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Clp Protease and OR Directly Control the Proteostasis of Phytoene Synthase, the Crucial Enzyme for Carotenoid Biosynthesis in Arabidopsis

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ABSTRACT
Phytoene synthase (PSY) is the crucial plastidial enzyme in the carotenoid biosynthetic pathway. However, its post-translational regulation remains elusive. Likewise, Clp protease constitutes a central part of the plastid protease network, but its substrates for degradation are not well known. In this study, we report that PSY is a substrate of the Clp protease. PSY was uncovered to physically interact with various Clp protease subunits (i.e., ClpS1, ClpC1, and ClpD). High levels of PSY and several other carotenogenic enzyme proteins overaccumulate in the clpc1, clpp4, and clpr1-2 mutants. The overaccumulated PSY was found to be partially enzymatically active. Impairment of Clp activity in clpc1 results in a reduced rate of PSY protein turnover, further supporting the role of Clp protease in degrading PSY protein. On the other hand, the ORANGE (OR) protein, a major post-translational regulator of PSY with holdase chaperone activity, enhances PSY protein stability and increases the enzymatically active proportion of PSY in clpc1, counterbalancing Clp-mediated proteolysis in maintaining PSY protein homeostasis. Collectively, these findings provide novel insights into the quality control of plastid-localized proteins and establish a hitherto unidentified post-translational regulatory mechanism of carotenogenic enzymes in modulating carotenoid biosynthesis in plants.

Key words: carotenoid, phytoene synthase, clp protease, OR, post-translational regulation, Arabidopsis

INTRODUCTION
Carotenoids play important roles in photosynthesis, photoprotection, phytohormone biosynthesis, and flower/fruit color development (Ruiz-Sola and Rodríguez-Concepción, 2012; Nisar et al., 2015; Yuan et al., 2015a; Sun et al., 2017). Despite great progress in characterizing carotenoid metabolic pathway enzymes, the control mechanisms that maintain carotenogenic enzyme proteostasis are largely unknown.

Phytoene synthase (PSY) is the crucial enzyme for carotenoid biosynthesis and directs carbon flow into the carotenoid biosynthetic pathway (Hirschberg, 1999; Wurtzel et al., 2012). Because of its role in governing carotenoid accumulation, PSY and its regulation have been subjected to intensive investigation.
Post-translational Regulation of PSY by Clp and OR

Identification of PSY-interacting proteins is a feasible strategy to explore proteins involved in its post-translational regulation in plastids (Yuan et al., 2015b; Zhou et al., 2015; Chayut et al., 2017). By employing co-immunoprecipitation (coIP) in conjunction with analysis by mass spectrometry (MS), we identified ClpC1 as a potential PSY-interacting protein. We provide evidence that Clp protease physically interacted with PSY to mediate PSY degradation. By contrast, OR as a major post-translational regulator of PSY promoted its stabilization. Together, Clp protease and OR maintain the homeostasis of PSY in the plastids to assure optimal enzyme protein abundance for adequate carotenoid biosynthesis in plants.

RESULTS

Identification of ClpC1 as a Potential PSY-Interacting Protein

To discover PSY-interacting proteins, we generated Arabidopsis expressing either 35S:PSY-GFP (Supplemental Figure 1) or 35S:GFP control. Proteins from 35S:PSY-GFP plants along with the 35S:GFP control lines were extracted from four biological replicates and used for the coIP experiments. The coIP products were separated by SDS-PAGE gels and identified by liquid chromatography–tandem MS analysis. A total of 202, 235, 238, and 163 proteins were identified from four biological replicates of colPs of Arabidopsis expressing PSY-GFP fusion protein, whereas 140, 168, 140, and 122 proteins were found from GFP-only controls in quadruplicates, respectively (Supplemental Table 1). Among these proteins, 31 were common to the PSY-GFP samples but absent in the controls (Supplemental Table 2). Interestingly, although the interaction between PSY and OR was recently demonstrated (Zhou et al., 2015), OR was not co-immunoprecipitated with PSY as the bait, which may indicate a transient interaction between these two proteins. Noticeably, geranylgeranyl reductase and a number of plastid chaperone proteins, i.e., HSP70 and Cpn60, were found as potential PSY-interacting proteins. Interactions between PSY, geranylgeranyl reductase, and geranylgeranyl synthase as well as associations between PSY and chaperones were reported in previous studies (Bonk et al., 1997; Ruiz-Sola et al., 2016), indicating the viability of the coIP approach in identification of the potential PSY-interacting proteins. In the current study, we focused on ClpC1, a key chaperone component of the Clp protease system (Desimone et al., 1997; Sjögren et al., 2014).

