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Control of plastidial metabolism by the Clp protease complex

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20 **Abstract**

21 Plant metabolism is strongly dependent on plastids. Besides hosting the
22 photosynthetic machinery, these endosymbiotic organelles synthesize starch, fatty
23 acids, amino acids, nucleotides, tetrapyrroles, and isoprenoids. Virtually all
24 enzymes involved in plastid-localized metabolic pathways are encoded by the
25 nuclear genome and imported into plastids. Once there, protein quality control
26 systems ensure proper folding of the mature forms and remove irreversibly
27 damaged proteins. The Clp protease is the main machinery for protein degradation
28 in the plastid stroma. Recent work has unveiled an increasing number of client
29 proteins of this proteolytic complex in plants. Notably, a substantial proportion of
30 these substrates are required for normal chloroplast metabolism, including
31 enzymes involved in the production of essential tetrapyrroles and isoprenoids such
32 as chlorophylls and carotenoids. The Clp protease complex acts in coordination
33 with nuclear-encoded plastidial chaperones for the control of both enzyme levels
34 and proper folding (i.e. activity). This communication involves a retrograde
35 signaling pathway, similarly to the unfolded protein response previously
36 characterized in mitochondria and endoplasmic reticulum. Coordinated Clp
37 protease and chaperone activities appear to further influence other plastid
38 processes, such as the differentiation of chloroplasts into carotenoid-accumulating
39 chromoplasts during fruit ripening.

40

41 **Keywords:** carotenoid, chaperone, chlorophyll, chloroplast, Clp protease,
42 chromoplasts, isoprenoid, proteostasis, tetrapyrrole, unfolded protein response

43 ## Introduction

44 Plastids are central metabolic factories for plant cells. Besides being the site of
45 carbon, nitrogen, and sulfur assimilation, they synthesize starch, fatty acids, amino
46 acids, nucleotides (purine and pyrimidine bases), tetrapyrroles (including heme
47 and chlorophyll), and isoprenoids (such as carotenoids, and the side chain of
48 chlorophylls). In fact, their classification into different plastid types largely depends
49 on the metabolites that they store: chloroplasts contain chlorophylls, chromoplasts
50 accumulate carotenoids, amyloplasts are starch-rich plastids, and elaioplasts are
51 specialized in storing lipids (Jarvis and Lopez-Juez, 2013; Rolland *et al.*, 2012;
52 Sakamoto *et al.*, 2008).

53 Similar to mitochondria, plastids are endosymbiotic organelles that retain their own
54 genome (plastome). The *Arabidopsis thaliana* plastome contains 45 RNA-coding
55 genes and 87 protein-coding genes, but only two of them are involved in processes
56 other than plastome gene expression or photosynthesis: *accD* encodes the
57 carboxytransferase beta subunit of the acetyl-CoA carboxylase complex (which
58 catalyzes the first committed step in fatty acid biosynthesis) and *clpP1* encodes a
59 proteolytic subunit of the stromal Clp (Caseinolytic protease) complex (Sato *et al.*,
60 1999). Therefore, virtually all proteins involved in plastidial metabolism (including
61 enzymes) are encoded by the nuclear genome and imported into plastids. Most of
62 these plastid-targeted proteins bear an N-terminal targeting signal called plastid
63 transit peptide. They are synthesized in the cytosol and transported into plastids in
64 an energy-dependent process mediated by molecular machines (translocons)
65 located in the outer and inner envelope membranes (Sakamoto *et al.*, 2008). At
66 the inner membrane their transit peptide is cleaved and the mature forms of the
67 imported proteins are properly folded, assembled, and sorted to their particular
68 subplastidial destination by a separate targeting process. Inside plastids, protein
69 quality control (PQC) systems formed by chaperones and proteases promote
70 correct protein folding and remove irreversibly damaged proteins. To ensure an
71 appropriate supply of functional proteins, retrograde (plastid-to-nucleus) signaling
72 pathways adjust nuclear gene expression to particular plastid needs, e.g. in
73 response to environmental challenges (Chan *et al.*, 2016; Grimm *et al.*, 2014).
74 Regulated proteolysis additionally adjust protein levels and hence activity to the

specific requirements of individual plastids (Nishimura *et al.*, 2016, 2017; Sakamoto, 2006).

The multimeric Clp complex is the main stromal protease

Among the several proteases found in plant plastids, the serine-type Clp protease complex is the main machinery for processive degradation of proteins in the plastid stroma (Nishimura *et al.*, 2016; Nishimura and van Wijk, 2015). One of the roles of this housekeeping protease appears to be the removal of proteins that do not fold properly or lose their native conformation and become misfolded in the stroma (Nishimura and van Wijk, 2015; Zybailov *et al.*, 2009). Because misfolded proteins tend to aggregate, the Clp protease complex might also prevent the formation of potentially toxic protein aggregates (Llamas *et al.*, 2017; Nishimura and van Wijk, 2015; Zybailov *et al.*, 2009). Additional PQC roles suggested for the Clp protease include the survey of proteins translocated into plastids, clearing proteins that remain unprocessed or misfolded after import but also removing proteins that aggregate and become stuck in the import channel (Flores-Perez *et al.*, 2016; Huang *et al.*, 2016; Sjogren *et al.*, 2014).

