular, worms depleted of HAF-1 have mis-regulated expression of UPR-related genes upon heat exposure and consequently show decreased vitality at high temperatures (Haynes et al., 2010). HAF-1 transporter is homologous to MDL1 mitochondrial peptide transporter of *Saccharomyces cerevisiae*. MDL1 also extrudes peptides produced within mitochondria by a FTSH protease, and the null mutant is sensitive to heat-stress (Young et al., 2001; Arnold et al., 2006; Jarolim et al., 2013). Taken together these findings point to the existence of putative Plastid Peptide Transporters (PPTs) belonging to the ABC transporters superfamily which, in Arabidopsis, consists of 130 proteins most of them still uncharacterized (Sánchez-Fernández et al., 2001; Kang et al., 2011).

Aims

In light of this, it can be hypothesised the existence of a chloroplast retrograde signalling pathway comparable to that described in mitochondria. Therefore, this work aims to:

1. Identify, in *Arabidopsis thaliana*, the ABC transporter that could mediate peptide extrusion from chloroplasts.
2. Detection and characterization of the putative peptide efflux from chloroplasts.
3. Characterize the biological function of such transporter, particularly in response to plastid protein homeostasis perturbations.

4. Attempt to link the function of the transporter with a putative retrograde signalling pathway generated by protein degradation within the chloroplast and mediated by the extrusion of the peptides.

Results

To computationally identify candidate plastid peptide transporters, the SwissProt protein sequence database of *A. thaliana* was interrogated by BLASTp searches employing as queries the amino acid sequences of both HAF-1 and MDL1. Hits were then arbitrarily filtered for BLAST scores higher than 100 and plastid localization according to SUBA5 database information. Among all the hits from both searches, three common uncharacterized candidates satisfied both criteria: TAP1 (Transporter associated with Antigen processing Protein) had the highest score, followed by NAP8 (Non-intrinsic ABC Protein 8 or TAP-related protein 1) and ATH12 (ABC Two Homolog 12). Our candidates clustered among peptide transporters in a multiple sequence alignment fed with protein sequences of known peptide transporters from bacteria to human and Arabidopsis ABCB auxin transporters, as control group (Nijenhuis and Hämmerling, 1996; Young et al., 2001; Wolters et al., 2005; Zhao et al., 2008; Haynes et al., 2010; Liesa et al., 2012; Lehnert et al., 2016; Nöll et al., 2017).

The coding sequences of the candidate proteins were cloned in frame with that of *GFP* or *RFP* genes under the control of the *CaMV 35S* constitutive promoter to obtain C-terminally tagged proteins. The coding sequence of *TIC20*, encoding for a subunit of the Inner membrane Translocon Complex of the chloroplast, was cloned as well as a marker of the plastid envelope. The produced constructs were then introgressed into wild-type plants by Agrobacterium-mediated transformation. Transformed plants were subsequently crossed to generate Arabidopsis lines co-expressing GFP or RFP tagged proteins as follows: *oeTAP1-GFP oeTIC20-RFP*, *oeTAP1-GFP oeNAP8-RFP*, *oeTAP1-GFP oeATH12-RFP*. Leaf tissue from these plants was then analysed via Confocal Laser Scanning Microscopy. The signal from TAP1-GFP appeared to be surrounding the fluorescence of plastid chlorophylls, resembling the RFP signal from the TIC20 chimaera. Moreover, NAP8-RFP and ATH12-RFP signals were ring-shaped and perfectly overlapped with that from TAP1-GFP. In accordance with these experimental observations, TAP1, NAP8 and ATH12 were found in the envelope fraction by a mass-spectrometry-based analysis of the chloroplast proteome (Ferro et al., 2010). Taken together, these observations are compatible with the role envisaged for TAP1, NAP8 and ATH12 as plastid envelope-located peptide transporters.

Single mutants bearing a T-DNA inserted in *TAP1* (AT1G70610), *NAP8* (AT5G03910) and *ATH12* (AT4G25450) gene loci were acquired from the Nottingham Arabidopsis Stock Centre. The single mutants were then crossed to yield all the possible combinations of double mutants and, subsequently, the triple mutant was generated. No obvious phenotypes were noticeable in any of the mutants when grown in standard conditions. The absence of phenotype, however, is in line with what was observed in the *mdl1Δ* and *haf-1* mutants in yeast and nematode, which behaved as the wild type in standard growth conditions (Young et al., 2001; Haynes et al., 2010).