PSY Physically Interacts with ClpC1 in Plastids

To confirm the interaction between PSY and ClpC1 inferred from the coIP results, we carried out yeast two-hybrid (Y2H) analysis using a split-ubiquitin membrane Y2H system (Obrdlik et al., 2004). This system has been proved suitable for studying PSY protein interactions (Zhou et al., 2015; Ruiz-Sola et al., 2016). As shown in Figure 1A, yeast growth on selective medium was observed when Nub-ClpC1 was mated with PSY-Cub, confirming that PSY physically interacted with ClpC1 in yeasts. As a negative control, we tested the interaction between ClpC1 and KAT1, an Arabidopsis K+ channel protein localized in the plasma membrane (Obrdlik et al., 2004), and observed no interaction in the Y2H assay (Figure 1A), indicating a specific interaction between ClpC1 and PSY.

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(Ruiz-Sola and Rodríguez-Concepción, 2012; Nisar et al., 2015; Yuan et al., 2015a). A number of regulatory genes (i.e., PIFs, HY5, RIN, and SISGR1) and factors are known to control PSY gene expression (Toledo-Ortiz et al., 2010; Martel et al., 2011; Kachanovsky et al., 2012; Luo et al., 2013; Toledo-Ortiz et al., 2014). However, knowledge of post-translational regulation of PSY remains obscure. Only very recently ORANGE (OR) proteins were found to physically interact with PSY and positively regulate its protein abundance and enzymatic activity in plastids (Zhou et al., 2015). PSY translation in Arabidopsis was shown to be controlled by differential 5' UTR splicing (Alvarez et al., 2016). Further investigation into the regulation of PSY abundance and identification of PSY proteolytic factors are critical to elucidating the delicate mechanisms that control PSY homeostasis for carotenogenesis in plants.

Intraplastid proteolysis is a key process in maintaining protein homeostasis in plastids. The ATP-dependent serine-type Clp protease system constitutes a central part of the plastid protease network. It is critically important for plastids to ensure optimal levels of functional proteins and to remove aggregated, misfolded, or unwanted proteins (Clarke, 2012; Nishimura and van Wijk, 2015). A plant-specific Clp component ClpF was recently hypothesized to form a ClpF–ClpS1 adaptor complex for substrate recognition and delivery of substrates of the Clp protease (Apitz et al., 2016; Pulido et al., 2013). However, only few proteins were confirmed to be the specific substrates of the Clp protease (Apitz et al., 2016; Pulido et al., 2013), indicating the viability of the coIP approach in identification of the potential Clp protease substrates. We provide evidence that Clp protease physically interacted with PSY to mediate PSY degradation. By contrast, OR as a major post-translational regulator of PSY promoted its stabilization. Together, Clp protease and OR maintain the homeostasis of PSY in the plastids to assure optimal enzyme protein abundance for adequate carotenoid biosynthesis in plants.

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To further verify PSY and ClpC1 interaction in vivo, we performed a bimolecular fluorescence complementation (BiFC) assay. When the N-terminal half of YFP fused to ClpC1 (ClpC1-YN) and the C-terminal half of YFP fused to PSY (PSY-YC) were co-expressed in tobacco (*Nicotiana benthamiana*) leaf epidermal cells, YFP signals were observed (Figure 1A). In contrast, no YFP signals were detected when ClpC1-YN was co-transformed with YC (Figure 1A), or when PSY-YC was with Tic40-YN, a chloroplast inner envelope protein used as a negative control (Supplemental Figure 2A). Such PSY and ClpC1 interaction occurred in chloroplasts, which is consistent with the plastid localizations of these proteins shown in previous studies (Desimone et al., 1997; Zhou et al., 2015). The BiFC results confirm direct interaction between PSY and ClpC1 in vivo.