Similar to other prokaryotic-type ATP-dependent plastidial proteases (such as FtsH and Lon), the Clp complex contains two differentiated domains: a barrel-shaped proteolytic core with the catalytic center positioned inside the chamber to prevent unspecific protein degradation and a chaperone ring that delivers the protein substrates. But unlike FtsH and Lon, in the Clp protease these two characteristic domains are separated into individual multiprotein subcomplexes (Figure 1). The subunit composition of the Clp protease is similar in all plastid types (D'Andrea *et al.*, 2018; Peltier *et al.*, 2004). The proteolytic core (ClpRP) is formed by two asymmetric rings (P and R) and stabilized by plant-specific ClpT1 and ClpT2 proteins (Kim *et al.*, 2015; Sjogren and Clarke, 2011). The P ring is formed by a 1:2:3:1 ratio of proteolytically active subunits ClpP3, ClpP4, ClpP5 and ClpP6. The R ring contains the plastome-encoded ClpP1 subunit but also non-catalytic ClpR1, ClpR2, ClpR3 and ClpR4 proteins in a 3:1:1:1:1 ratio (Nishimura *et al.*, 2016; Nishimura and van Wijk, 2015; Olinares *et al.*, 2011; Peltier *et al.*, 2004). Proteins entering the catalytic ClpRP barrel are assumed to be degraded into

107 peptide fragments (5-10 residues) that are ejected from the core cavity through
108 lateral pores (Peltier *et al.*, 2004).

109 Substrate access to the proteolytic core is controlled by a hexameric ring
110 presumably formed by homooligomers of the Hsp100-type chaperones ClpC1,
111 ClpC2, and ClpD (hence referred to it as ClpCD). Mutants defective in ClpC1 are
112 pale-green but knock-out lines of ClpC2 or ClpD show a wild-type phenotype,
113 supporting a major housekeeping role for the former. These chaperones
114 (particularly ClpC1) can directly recognize substrates and unfold them for
115 continuous translocation into the proteolytic chamber in an ATP-dependent
116 manner (Nishimura *et al.*, 2016; Nishimura and van Wijk, 2015; Peltier *et al.*, 2004).
117 In other cases, substrates are recognized by adaptors and then delivered to the
118 chaperones (Figure 1). Several pathways involving such adaptors are known. One
119 of them involves the recognition of client proteins by a binary adaptor formed by
120 ClpF and ClpS1 (Nishimura *et al.*, 2015). The current model proposes that ClpF,
121 ClpS1, or a ClpF-ClpS1 complex recognizes the protein client and forms a ClpF-
122 ClpS1-substrate complex that then binds to the Hsp100 chaperone ring of the Clp
123 protease complex. A second pathway involves another chaperone type, Hsp70,
124 which can interact with ClpC1 to synergistically promote the unfolding process (Shi
125 and Theg, 2010; Su and Li, 2010). Hsp70 chaperones use J-proteins as adaptors
126 that guide them to protein clients and confer substrate specificity. After recognition
127 of specific proteins by particular J-protein adaptors, the substrates are delivered to
128 Hsp70, which then interacts with ClpC1 for unfolding and subsequent ClpRP-
129 mediated degradation (Pulido *et al.*, 2016; Pulido *et al.*, 2013). The possibility of
130 additional ways of targeting proteins to ClpC1, ClpC2, and ClpD chaperones
131 remains open (Pulido *et al.*, 2017). It is generally assumed that specific signals for
132 degradation (named degrons) might be exposed in protein substrates that need to
133 be removed. The observation that the N-terminus of proteins is a major
134 determinant of protein turnover in prokaryotic and eukaryotic organisms led to
135 propose the N-end rule, which states that particular N-terminal residues act as
136 triggers for whole protein degradation. However, the identification of degrons and
137 the relevance of the N-end rule for the recognition of Clp protease targets by the
138 ClpCD ring chaperones or their adaptors (J-proteins/Hsp70, ClpF/ClpS1) remains
139 an open question (Rowland *et al.*, 2015).

140

141 **Several metabolic enzymes are clients of the Clp protease**

142 Despite the central relevance of the Clp protease in PQC and plastidial
143 proteostasis, only a few proteins have been conclusively shown to be degraded by
144 this proteolytic complex. As true Clp protease clients, they (a) show increased
145 protein levels (but not transcripts) in mutants defective in Clp protease activity, (b)
146 interact with chaperones of the ClpCD ring or their adaptors, and (c) are degraded
147 at a slower rate in mutants of the chaperone or/and catalytic domains of the Clp
148 complex. Interestingly, most of the proteins experimentally demonstrated to meet
149 these criteria have a role in plastidial metabolism. Besides the thylakoid membrane
150 copper transporter PAA2/HMA8, a protein with stromal domains that is degraded
151 by the Clp protease by mechanisms that are not yet fully understood (Tapken *et*
152 *al.*, 2015), four enzymes have been found to be Clp substrates in Arabidopsis
153 (Figure 1). The identified enzymes and the pathways in which they participate are
154 described below. We also discuss other plastidial enzymes that accumulate upon
155 interference with Clp protease activity but are not yet confirmed to be direct clients
156 of this proteolytic complex.

157

158 *Glutamyl-tRNA reductase and tetrapyrrole biosynthesis.*

159 The enzyme glutamyl-tRNA (GluT) reductase (GluTR) catalyzes an early step in
160 the tetrapyrrole biosynthesis pathway (Figure 1). GluT, produced after ligation of
161 Glu to tRNA(Glu), is reduced to Glu-1-semialdehyde by GluTR and then
162 transaminated to 5-aminolevulinic acid (ALA), the first committed precursor of the
163 pathway. Tetrapyrrole products are central for chloroplast metabolism as they are
164 required for photosynthesis (chlorophylls), nitrogen and sulfur assimilation
165 (siroheme), redox reactions (heme), photoperception (phytochromobilin), and
166 even retrograde signaling (Chan *et al.*, 2016; Grimm *et al.*, 2014). Tetrapyrrole
167 biosynthesis is tightly regulated to balance the production of chlorophylls and heme
168 and hence prevent the accumulation of metabolic intermediates that can potentially
169 cause severe photooxidative damage. Thus, chlorophyll biosynthesis needs to be
170 downregulated in darkness to prevent excessive accumulation of
171 protochlorophyllide in the dark (Meskauskiene *et al.*, 2001).