Chloroplasts were isolated from wild-type and mutant plants through Percoll gradients (Seigneurin-Berny et al., 2008). Isolated chloroplasts were then incubated at 45° C to perturb the protein homeostasis, by promoting protein misfolding and degradation, and possibly triggering peptide efflux. ATP was added to the samples to support the ATP-dependent peptide transport. As a control, wild-type chloroplasts were incubated at 25° C with ATP or, at 45° C in the absence of ATP. After the treatment, chloroplasts were pelleted and supernatants were collected. Samples were cleaned through a 30 kDa cut-off filter and successively processed with a solid-phase extraction column, to allow enrichment in low molecular weight peptides. Finally, peptide concentration in each sample was evaluated by UV spectrometry at 280 nm. When treated at 45° C with ATP, wild-type chloroplasts produced a peptide extrusion of about twice the amount of the control samples, in whose no heat or ATP was applied. The lack of one or two of the transporters did not significantly affect the level of retrieved peptides in the supernatants. Instead, when subjected to the same treatment, chloroplasts were deprived of all three transporters and extruded half the amount of peptides in comparison with wild-type chloroplasts. The peptide efflux from triple mutant was comparable to the

one from untreated wild-type chloroplasts, in the presence of ATP, or treated chloroplasts without ATP. These results suggest that the identified putative plastid-located peptide transporters promote peptide efflux upon heat treatment. Moreover, all three TAP1, NAP8 and ATH12 transporters contribute to the total peptide efflux from chloroplasts upon heat exposure in an ATP-dependent manner, showing functional redundancy and possible substrate overlap.

Peptides recovered from heat-treated wild-type chloroplasts were analysed by mass spectrometry, to attempt sequence identification. The average total number of detected peptides from 2 independent experiments was 10629, derived from 481 different proteins. 99,2% of the peptides mapped on the amino acid sequences of plastid proteins, indicating that the chloroplast samples were virtually pure. The majority of the plastid-derived peptides (57,1%) originated from thylakoid proteins. Luminal proteins contributed to the production of 18,9% of the peptides, whereas the stromal proteins supplied 17,7%. The remaining portions of peptides were attributed to proteins localised in plastoglobules (1%), envelope (0,5%) or unsorted ones (4,8%). About 50% of the peptides belonged to the Photosystem II proteins, the subunits of its antenna complexes or the oxygen-evolving complex. Among all recovered peptides in wild-type heat-treated plants, the length ranged from 8 to 25 amino acids, with the average peptide being 15 amino acids long.

However, the diminished peptide extrusion from triple mutant chloroplasts could be attributed to differences in the protein degradation rate rather than peptide extrusion. To rule out this possibility, the relative abundance of proteins was monitored in heat-treated chloroplasts in the presence of ATP through western blot. Chloroplasts from the wild type and the triple mutant were sampled before the treatment, after 30 minutes and at the end of the incubation. Samples incubated with ATP but not exposed to heat and chloroplasts heat-treated without ATP were included as a control. PSBO and LHCB4 proteins were selected as targets since a high amount of their peptides was found in the previous analysis. The relative abundance of both proteins decreased gradually during the treatment in both chloroplast populations, indicating that the diminished peptide efflux in the triple mutant chloroplasts is to be attributed to the absence of the transporters rather than impaired proteolysis.

Since TAP1, NAP8 and ATH12 ABC transporters appeared to contribute to the plastid peptide efflux upon heat treatment, it could be possible that the heat-sensitive phenotype of *mdl1Δ* mutant could be rescued by the introduction of *TAP1*, *NAP8* and/or *ATH12* genes. To test the hypothesis, the wild-type yeast strain BY4741 was transformed with DNA recombination cassettes to yield *mdl1Δ TAP1*, *mdl1Δ NAP8* and *mdl1Δ ATH12* transgenic strains and the null mutant *mdl1Δ*. The wild type and the obtained mutant strains were incubated in growing conditions (28° C, 250 rpm) until the exponential phase was reached. Then, cell cultures were divided into two subcultures (Mock and Heat). Before splitting, aliquots were collected, serial diluted and plated on a complete medium as T0 samples. The Mock cultures were incubated in the previous growing conditions as a control. The Heat cultures were incubated at 45° C instead. After 2 hours both cultures were sampled, and cells were plated on Petri dishes kept at 28° C for 3 days to allow colony formation. The obtained colonies were then manually counted to compare the T0 sample with the Mock and the Heat ones to estimate cell growth (expressed as a ratio) in the two conditions. All the yeast strains grew similarly when incubated at 28° C while the temperature raise at 45° C caused cell loss in all cultures. However, the *mdl1Δ* Heat sample produced about -60% of colonies relative to the T0 sample whereas, the wild type and, importantly, all the transgenic strains resulted in about -30% of negative growth.