**PSY Also Interacts with Other Clp Protease Subunits Involved in Substrate Selection**

Substrate selection by the Clp protease occurs through the ClpC/D chaperones and adaptor proteins like ClpS (Clarke, 2012; Nishimura and van Wijk, 2015; Nishimura et al., 2015). To find out whether other Clp protease subunits involved in substrate selection were also physically associated with PSY, we examined the interactions of PSY with ClpC2, ClpD, and ClpS1. By Y2H assay, we found that ClpD, ClpC2, and ClpS1 all interacted with PSY (Figure 1B). These PSY-interacting subunits neither interacted with the empty vector control nor with the KAT1 negative control in the Y2H analysis (Figure 1B). To further confirm their interactions in vivo, we selected ClpD and ClpS1 to examine their interactions with PSY in tobacco leaves using the BiFC assay. Strong YFP signals were observed in chloroplasts when ClpD-YN and ClpS1-YN were individually co-expressed with PSY-YC in tobacco leaves (Figure 1B). These results indicate direct interactions between PSY and these Clp protease subunits in vivo.

We also tested our systems with two proteins, PRLI-interacting factor L (pTAC17; AT1G80480) and ClpF (AT2G03390), which were identified as ClpS1 substrates from affinity enrichment and form a binary ClpF–ClpS1 adaptor complex (Nishimura et al., 2013, 2015). Interactions with ClpS1 were confirmed by BiFC in tobacco leaves with both proteins, supporting the suitability of BiFC to study Clp–substrate interactions (Figure 1B). However, both proteins showed strong autoactivation of reporter genes in yeasts, rendering their analysis by Y2H impossible (see Supplemental Figure 2B).

In addition, we tested the interaction between PSY and HSP70 (AT5G49910), a protein identified from the coIP experiments and known to be involved in folding of DXS (Pulido et al., 2013, 2016). PSY was also found to directly interact with HSP70 both in Y2H and in BiFC assays (Figure 1C).
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The PSY protein levels in 3-week-old leaves of these clp mutants and WT control were examined by western blot analysis. In comparison with WT, PSY protein levels were greatly enhanced in clpc1, clpr1-2, and clpp4, but remained similar in the other mutants (Figure 2B). Quantification of PSY protein levels showed approximately 10-fold increases in the clpc1, clpr1-2, and clpp4 mutants compared with the WT control (Figure 2C).

PSY transcript levels were also measured using real-time RT–PCR. No significant differences were observed between WT and the clp mutant lines (Figure 2D). The result indicates that deficiency of the Clp subunits in clpc1, clpp4, and clpr1-2 did not affect PSY transcription. The data also confirm that the observed increase of PSY protein levels in those clp mutants was not a consequence of enhanced gene expression, but occurred post-translationally.

To rule out that the PSY protein accumulation in clpc1, clpr1-2, and clpp4 was a consequence of chloroplast defect, we examined PSY protein levels in some unrelated chlorotic mutants including rps5 (Zhang et al., 2016), xk1 (Hammerlin et al., 2006), toc33 (Jarvis et al., 1998), glk1/glk2 (Waters et al., 2008), and fd2 (Voss et al., 2008). Increased PSY protein levels were not observed in these mutants (Figure 2E). The results indicate that the elevated PSY levels in the clp mutants were due to the loss of Clp activity but not chlorosis. In addition, since PSY was found to directly interact with HSP70-2 (Figure 1C), PSY protein level was also examined in hsp70-2. A slightly elevated PSY protein level was observed in the hsp70-2 mutant in comparison with WT control (Figure 2E). The accumulation of PSY in hsp70-2 suggests a possible role of HSP70 in the Clp-mediated PSY homeostasis.