172 As a key regulatory enzyme of the tetrapyrrole pathway, GluTR is controlled at
173 multiple levels. Among them, interaction of the N-terminal region of GluTR with the
174 GluTR binding protein (GBP) results in association of part of the stromal enzyme
175 to the thylakoid membrane (Czarnecki *et al.*, 2011). It is proposed that this
176 mechanism allows two separate pools of GluTR for the separate production of ALA
177 for heme in the thylakoid membrane and for chlorophylls in the stroma. The N-
178 terminal domain of GluTR harbors aggregation-prone motifs, but interaction with
179 the plastidial chaperone SRP43 efficiently prevents aggregation, thus enhancing
180 the stability of the protein and promoting its localization to the thylakoid membrane
181 (Wang *et al.*, 2018). Furthermore, this N-terminal region can also interact with the
182 ClpF and ClpS1 adaptors and the ClpC1 chaperone for Clp protease-mediated
183 degradation of the enzyme (Apitz *et al.*, 2016; Nishimura *et al.*, 2015; Nishimura *et*
184 *al.*, 2013). A truncated GluTR protein lacking the N-terminal region remains in the
185 stroma and is more stable during prolonged darkness, causing an enhanced
186 accumulation of protochlorophyllide and a necrotic phenotype upon reillumination
187 (Apitz *et al.*, 2016). Consistent with the role of the N-terminal domain as a degron,
188 the rate of GluTR proteolysis in the dark is increased in the absence of GBP (i.e.
189 when the degron is exposed) but decreased in mutants impaired in ClpC1 or the
190 proteolytic core subunit ClpR2 (Apitz *et al.*, 2016). While GluTR accumulates at
191 higher levels in ClpC1 and ClpS1 mutants, the degradation rate of the enzyme in
192 the dark appears not to be altered when ClpS1 is missing. It is therefore concluded
193 that ClpC1 might have a more relevant role than ClpS1 (or ClpF) to recognize and
194 direct GluTR into the Clp proteolytic core for degradation (Apitz *et al.*, 2016). A
195 proposed model (Wang *et al.*, 2018) suggests that the interaction of SRP43 with
196 unfolded GluTR after plastid import might ensure proper enzyme folding and
197 prevent aggregation. Folded GluTR could then interact with GBP for thylakoid
198 membrane targeting. It is likely that competition for the N-terminal domain between
199 GluTR interactors (SPR43 and GBP) and Clp adaptors or/and chaperones, but
200 also structural changes triggered by metabolite binding, misfolding or aggregation,
201 might play a role in determining the degradation rate of this important enzyme.
202 Strikingly, the N-terminus is relevant for the Clp-mediated degradation of GluTR
203 not only in plants but also in bacteria (Wang *et al.*, 1999), suggesting a remarkable
204 evolutionary conservation of the mechanisms controlling GluTR turnover.

205

206 *Chlorophyllide a oxygenase and chlorophyll biosynthesis.*

207 The final steps of chlorophyll biosynthesis (Figure 1) involve the incorporation of
208 molecular oxygen into the C7-methyl group of the tetrapyrrole ring (porphyrin) of
209 chlorophyllide *a* to produce chlorophyllide *b* followed by the esterification of
210 isoprenoid-derived phytyl diphosphate to both chlorophyllides to form chlorophylls
211 *a* and *b*, respectively (Kim *et al.*, 2013b). Chlorophyll *a* occurs in antenna
212 complexes and reaction centers, whereas chlorophyll *b* is absent from reaction
213 centers. Chlorophyll *b* levels influence the antenna size of photosystem II and are
214 controlled by the stability of chlorophyllide *a* oxygenase (CAO), the enzyme that
215 catalyzes the conversion of chlorophyllide *a* to *b* (Sakuraba *et al.*, 2007; Yamasato
216 *et al.*, 2005). Degradation of CAO when chlorophyll *b* is present requires the N-
217 terminal domain (or A domain) of the enzyme (Yamasato *et al.*, 2005) and is
218 impaired in ClpC1-defective mutants (Nakagawara *et al.*, 2007). The current model
219 is that chlorophyll *b* may modulate the structure of the A domain either by direct
220 interaction or via other factors. The induced change of conformation would then
221 expose a degron for ClpC1-mediated unfolding and eventual ClpRP-mediated
222 degradation of the whole enzyme (Sakuraba *et al.*, 2009). CAO localizes in
223 thylakoid and envelope membranes but lacks obvious membrane-spanning
224 domain (Eggink *et al.*, 2004; Yamasato *et al.*, 2005). It is therefore possible that
225 interaction of the N-terminal domain of CAO with ClpC1 (or an adaptor) occurs at
226 the periphery of these plastidial membranes.

227

228 *Deoxyxylulose 5-phosphate synthase and isoprenoid biosynthesis.*

229 The isoprenoid phytol chain of chlorophylls is synthesized from precursors derived
230 from the stromal methylerythritol 4-phosphate (MEP) pathway. In fact, a strict
231 coordination between the tetrapyrrole and the MEP pathways is essential to
232 prevent the formation of oxidative metabolic intermediates (Kim *et al.*, 2013b).
233 MEP-derived prenyl diphosphates isopentenyl diphosphate (IPP) and dimethylallyl
234 diphosphate (DMAPP) are also used for the production of other plastidial
235 isoprenoids involved in photosynthesis (tocopherols and carotenoids as
236 photoprotectants, and prenylquinones such as plastoquinone and phylloquinones