ppts triple mutant plants, being unable to generate a peptide efflux from chloroplasts, could also be more sensitive to high temperatures. To test this, the photosynthetic efficiency (F_v/F_m) of heat-treated plants was evaluated since this parameter has been reported to be a good indicator of plant health and to correlate with heat tolerance/sensitivity (Allakhverdiev et al., 2008; Chen et al., 2018). 15 days old plants grown in standard conditions were subjected to two different heat treatments. At first, plants were incubated at 45° C for 2 hours in the dark (HS). In a second treatment, plants were first acclimated (1,5 hours at 37° C in the dark and 2 hours of recovery in standard conditions) before the incubation (ACHS). To measure reference values, a 2-hour-long incubation in the dark without

shifts in temperature was performed (Mock). The *ftsh11* mutant was included in the analysis, as it lacks a transmembrane protease involved in thermotolerance and protein quality control (Chen et al., 2006; Chen et al., 2018; Adam et al., 2019). Wild-type photosynthetic efficiency decreased strongly after the HS treatment, while acclimation allowed it to endure better heat incubation, mitigating the impairment of photosynthetic parameters. The F_v/F_m values reduction was enhanced in the *ftsh11* mutant in both treatments. Interestingly, the *ppts* triple mutant appeared less capable to recover after heat exposure than the wild type in both conditions.

To challenge the *ppts* triple mutant with alternative-to-heat plastid protein homeostasis perturbations, the sensitivity to methyl viologen (MV) was evaluated as well. Also known as Paraquat herbicide, MV is an oxidative agent that induces protein misfolding by promoting ROS development and carbonylation (Manning-Bog et al., 2002; Nyström, 2005; Pulido et al., 2017). Wild-type, *ppts* and *ftsh11* plants were sown on MS synthetic medium containing 1 or 2 μM MV and their photosynthetic efficiency were evaluated after 9 days of growth. The wild type was marginally affected by the presence of MV, as its photosynthetic efficiency decreased slightly compared to that observed in the mock. On the other hand, the *ftsh11* mutant was significantly more sensitive to the chemical treatment. Remarkably, if the photosynthesis of *ppts* triple mutant was just slightly diminished at 1 μM MV relative to the wild type, it was greatly affected in 2 μM MV-treated samples.

The *ppts* triple mutant was found to be sensitive to both heat and MV treatments, which are known to induce protein instability (Manning-Bog et al., 2002; Feller, 2010). In *A. thaliana*, the nuclear-encoded plastid-located CLPB3 chaperone is up-regulated when the chloroplast protein homeostasis is challenged (Myouga et al., 2006; Lee et al., 2007; Llamas et al., 2017). Indeed, the CLPB3 plastid chaperone has the important function of resolving aggregates of unfolded proteins in the stroma to avoid their toxic accumulation (Llamas et al., 2017; Parcerisa et al., 2020). Hence, CLPB3 relative abundance was evaluated as a marker of ongoing protein homeostasis maintenance and possible chloroplast UPR molecular marker. First, the amount of CLPB3 was probed via immunoblotting in total protein samples harvested from 15 days old wild-type and *ppts* triple mutant plants that were grown on soil in standard conditions. Notably, on average, the amount of CLPB3 in the ppts triple mutant was about 65% more accumulated relative to the wild type. It was noticed that chloroplasts from the ppts triple mutant were still able to perform proteolysis upon heat exposure despite the hampered peptide efflux. In yeast, it was demonstrated that mitochondria constantly extrude peptides resulting from normal protein turnover (Augustin et al., 2005). Therefore, taken together, these results suggest that the absence of PPTs could be per se a source of plastid proteostasis alteration, possibly due to the not-extruded peptides derived from plastid protein turnover.

Next, the ability to up-regulate CLPB3 was evaluated in response to stress. Wild-type and triple mutant plants grown in standard conditions were moved in a pre-heated incubator at 45° C for 2 hours to deliver the heat shock. After the treatment, plants were allowed to recover back to standard conditions. Plants were sampled before the heat shock (preHS), immediately after the treatment (2h HS), after 1 hour of recovery (1h Rec) and after 2 additional hours (3h Rec). Total proteins were then extracted, fractionated on SDS-PAGE and probed with CLPB3-specific antibodies. In the wild type, CLPB3 gradually increased its accumulation in all the considered time points reaching, in the 3h Rec time point, about 3-fold the amount of the preHS sample. Instead, the ppts triple mutant failed to over-accumulate CLPB3 during the same time window. Importantly, CLPB3 in the treated mutant decreased to the preHS sample by about 70%. To understand whether gene expression was altered as well, the expression of the CLPB3 gene was tested by RT-qPCR at the preHS and 1h Rec time points. Remarkably, the detected amount of CLPB3 transcripts in both wild type and *ppts* triple mutant were comparable, which increased about 4-fold after 1 hour of recovery from the heat treatment. According to these results, CLPB3 is steadily up-regulated in the wild type upon exposure of the plants to heat. Such up-regulation is lost in the ppts triple mutant at the protein level, whereas transcripts accumulation appeared unperturbed, thus providing pieces of evidence that PPTs could be involved in

the positive post-transcriptional regulation of CLPB3 upon stresses which eventually confers higher tolerance to elevated temperatures.