PSY Protein Turnover Rate Is Reduced in clpc1

The Clp protease in plastids is responsible for degradation of misfolded or unwanted proteins (Kato and Sakamoto, 2010; van Wijk, 2015). Various stresses such as heat cause proteins to lose their native conformation and to form aggregated or misfolded polypeptides (Pulido et al., 2016). To examine whether the Clp protease was responsible for degradation of aggregated or misfolded PSY, we monitored PSY protein turnover rate following heat treatment in Arabidopsis leaves. Since loss of function of individual Clp complex subunits typically results in decreased proteolytic activity of the whole complex (Nishimura and van Wijk, 2015) and ClpC1 is the principal chaperone component of the chloroplast Clp protease (Zheng et al., 2002; Sjögren et al., 2014), the clpc1 mutant was used for this study.

Three-week-old Arabidopsis plants of WT and clpc1 grown at 23°C were transferred to 42°C and rosette leaves were collected at different time points for western blot analysis. As shown in Figure 3A and 3B, PSY protein level rapidly decreased in the WT control but remained relatively high in the clpc1 mutant. At 45 min of treatment, PSY level was reduced to about 30% in the WT control but remained similar in the clpc1 mutant. The low PSY degradation rate in clpc1 indicates a slow proteolytic removal of PSY protein, demonstrating that proper Clp protease activity was required for maintaining PSY homeostasis.
Carotenoid Pathway Activity Is Affected in \textit{clp}\textsubscript{1} and \textit{clpr1-2}

To examine whether carotenoid biosynthesis and accumulation were affected by impairment of Clp protease activity in \textit{Arabidopsis}, we examined pigment formation and carotenoid pathway activity in the \textit{clp} mutants. Consistent with the mutant plant phenotypes (Figure 2A), chlorophyll and total carotenoid contents of several \textit{clp} mutants were not significantly different from WT except \textit{clpc1}, \textit{clpp4}, and \textit{clpr1-2}, in which the carotenoid contents were significantly reduced, especially in \textit{clpp4} with white tissue (Figure 4A and 4B). Interestingly, the immediate product of PSY, phytoene, was found to accumulate in \textit{clpd}, but was absent in WT and all the other \textit{clp} mutants examined (Figure 4B). Constant ratios of carotenoids to chlorophylls were observed in most mutants, which mirrored the defined pigment stoichiometry in light-harvesting complex proteins (Figure 4C).

With the exception of \textit{clpd}, phytoene usually does not accumulate in leaves and is promptly metabolized into downstream carotenoids. However, upon treatment with norflurazon (NFZ), an inhibitor of phytoene desaturase, phytoene accumulates and its level directly reflects PSY activity and, thus, carotenoid pathway activity (Rodríguez-Villalón et al., 2009; Látari et al., 2015; Zhou et al., 2015). Therefore, we measured the accumulation of phytoene in leaves of 3-week-old plants treated with NFZ by high-performance liquid chromatography (HPLC) (green leaves from \textit{clpp4} were included). In comparison with the WT control, \textit{clpc1} and \textit{clpr1-2} showed significantly increased phytoene levels whereas the other \textit{clp} mutants accumulated similar levels of phytoene following NFZ treatment (Figure 4D). These results suggest that \textit{clpc1} and \textit{clpr1-2} exhibited increased PSY activity, and the accumulated phytoene in the \textit{clpd} leaves was not caused by increased synthesis. We also measured the levels of the rest carotenoids and found similar
carotenoid accumulation patterns both following NFZ treatment and without NFZ treatment in the clp mutants (Supplemental Figure 4).