237 for electron transport) and signaling (including several types of retrograde signals,
238 hormones, and volatiles for environmental interactions) (Rodriguez-Concepcion
239 and Boronat, 2015). The control of protein turnover has a major impact on MEP
240 pathway enzymes including deoxyxylulose 5-phosphate (DXP) synthase (DXS),
241 DXP reductoisomerase (DXR), hydroxymethylbutenyl 4-diphosphate (HMBPP)
242 synthase (HDS), and HMBPP reductase (HDR) (Figure 1). All these enzymes
243 accumulate at higher levels in Arabidopsis plants with decreased Clp proteolytic
244 activity such as mutants defective in either ClpC1 or ClpRP subunits (Table 1)
245 (Flores-Pérez *et al.*, 2008; Kim *et al.*, 2013a; Kim *et al.*, 2009; Nishimura *et al.*,
246 2013; Rodriguez-Villalon *et al.*, 2009; Rudella *et al.*, 2006; Sauret-Güeto *et al.*,
247 2006; Welsch *et al.*, 2018; Zybailov *et al.*, 2009). However, only DXS has been
248 experimentally shown to be a direct Clp substrate based on the three criteria
249 described above (Figure 1). DXS can either directly interact with ClpC1 or be
250 delivered to this chaperone via J20, a J-protein adaptor of Hsp70 (Pulido *et al.*,
251 2016), as detailed below. DXR is more stable and does not rely on J20 for
252 degradation (Perello *et al.*, 2016; Pulido *et al.*, 2016). Instead, removal of excess
253 DXR takes place by packaging the enzyme in vesicles formed by engulfing stromal
254 fractions that likely retain Clp protease activity (Perello *et al.*, 2016). The
255 accumulation of MEP pathway enzymes does not change in mutants defective in
256 ClpF or ClpS1 adaptors with only one exception: HDS (Nishimura *et al.*, 2015;
257 Pulido *et al.*, 2016). However, HDS levels do not increase but decrease in the
258 mutants (Table 1) (Nishimura *et al.*, 2015), i.e. the opposite that it would be
259 expected if the ClpF/ClpS1 pathway delivers this enzyme to the Clp complex.
260 Beyond Arabidopsis, work in other systems has shown that the accumulation of
261 DXS and other MEP pathway enzymes is directly or indirectly controlled by the Clp
262 protease in tobacco (*Nicotiana tabacum*) chloroplasts (Moreno *et al.*, 2018),
263 tomato (*Solanum lycopersicum*) chromoplasts (D'Andrea *et al.*, 2018), the plastid-
264 like apicoplasts of the malaria parasite *Plasmodium falciparum* (Florentin *et al.*,
265 2017) and even bacterial cells (Ninnis *et al.*, 2009).

266 Consistent with the major contribution of DXS to the control of the MEP pathway
267 flux (Pokhilko *et al.*, 2015; Wright *et al.*, 2014), its activity is regulated by several
268 post-translational mechanisms (Rodriguez-Concepcion and Boronat, 2015),
269 including Clp-mediated degradation (Flores-Perez *et al.*, 2008; Llamas *et al.*, 2017;

270 Perello *et al.*, 2016; Pulido *et al.*, 2016; Welsch *et al.*, 2018). A shortage of MEP
271 pathway products upregulates DXS enzymatic activity and promotes the
272 accumulation of higher protein levels (Ghirardo *et al.*, 2014; Han *et al.*, 2013;
273 Pokhilko *et al.*, 2015). It is possible that binding of IPP or DMAPP to DXS causes
274 a conformational change that inactivates the enzyme and renders it more
275 susceptible to degradation (Pokhilko *et al.*, 2015). Stress episodes can also disrupt
276 the native structure of DXS, resulting in protein misfolding, and eventually
277 aggregation in chloroplasts. The high aggregation propensity of DXS was also
278 observed in cyanobacteria, the evolutionary ancestors of chloroplasts (Kudoh *et*
279 *al.*, 2017). Work in Arabidopsis has unveiled a PQC mechanism to deal with the
280 unwanted accumulation of inactive DXS proteins (Figure 1). The J-protein adaptor
281 J20 specifically binds misfolded forms of DXS and delivers them to the Hsp70
282 chaperone for either proper folding or degradation (Pulido *et al.*, 2013). The fate of
283 Hsp70-bound enzyme appears to depend on the relative abundance of Hsp100
284 chaperones that synergistically contribute to the unfolding process. Under normal
285 growth conditions, inactive Hsp70-bound DXS proteins are delivered to ClpC1 and
286 unfolded prior entrance into the ClpRP proteolytic chamber to be degraded.
287 Environmental episodes causing protein folding stress (e.g. heat) do not cause
288 changes in the levels of ClpC1 but induce the accumulation of ClpB3, another
289 plastidial member of the Hsp100 family. Unlike ClpC1 and the other Hsp100
290 chaperones associated to the ClpCD complex, ClpB3 lacks the IG(F/L) tripeptide
291 (also named P-loop motif) required for interaction with the ClpRP core subunits but
292 harbors a domain that allows efficient interaction with Hsp70 chaperones (Flores-
293 Perez *et al.*, 2016; Kim *et al.*, 2001; Peltier *et al.*, 2004; Pulido *et al.*, 2016).
294 Therefore, stress episodes activate the ClpB3-dependent pathway to unfold
295 Hsp70-bound DXS into the stroma for spontaneous refolding and reactivation
296 (Pulido *et al.*, 2016). This model implies that although the refolding strategy (via
297 ClpB3) is likely favored, Clp-mediated degradation (via ClpC1) is the housekeeping
298 pathway to remove inactive DXS. Most likely, both share the same goal, i.e. to
299 prevent protein aggregation in the plastid and maintain the enzymes in a
300 catalytically active form. The observation that double mutants defective in ClpB3
301 and ClpR2 were unable to survive under autotrophic conditions (Zybailov *et al.*,
302 2009) illustrates the key relevance of the seemingly antagonistic refolding and
303 degradation pathways for plant life. The common regulation of both the MEP and

304 the tetrapyrrole pathways by the Clp protease (Figure 1) likely contributes to finely
305 balance the supply of metabolic precursors for the production of chlorophylls (Kim
306 *et al.*, 2013b).