In the effort to provide a plastid-specific perturbation of the chloroplast protein homeostasis, a genetic approach was employed by introgressing mutations in plastid proteases into the *ppts* triple mutant background. In these conditions, possible genetic interactions between the hampered plastid proteolytic machinery and the putative peptide-mediated response could be revealed. Three mutants in plastid proteases had been selected for this purpose. The *ftsh11* mutant was described in the previous section. The highly variegated *ftsh2* mutant lacks a thylakoidal transmembrane protease located on the thylakoid membranes (Takechi et al., 2000; Dogra et al., 2019). Finally, the virescent *clpr1* has the CLP multisubunit stromal protease hampered since it lacks a non-catalytic subunit of the core enzyme (Pulido et al., 2016; Llamas et al., 2017; Pulido et al., 2017). Quadruple mutants were generated by manual crossing and isolated through PCR-based screening. The obtained quadruple mutants displayed no major differences from the phenotypes observed in the parental single mutants. Nevertheless, their photosynthetic efficiencies were measured to evaluate if the functionality of chloroplasts was further perturbed by the concomitant absence of the transporters. Measurements were performed on plants grown on soil in standard conditions for 15 days. Interestingly, all of them displayed a modest decrease in the F_v/F_m values relative to the single mutants, indicating the existence of genetic interactions between the protease activity and the peptide extrusion in chloroplasts. To test whether CLPB3 accumulation was somehow affected also in these conditions, its relative abundance was evaluated as well. As previously observed, the *ppts* triple mutant accumulated more CLBP3 relative to the wild type. The same was observed for all three mutants in plastid proteases. This was already detected by mass spectrometry analyses in mutant backgrounds defective in the proteolytic activity mediated by the protease of interest (Kim et al., 2013a; Adam et al., 2019; Dogra et al., 2019). Remarkably, CLPB3 was found to be less accumulated in *ppts fish* mutants, whereas in *ppts clpr1* background it was more abundant. Such a result further supports the interactions in place between protein degradation, peptide extrusion and the activation of protein quality control systems.

Conclusions and Perspectives

In conclusion, the data here reported can delineate a working model in which PPTs are peptide transporter located on the chloroplast envelope. Under standard growing conditions, PPTs could contribute to the removal of peptides originated by the normal protein turnover from the stroma. Upon challenges to the chloroplast protein homeostasis, peptide production is increased due to a higher proteolysis rate of the accumulating damaged proteins. The resulting peptides are then extruded from chloroplasts into the cytoplasm by PPTs. This event has somehow a role in regulating CLPB3 post-transcriptionally leading to its higher accumulation in chloroplasts. The failed CLPB3 upregulation could be responsible for the higher sensitivity of *ppts* triple mutant to the chloroplast protein homeostasis.

From a future perspective, the purification of PPT proteins would be instrumental to test their activity also *in vitro* by coupling the ATP hydrolysis with the presence of their substrate (*i.e.* peptides) as recently reported (Saxberg et al., 2021). Currently, it is not clear how the peptide efflux contributes to CLPB3 regulation after heat treatment. Additionally, it remains to investigate if PPTs activity influences nuclear gene expression. The transcriptome analysis of the *ppts* triple mutant both under standard conditions and after heat treatment will be performed to identify PPTs-dependent genes and to correlate the PPTs-mediated peptide efflux with the retrograde signalling. As mentioned, the observations through confocal microscopy of mesophyll tissue co-expressing PPTs fluorescence-tagged chimerae, revealed, in addition to their plastid-localisation, that PPTs are close suggesting possible interactions. Indeed, PPTs, being half-transporters and, thus, requiring a partner molecule to produce a complete functional transporter, could either form homodimers, as in the case of MDL1 and HAF-1 or heterodimers, as occurs for TAP1 and TAP2 in *H. sapiens* (Nijenhuis and Hämmerling, 1996; Young et al., 2001; Haynes et al., 2010). The results obtained from both the peptide-efflux detection and the yeast complementation assay support the idea that PPTs form homodimers, as the presence of just one of the proteins was sufficient to either produce an efflux from chloroplasts or to complement the yeast heat sensitivity (Young et al., 2001; Haynes et al., 2010). Nevertheless, these observations provide indirect pieces of evidence for this conclusion which will be reinforced by a Split-ubiquitin screen (Grefen et al., 2007).

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