Overaccumulated PSY in clpc1 and clpr1-2 Is Partially Enzymatically Active

To corroborate the results from carotenoid pathway activity measurements, in vitro PSY activity of clpc1 and clpr1-2 along with clpd was examined. Active PSY is membrane associated and usually undetectable in the stromal fractions of chloroplasts in Arabidopsis (Welsch et al., 2000; Lätari et al., 2015), unlike in maize and tomato chromoplasts where soluble PSY1 is enzymatically active (Fraser et al., 1999, 2000; Shumskaya et al., 2012). Because other pathway enzymes compete for the substrate GGPP (e.g., chlorophyll biosynthesis), determination of PSY activity in vitro requires separation of stroma and membranes through plastid fractionation (Welsch et al., 2000; Ruiz-Sola et al., 2016; Zhou et al., 2017).

The plastid fractionation for PSY activity assay also allowed determination of the enzyme protein distributions in chloroplast membrane and stromal fractions. While DXS was exclusively found in the chloroplast stroma in these mutants, the PSY substrate-delivering enzyme GGPS was present in both stroma and membranes (Figure 5A). Unchanged GGPS protein levels with similar GGPS activities were observed in the membrane fractions (Figure 5A and Supplemental Figure 5). In contrast, PSY protein was only detected in the membrane fractions (Figure 5A). Consistent with the observed PSY overaccumulation in clpc1 and clpr1-2 and unchanged PSY levels in clpd (Figure 2B), high levels of PSY protein were noted in clpc1 and clpr1-2 in comparison with WT and clpd (Figure 5A).

In agreement with carotenoid pathway activity measurements in leaves, increased PSY activity was detected in clpc1 and clpr1-2 while clpd along with clpc2 and clps1 exhibited PSY activity similar to that of WT (Figure 5B). Remarkably, the increases in PSY activity and PSY protein levels were not proportional in the clpc1 and clpr1-2 mutants. The specific enzyme activities (i.e., normalized to PSY protein levels) were 53% and 31% lower for clpc1 and clpr1-2, respectively, than WT (Figure 5C). This indicates that proportions of the overaccumulated PSY were enzymatically inactive. Immunoblots of chloroplast subfractions confirmed that the inactive fractions of PSY remained membrane associated and were not dislocated into...
Post-translational Regulation of PSY by Clp and OR

The results suggest that several proteins in the carotenoid biosynthetic pathway could be the targets of Clp protease activity. Whether OR could promote proper PSY folding to maintain enzymatic activity and counteract PSY degradation by the Clp protease, we introduced OR in a Clp-defective background by crossing clpc1 with an AtOR-overexpression line (Yuan et al., 2015). The clpc1 × AtOR F3 plants that were double homozygous for clpc1 and the OR transgene were generated. Examination of the OR protein levels in the F3 plants revealed slightly lower OR abundance than in the AtOR line used for cross (Figure 7C). In vivo PSY activity was assessed by measuring phytoene accumulation following NFZ treatment in the 3-week-old leaf samples of clpc1 × AtOR plants along with WT, clpc1, and the AtOR overexpressor.

Consistent with the result obtained above, more phytoene was observed in clpc1 than in WT (Figure 7D). Similarly, more phytoene was detected in the AtOR overexpression line than WT as previously observed (Zhou et al., 2015). However, a higher level of phytoene accumulation was obtained in the NFZ-treated clpc1 × AtOR line than the AtOR overexpressor or clpc1 (Figure 7C). The results suggest that OR increased the enzymatically active proportion of PSY in clpc1, possibly through promoting PSY folding or preventing its misfolding/aggregation.

**DISCUSSION**

**PSY Is a Substrate of Clp Protease**

PSY catalyzes the critical step in carotenogenesis and directs isoprenoid carbon flow into the carotenoid biosynthetic pathway (Ruiz-Sola and Rodríguez-Concepción, 2012; Nisar et al., 2015; Sun et al., 2017). While multiple levels of regulation govern PSY protein amounts and enzymatic activity, post-translational regulation of PSY including proteolysis of excessive or dysfunctional PSY protein is important in maintaining PSY proteostasis in plastids. In this study, we reveal that PSY was a substrate of the Clp protease and demonstrate a new post-translational control mechanism of PSY homeostasis.