307

308 *Phytoene synthase and carotenoid biosynthesis.*

309 The activity of the MEP pathway enzymes generates prenyl diphosphates used by
310 the enzyme phytoene synthase (PSY) to produce phytoene, the first committed
311 intermediate of the carotenoid pathway. Carotenoids are essential for
312 photoprotection of the photosynthetic apparatus against excess light, act as
313 precursors of hormones and retrograde signals, and function as natural pigments
314 in non-photosynthetic organs such as flowers and fruits (Rodriguez-Concepcion *et*
315 *al.*, 2018). Yeast two-hybrid experiments followed by bimolecular fluorescence
316 complementation assays in plant cells showed that PSY, the main rate-determining
317 enzyme of the pathway (Fraser *et al.*, 2002), can physically interact with ClpS1 and
318 several Clp protease subunits, including ClpC1 and ClpD (Welsch *et al.*, 2018).
319 However, only the absence of ClpC1 results in increased enzyme levels as a
320 consequence of reduced PSY turnover rate, suggesting a dispensable contribution
321 of ClpS1 or ClpD in the delivery of the enzyme to ClpRP-mediated degradation
322 (Figure 1). As expected, PSY enzyme levels are also increased in mutants
323 defective in individual subunits of the ClpRP proteolytic core such as ClpP4 and
324 ClpR1. PSY stability further increases in the presence of ORANGE (OR), a J-like-
325 protein with chaperone activity that also promotes PSY enzymatic activity (Park *et*
326 *al.*, 2016; Pulido and Leister, 2018; Welsch *et al.*, 2018; Zhou *et al.*, 2015). OR
327 might play a similar role as ClpB3 in the case of DXS, i.e. promote correct PSY
328 folding (and hence enzymatic activity) and prevent its misfolding and eventual
329 aggregation by the Clp protease (Figure 1).

330 Similar to PSY, other carotenoid biosynthetic enzymes overaccumulate in mutants
331 defective in ClpC1 or ClpRP core subunits (Figure 1) (Welsch *et al.*, 2018). While
332 further experiments will be required to conclude whether these enzymes are true
333 Clp protease targets, the available evidence suggests that Clp-dependent
334 mechanisms contribute to coordinate the MEP pathway and the carotenoid
335 pathway by influencing the accumulation of multiple enzymes of both pathways.
336 Besides possible secondary effects eventually impacting enzyme levels, this

337 control mechanism involves the direct degradation of some enzymes, including
338 those catalyzing the main rate-determining steps (DXS and PSY). Because the Clp
339 protease also regulates the production of chlorophylls via direct control of GluTR
340 and CAO turnover (Figure 1), this proteolytic complex might further coordinate
341 chlorophyll and carotenoid biosynthesis for an efficient photosynthetic function in
342 chloroplasts. Recent results further suggest that the Clp protease might ensure a
343 proper supply of MEP-derived precursors for carotenoid biosynthesis in different
344 plastid types. Downregulation of Clp protease activity during tomato ripening led to
345 increased levels of both DXS and PSY enzymes in the carotenoid-accumulating
346 chromoplasts of ripe fruit (D'Andrea *et al.*, 2018). Importantly, Clp-defective fruits
347 developed aberrant chromoplasts with disorganized membranes and displayed an
348 orange color (instead of red) because they accumulated enhanced levels of the
349 pro-vitamin A carotenoid β-carotene (D'Andrea *et al.*, 2018). These data indicate
350 that Clp protease activity controls not only carotenoid biosynthesis (via DXS and
351 PSY stability) but also storage (via plastidial ultrastructure). It is conceivable that
352 during normal fruit ripening Clp protease function might facilitate the turnover and
353 removal of many other enzymes and structural proteins that lose their functionality
354 or that are not further required as chloroplasts differentiate into chromoplasts.

355

356 *Other enzymes and metabolic pathways.*

357 Genetic interference with the accumulation of Clp subunits perturbs the
358 stoichiometry in the complex and negatively impacts its protein degradation
359 activity. Based on the premise that decreased Clp proteolytic activity should lead
360 to increased accumulation of its protein targets, quantitative proteomic approaches
361 have been employed for the identification of potential Clp protease clients. Initially,
362 the steady state proteome of wild-type Arabidopsis plants was compared with that
363 of mutants defective in particular subunits of the complex (Kim *et al.*, 2013a; Kim
364 *et al.*, 2009; Rudella *et al.*, 2006; Sjogren *et al.*, 2006; Stanne *et al.*, 2009; Zybailov
365 *et al.*, 2009). While components of metabolic pathways impacting the production
366 of starch, fatty acids, amino acids, isoprenoids, and thiamine were found to be
367 upregulated in the mutants, this strategy per se could not distinguish between
368 primary and downstream effects (e.g. loss of photosynthetic activity and
369 downregulation of Calvin cycle enzymes) associated with the reduction of Clp

370 protease activity (Nishimura and van Wijk, 2015). Further biochemical approaches
371 using mutants defective in the adaptors ClpF and ClpS1 went a step forward and
372 identified GluTR as a target of the complex (Nishimura *et al.*, 2015; Nishimura *et*
373 *al.*, 2013). Other potential clients identified in these works are the enzymes
374 deoxyarabinoheptulosonate 7-phosphate synthase (DHS) and chorismate
375 synthase (CS), which catalyze the first and last reaction of the shikimate pathway,
376 respectively. While DHS isoforms were consistently found to be upregulated in
377 mutants defective in ClpC1 and ClpRP core subunits, the results for CS are much
378 less clear (Table 1). The product of the shikimate pathway, chorismate, is
379 converted into aromatic amino acids such as Phe or Tyr, which are also precursors
380 for the biosynthesis of phenylpropanoids (e.g. lignins, flavonols, and anthocyanins)
381 and isoprenoids such as tocopherols, plastoquinone, and phylloquinones (Figure
382 1).