Clp protease is a major protease system in plastids (Clarke, 2012; Nishimura and van Wijk, 2015). The levels of many chloroplast...
proteins are expected to be controlled by the Clp protease, but the identities of direct targets of Clp protease remain few. Comparative proteomic analyses of differentially expressed proteins between Arabidopsis clp mutants and WT identified some upregulated proteins as potential Clp substrates, although many may be due to secondary effects upon loss of the Clp activity (Nishimura et al., 2013; Nishimura and van Wijk, 2015). A recent study confirmed one of these, the first enzyme in 5-aminolevulinic acid biosynthesis glutamyl-tRNA reductase (GluTR), as a direct substrate of the Clp protease (Apitz et al., 2016). Here, we discovered that PSY directly interacted with the adaptor ClpS1 and chaperones ClpC/D (Figure 1). PSY protein levels were greatly increased following the reduction of Clp protease activity in clpc1, clpp4, and clpr1-2 (Figure 2). Moreover, the PSY protein degradation rate was reduced when Clp protease was not properly functioning (Figure 3). These data corroborate PSY as a target of the Clp protease, adding PSY to the substrate list of Clp protease.

Determination of the substrate selection and delivery mechanisms to proteolyze unwanted proteins in plastids remains a challenge (Nishimura and van Wijk, 2015; van Wijk, 2015). A J-protein/Hsp70-dependent pathway for substrate recognition and delivery has been shown recently for DXS (Pulido et al., 2013, 2016). A J-protein adapter, J20, was found to specifically recognize the inactive forms of DXS and deliver them to Hsp70 chaperones either for proper folding via interaction with ClpB3 (a plastidial Hsp100 chaperone) or for unfolding by ClpC1 for degradation by the Clp protease. We observed a direct interaction between PSY and Hsp70 (Figure 1C), which agrees with the co-existence of PSY and chaperones in high molecular weight complexes in chloroplasts (Bonk et al., 1997). Moreover, we observed slightly elevated PSY protein levels in hsp70-2 mutant (Figure 2E), suggesting the involvement of Hsp70 in PSY proteostasis. OR is known to directly interact with PSY degradation signal (an N-degron) for degradation by Clp protease. It is inconclusive whether the N-end rule generally applies to plastid protein proteolysis in plants (Apel et al., 2010; Rowland et al., 2015; Pulido et al., 2016). Here we found that PSY physically interacted with ClpS1. We further examined this interaction by Y2H and found that a 13-amino-acid stretch of PSY (PSY71–83) with a putative N-degron interacted with ClpS1 (Supplemental Figure 7). These results suggest a possible role for the ClpS1 adapter in recognizing and delivering PSY to Clp protease for degradation. However, as PSY protein did not overaccumulate in clps1, other adapter(s) or chaperones are likely more crucial and/or used to deliver PSY to the Clp protease.

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(Zhou et al., 2015). This raises the question as to whether OR has a J20-analogous function on PSY. However, OR and J20 are different in a number of ways (Pulido et al., 2013; Zhou et al., 2015). In contrast to J20, OR is not a J-protein (Lu et al., 2006). While unfolded DXS protein overaccumulates in j20, PSY is almost absent in the or mutants. Moreover, J20 overexpression results in reduced DXS levels while OR overexpression increases PSY abundance and activity. Furthermore, no direct interaction was observed between OR and Hep70 (Supplemental Figure 8). Thus, it is currently unknown as to whether there is a J-protein that specifically recognizes PSY for a J-protein/Hsp70-dependent pathway.

Our data suggest that the ClpC1 direct pathway might play a key role in selecting and delivering PSY to the Clp core complex. ClpC is the principal chaperone of Clp protease with ClpC1 contributing greatly to substrate unfolding (Sjögren et al., 2014). The ClpC1 chaperone was recently proposed to be more essential in specifically recognizing and directing GluTR1 to the Clp core complex for GluTR1 turnover (Apitz et al., 2016). Here we found that the ClpC1 chaperone physically interacted with PSY and was required for PSY degradation by the Clp protease. Lack of ClpC1 resulted in PSY overaccumulation (Figure 2) and slow turnover (Figure 3), indicating the important role of ClpC1 for the Clp-mediated PSY degradation.