383 More recently, induced downregulation of Clp complex subunits in tobacco led to
384 propose additional enzymes as potential Clp protease clients based on their
385 overaccumulation at early time points after triggering repression of Clp proteolytic
386 activity (Moreno *et al.*, 2018). Among them, two enzymes involved in thiamine
387 biosynthesis appear as good candidates: THI1 and THIC (Figure 1). These two
388 enzymes catalyze the first steps in the biosynthesis of the two moieties required
389 for the production of thiamine, thiazole (THI1) and pyrimidine (THIC) (Goyer,
390 2010). Supporting the conclusion that they might be true Clp protease targets, the
391 Arabidopsis THI1 and THIC proteins are accumulated at higher levels in mutants
392 defective in ClpC1 or ClpR2 (Table 1) (Nishimura *et al.*, 2013; Zybailov *et al.*,
393 2009). In the case of THI1, ClpS1 mutants contained increased protein levels in
394 one study (Nishimura *et al.*, 2013) but decreased in another (Nishimura *et al.*,
395 2015), preventing to reach a conclusion on the possible role of ClpS1 in delivering
396 this enzyme to degradation. In any case, it is interesting to note that thiamine
397 diphosphate (vitamin B1) serves as a cofactor for many enzymes (including DXS;
398 Figure 1) and hence represents an important node in the control of the plant cell
399 metabolism.

400

401 **Plastidial proteostasis requires communication with the nucleus**

402 Highly reproducible patterns deduced from quantitative proteomics analyses of
403 Clp-defective mutants include a strong reduction of photosynthetic capacity (in part
404 due to loss of thylakoid membrane homeostasis) and a systematic upregulation of
405 all stromal chaperone systems, with most consistent increases typically observed
406 for the stromal Hsp100 chaperone ClpB3 (Table 1). It was proposed that
407 accumulation of ClpB3 and stromal protein folding machineries in the mutants was
408 a response to deal with protein aggregation likely occurring when Clp protease
409 activity was defective (Nishimura and van Wijk, 2015; Zybailov *et al.*, 2009). By
410 downregulating the expression of the plastome-encoded ClpP1 subunit of the
411 ClpRP core in the unicellular alga *Chlamydomonas reinhardtii*, it was observed that
412 these chaperones and small heat shock proteins such as Hsp21 increased at both
413 transcript and protein levels (Ramundo *et al.*, 2014). A role for retrograde signaling
414 was later suggested in plants to increase the supply of nuclear-encoded plastid-
415 targeted chaperones when loss of Clp protease activity caused protein folding
416 stress in the plastid (Nishimura and van Wijk, 2015). More recently, the existence
417 of a mechanism upregulating the expression of nuclear genes encoding plastid-
418 targeted chaperones such as Hsp21, Hsp70, and ClpB3 was demonstrated in
419 Arabidopsis (Llamas *et al.*, 2017). The mechanism was designated chloroplast
420 unfolded protein response (cpUPR), based on its conceptual similarity to those
421 previously reported in mitochondria and endoplasmic reticulum (Hwang and Qi,
422 2018; Lin and Haynes, 2016). Although the term “unfolded” might be misleading
423 and it would be most appropriate to name it protein folding stress response, we will
424 keep using the term cpUPR to be consistent with the literature.

425 Studies using DXS as a model showed that plastid proteostasis involves a dynamic
426 balance between degradation and repair of structurally compromised proteins. In
427 non-stressed conditions, the Clp protease (via ClpC1) removes DXS proteins that
428 become non-functional (e.g. when losing their native conformation). However,
429 protein folding stress can occur when Clp protease activity becomes compromised
430 and cannot deal with the accumulation of misfolded proteins. For example,
431 malfunctioning of the plastome gene expression system can lead to altered levels
432 of the catalytic ClpP1 subunit (Flores-Pérez *et al.*, 2008), causing reductions in Clp
433 protease activity (because of distortions in the stoichiometry of the complex) that
434 eventually lead to protein aggregation (Llamas *et al.*, 2017). Protein folding stress

435 in the plastid somehow sends an unknown retrograde signal to upregulate the
436 expression of the nuclear gene encoding the transcription factor HsfA2 (Llamas *et*
437 *al.*, 2017). HsfA2 directly binds the promoters of genes encoding ClpB3 and other
438 plastidial chaperones, inducing their expression. As a result, more of these
439 chaperones are made and sent to plastids to alleviate protein folding stress in this
440 organelle. Higher levels of plastidial ClpB3 then promote the disaggregation of
441 DXS, i.e. the recovery pathway. It is expected that upregulating the supply of
442 plastidial chaperones would also increase the global folding capacity of the plastid,
443 which should contribute to effectively remove protein aggregates, promote correct
444 folding, and restore proteostasis.

445 Consistent with the conclusion that the basic PQC mechanisms are conserved
446 among different plastid types, the downregulation of Clp protease activity during
447 tomato fruit ripening also resulted in increased accumulation of chaperones in the
448 chromoplasts of ripe fruit (D'Andrea *et al.*, 2018). Specifically, genes encoding
449 tomato homologues of ClpB3 and OR were upregulated in Clp-defective fruit,
450 leading to increased levels of the corresponding proteins and subsequent
451 protection of DXS and PSY enzymes, respectively, against degradation.
452 Interestingly, OR chaperones not only promote PSY stability and enzymatic activity
453 but also act as positive regulators of chromoplast differentiation and repressors of
454 carotenoid degradation (Chayut *et al.*, 2017; Park *et al.*, 2016; Zhou *et al.*, 2015).
455 It is proposed that Clp protease-regulated expression of nuclear genes encoding
456 chaperones such as OR and ClpB3 might be a compensatory mechanism
457 triggered when Clp protease activity is compromised during fruit ripening
458 (D'Andrea *et al.*, 2018). When Clp protease activity becomes limiting, the resulting
459 defective protein turnover might impair chromoplast differentiation. The
460 accumulation of non-functional proteins, however, would trigger a cpUPR to
461 eventually mitigate protein folding stress (e.g. via ClpB3) and restore chromoplast
462 development (e.g. via OR). The observation that, similar to that described in
463 Arabidopsis (Llamas *et al.*, 2017), tomato HsfA2 regulates the expression of
464 plastidial chaperones to increase the protection against protein folding stress
465 (Fragkostefanakis *et al.*, 2015), suggests that several components of the organelle
466 UPR might be conserved among different species. A better understanding of how
467 the Clp protease coordinates with chaperones such as ClpB3 and OR in different

468 plastid types should contribute to more rational engineering approaches for
469 improved production of carotenoids and other plastidial metabolites.