Previous studies suggest that the Clp protease controls protein levels of a number of other enzymes required for isoprenoid metabolism. The key enzymes DXS and DXR in the MEP pathway are augmented in the clp mutants (Flores-Pérez et al., 2008; Zybaïlov et al., 2009; Nishimura et al., 2013). Similarly, the MEP pathway enzyme hydroxymethylbutenyl-4-diphosphate synthase (HDS) overaccumulates in the clp mutants (Kim et al., 2009, 2013, 2015). Recently, Pulido et al. (2016) showed that the Clp protease plays a primary role in DXS proteolysis. We found that in addition to PSY, carotenogenic enzyme proteins PDS, ZDS, BCH, LUTS, and ZEP also overaccumulated in the clpc1, clpp4, and clpr1-2 mutants, adding them to the list of potential Clp protease targets. Clearly, a coordinated proteolytic control of both MEP and carotenoid biosynthesis pathways represents an important mechanism in modulating the steady state of carotenoids in cells.

Both Enzymatically Active and Inactive Forms of PSY Accumulate in clpc1 and clpr1-2

Examination of the specific PSY enzyme activities (i.e., normalized to PSY protein levels) indicates a co-existence of both enzymatically active and inactive PSY forms in clpc1 and clpr1-2 (Figure 5). A large proportion of the overaccumulated PSY protein in the clp mutants was enzymatically inactive, indicating the accumulation of aggregated or misfolded PSY forms. Previous reports show that PSY is present both as membrane-associated active and stromal non-active forms (Schlez et al., 1996; Welsch et al., 2000; Lätari et al., 2015). The translocation of stromal, inactive into membrane-localized, active PSY was observed in de-etiolating seedlings (Welsch et al., 2000). This probably reflects a developmentally regulated solubilization of PSY, allowing its reactivation through membrane association during chloroplast formation. Similarly, a partial stromal relocation of an inactive PSY population may result from surplus abundance of PSY through overexpression (Lätari et al., 2015). In both clpc1 and clpr1-2, PSY quantitatively accumulated in the membrane fractions; thus, inactive PSY populations were not dislocated into the stroma. Apparently, inactive PSY aggregates remain membrane associated.

A proportion of the overaccumulated PSY protein in the clp mutants was also enzymatically active, similarly as shown for DXS in clpc1 (Pulido et al., 2013, 2016). However, in contrast to DXS in clpc1 where the increased DXS correlates with equivalently higher enzymatic activity (Pulido et al., 2013, 2016), only about half of the PSY in clpc1 was enzymatically active. High DXS activity in clpc1 was explained by accumulation of chaperones to prevent DXS aggregation (Pulido et al., 2016). Indeed, we observed an increased level of Hsp70 in clpc1 and a few other clp mutants (Supplemental Figure 9), and had indications for a contribution of HSP70-2 to PSY proteostasis (Figures 1C and 2C). However, the major differences in the proteostatic mechanisms between PSY and DXS might be due to different localizations. While DXS is soluble in the stroma, PSY requires membrane association for activity (Welsch et al., 2000). Membrane integral or associated proteins are known to require a chaperone-assisted release into membranes upon plastid import (Falk and Sinning, 2010; Liang et al., 2016). Compared with soluble refolding processes applicable to DXS, proper folding control of membrane proteins poses a particular challenge to protein homeostasis (Liang et al., 2016). Thus, degradation rather than refolding of misfolded or aggregated PSY might essentially contribute to PSY proteostasis. Chaperones including OR may facilitate PSY folding to make a fraction of PSY active in clpc1.