470

471 Open questions

472 While significant progress has been achieved in the last few years on the
473 identification of Clp protease protein substrates, we still have an incomplete map
474 of the plastidial pathways impacted by this major proteolytic machinery. In addition,
475 substrates involved in the storage of plastidial metabolites remain to be identified.
476 For example, Clp-defective mutants typically show increased levels of proteins
477 associated to plastoglobules, which are thylakoid-derived compartments that store
478 isoprenoids and other lipids (van Wijk and Kessler, 2017). However, it is unclear
479 whether this is a primary effect of reducing the turnover of Clp protein substrates
480 or a secondary consequence derived from interferences with thylakoid membrane
481 homeostasis or stress responses (Nishimura and van Wijk, 2015). Recent
482 approaches using inducible lines have been useful to distinguish early from late
483 accumulating proteins (Moreno *et al.*, 2018; Ramundo *et al.*, 2014). Clp-trapping
484 strategies similar to those used in bacteria (Flynn *et al.*, 2003; Trentini *et al.*, 2016)
485 were expected to also be useful to identify bona fide plant targets, despite the
486 challenges derived from the much higher complexity and essential nature of the
487 plastidial Clp protease. Recent experiments using *in vivo* tagged core complexes
488 containing inactive ClpP3 and ClpP5 variants in Arabidopsis could not identify new
489 Clp protease clients, proposedly because the bottleneck for degradation might not
490 be the catalytic activity of the ClpRP core but substrate recognition and unfolding
491 (Liao *et al.*, 2018). *In vivo* substrate trapping through partial inactivation of ClpC1,
492 ClpC2 or ClpD might therefore provide an alternative, more efficient strategy to
493 identify protein clients.

494 To date, only ClpC1-dependent substrates have been described, suggesting that
495 the other chaperones of the ClpCD ring (ClpC2 and ClpD) are partially redundant
496 (Kovacheva *et al.*, 2007; Nishimura *et al.*, 2015). Strikingly, ClpD is found
497 exclusively in stroma, whereas ClpC1 and ClpC2 are also present in association
498 with envelope membranes for PQC of imported proteins (Sjogren *et al.*, 2014). A
499 related question is how the substrates of the Clp protease are selected for
500 degradation, while preventing the removal of functional proteins and off-targets.

501 While the relevance of the N-end rule for chloroplast protein turnover remains
502 unclear (Rowland *et al.*, 2015), it is likely that the first step in the degradation
503 pathway could be exposure of a degron after a conformational change caused by
504 metabolite binding (as in the case of CAO) or by misfolding (as in the case of
505 GluTR, DXS and PSY). Degrons could also be exposed after removal of proteins
506 bound to the motifs required for degradation (as in the case of GluTR). Then, the
507 degron could be recognized by the ClpF/ClpS1 tandem or the J-protein/Hsp70
508 system and delivered to ClpC1 or any other Hsp100 component of the Clp
509 chaperone ring for unfolding and ClpRP-mediated proteolysis. Rather than a
510 particular amino acid sequence, degrons might comprise a secondary structure
511 and/or a post-translational modification (phosphorylation, acetylation, etc), which
512 enormously complicate their identification. For instance, arginine phosphorylation
513 was found to direct substrates for degradation by the Clp protease in bacteria
514 (Trentini *et al.*, 2016). Furthermore, it is likely that some enzymes could be
515 degraded by different proteases in the plastid (Nishimura *et al.*, 2016). Considering
516 the protease network as a whole (Majsec *et al.*, 2017) would also be required to
517 fully understand how plastidial metabolism is regulated inside plastids in response
518 to developmental and environmental cues.

519 Finally, the molecular pathway connecting Clp protease defects with enhanced
520 expression of genes for plastid-targeted chaperones remains unknown. Recent
521 results have shown that GUN1, a central regulator of plastid to nucleus
522 communication, is degraded by the Clp protease via ClpC1 (Wu *et al.*, 2018).
523 Another protein involved in tetrapyrrole-dependent retrograde signaling, GUN5, is
524 also upregulated in Arabidopsis mutants defective in ClpC1 and Clp protease
525 activity (Table 1). A role for the Clp protease in controlling the turnover of
526 retrograde signaling mediators such as GUN1 and GUN5 is expected to represent
527 a powerful tool in the communication of plastidial protein folding stress to the
528 nucleus. However, neither GUN1 nor GUN5 appear to be required for the
529 upregulation of genes encoding plastidial chaperones such as Hsp21, Hsp70, and
530 ClpB3 that occurs soon after triggering protein aggregation in chloroplasts by
531 interfering with Clp protease activity (Llamas *et al.*, 2017). Alternative good
532 candidates to participate in this process are the DNA-interacting factors WHIRLY
533 (pTAC1/WHY1 and pTAC11/WHY3) and pTAC12/HEMERA, which have been

534 located in plastidial transcriptionally active chromosomes (pTACs) or nucleoids but
535 also in the nucleus (Melonek *et al.*, 2016). These proteins are more abundant in
536 Clp-defective plants (Table 1) (Moreno *et al.*, 2018) and display the same size in
537 chloroplasts and nuclei, suggesting that their accumulation in plastids with a
538 compromised Clp proteolytic activity might cause their translocation to the nucleus
539 to regulate nuclear gene expression. Future work should contribute to confirm this
540 hypothesis and identify other dual-localized factors potentially involved in the
541 cpUPR-associated retrograde signaling pathway that allows plants to overcome
542 protein folding stress in the plastid.

543

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TABLES

Table 1. Levels of plastidial proteins in Clp-defective Arabidopsis mutants.

			Zybailov et al., 2009	*Kim et al., 2009*	*Kim et al., 2013*	*Nishimura et al., 2013*			*Nishimura et al., 2015*	
	Accession	**Name**	***clpr4-1***	***clpp3-1***	***clpc1-1***	***clps1 clpc1-1***	***clps1***	***clps1***	***clpr2-1***	***clpf1-1***
MEP pathway	At4g15560	DXS	nd	nd	**higher**	**22,88**	nd	nd	nd	nd
	At5g62790	DXR	3,53	**2,06**	**2,05**	1,73	0,78	1,09	1,63	1,06
	At2g02500	MCT	nd	3,48	nd	nd	nd	0,90	**higher**	1,06
	At2g26930	CMK	nd	0,71	nd	nd	nd	0,70	nd	0,25
	At1g63970	MDS	nd	1,61	nd	nd	nd	0,78	nd	0,81
	At5g60600	HDS	**5,10**	**3,54**	**2,05**	**2,58**	0,71	**0,63**	**9,25**	**0,50**
	At4g34350	HDR	nd	nd	**3,38**	**higher**	1,12	1,02	nd	0,50
Shikimate pathway	At4g33510	DHS2	nd	**3,30**	**higher**	**higher**	nd	0,37	3,01	0,74
	At4g39980	DHS1	nd	2,65	nd	nd	nd	nd	8,16	nd
	At1g48850	CS	nd	nd	2,33	1,06	1,24	1,03	nd	1,27
Thiamine pathway	At5g54770	THI1	2,37	1,63	**17,02**	**4,26**	**2,25**	**0,69**	**5,35**	**0,54**
	At2g29630	THIC	nd	0,42	**6,98**	**2,41**	0,57	0,68	5,01	0,74
Transcription factors	At1g14410	WHY1	**5,30**	nd	nd	nd	nd	1,76	2,67	nd
	At2g02740	WHY3	nd	**7,37**	nd	**higher**	nd	0,57	nd	0,51
	At2g34640	HEMERA	nd	1,55	nd	nd	nd	nd	nd	nd
Other proteins	At3g59400	GUN4	nd	0,90	nd	nd	nd	0,88	nd	1,01
	At5g13630	GUN5	6,71	1,35	**3,46**	**3,52**	**0,32**	1,53	**7,04**	0,77
	At5g15450	ClpB3	**4,46**	**4,84**	**3,39**	**2,62**	**1,62**	**0,60**	**3,67**	**0,55**

Numbers indicate the ratio of protein levels in the indicated Arabidopsis mutant relative to the wild-type (fold-change)

Colors indicate when protein levels in the mutant are statistically higher (blue) or lower (red) compared to the wild-type

higher, proteins detected only or preferentially in the mutant

nd, not detected

827 **FIGURE LEGENDS**

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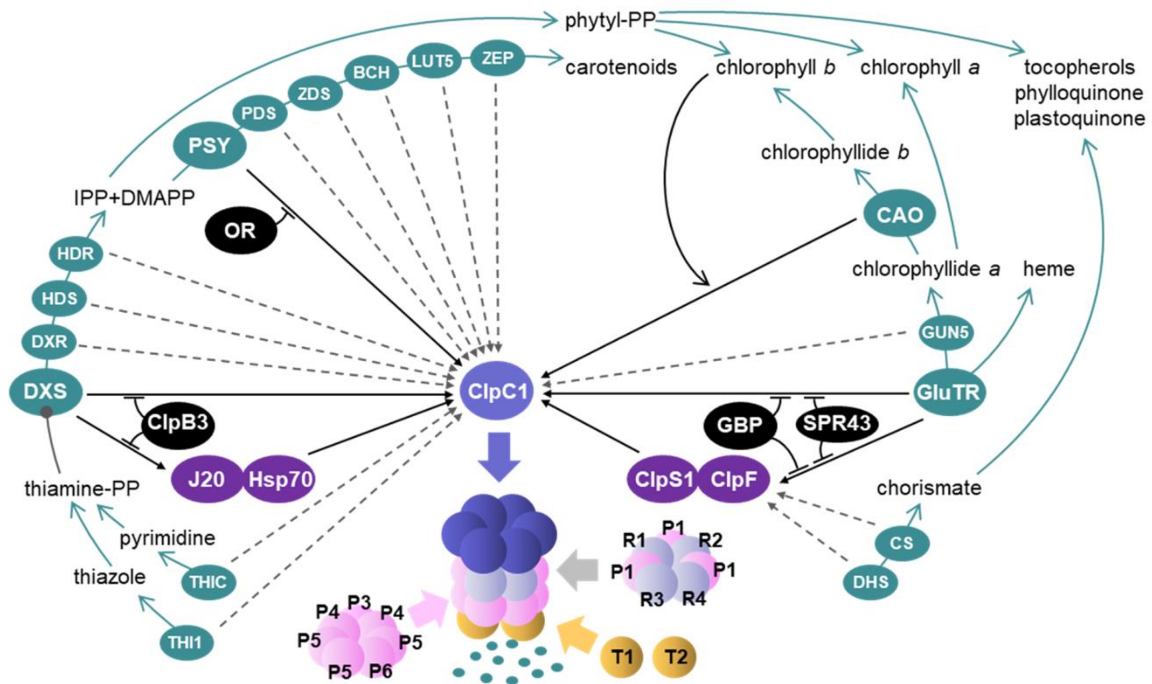

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830 **Figure 1. Degradation of plastidial enzymes by the Clp protease complex.**
831 Enzymes are indicated in green, adaptor systems are represented in purple, and
832 proteins that prevent degradation are shown in black. Green arrows represent
833 metabolic pathways. Solid black arrows mark confirmed interactions and dashed
834 black arrows indicate potential interactions. The structure of the Clp protease
835 complex and its components is also shown.