Carotenoid Patterns in the clp Mutants

The overaccumulation of the crucial enzymes for both MEP and carotenoid biosynthetic pathways disagrees with the reduced pigment content observed in clpc1 and clpr1-2 (Figure 4B). Both DXS and PSY accumulated with higher total activities (Pulido et al., 2016; Figure 5). Moreover, downstream carotenogenic enzymes such as PDS and ZDS also accumulated in clpc1 and clpr1-2 (Figure 6). Desaturation intermediates such as phytoene and phytofluene were absent, which supports unrestricted pathway flow. Therefore, processes other than the synthesis of carotenoids are likely to account for the reduced carotenoid levels in these clp mutants. Clp protease subunits are known to differentially affect the homeostasis of many plastid proteins (Kim et al., 2009; Moreno et al., 2017). Defects in ClpC1 and ClpR1-2 affect chloroplast development (Sjögren et al., 2004; Kim et al., 2009). Since leaf carotenoid contents are determined also by sequestering structures in addition to biosynthesis, it is possible that the lower levels of carotenoid-binding proteins (light-harvesting complex proteins) as shown in the clpc1 mutant (Sjögren et al., 2004) restrain carotenoid accumulation, leading to reduced carotenoid levels in clpc1 and clpr1-2.

Surprisingly, in contrast to all other clp subunit mutants investigated in this work, leaves of clpd accumulated phytoene (Figure 4B). However, the accumulated phytoene was not due to increased PSY activity as in vitro PSY activity in clpd was similar to that in WT (Figure 5B). Active PDS requires
Based on the data obtained, we propose a model for Clp protease and OR in governing the balance between PSY turnover and activity in plastids (Figure 8). OR as a membrane protein physically interacts with PSY to promote PSY membrane association in its active form for carotenogenesis. Inactive misfolded and/or aggregated forms of PSY are likely recognized by ClpC1, either directly or upon recognition and delivery by ClpS1, and unfolded prior to proteolysis by the core Clp protease components. Upon increased OR abundance in the AtOR-overexpressing tissues, PSY protein is maintained in an active, membrane-associated state and thus prevented from proteolysis, while absence of OR results in PSY degradation, a regulation that occurs solely post-translationally. The suggested regulatory mechanism shares similarities with the post-translational regulation of tetrapyrrole and thus chlorophyll biosynthesis, catalyzed by GluTR (Apitz et al., 2016). The similar post-translational regulations of crucial enzymes for the synthesis of major photosynthetic pigments, GluTR for tetrapyrrole and PSY for carotenoid biosynthesis, might contribute to an efficient coordinated supply of stoichiometrically balanced amounts for accurate assembly of photosynthetic complexes in chloroplasts.

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**METHODS**

**Plant Materials**

*Arabidopsis thaliana* WT (ecotype Columbia-0) and mutant lines as well as *N. benthamiana* plants used for transformation were grown in soil under 14 h light/10 h dark at 23°C. The T-DNA insertion mutant lines used here included clps1, clpc1, clpc2, clpd, clpr1-2, and clpt1/t2 (Pulido et al., 2016). The clp4 antisense line was generated by introducing ClpP4 antisense construct into *Arabidopsis* (ecotype Columbia-0). The clp1 was also crossed with an AtOR overexpressor (Yuan et al., 2015b; Zhou et al., 2015) to produce clp1 × AtOR homozygous line. The PSY-GFP transgenic lines in WT and OR-overexpressing backgrounds were produced by introducing 3SS:PSY-GFP construct into *Arabidopsis* WT and the OR-overexpressing transgenic plants. The rps5, xk-1, glk1/glk2, toc33, fad2, and hsp70-2 mutants were either ordered from ABRC or obtained from collaborators.

**Co-immunoprecipitation**

CoIP was conducted with quadruplicate biological replicates as described previously (Zhou et al., 2015). In brief, proteins were extracted from *Arabidopsis* plants expressing 3SS:PSY-GFP or 3SS: GFP, mixed with magnetic beads conjugated to anti-GFP antibodies (Miltenyi Biotec, Auburn, CA), and incubated on ice for 30 min. Protein complexes containing PSY-GFP and GFP were purified in μ columns by washing four times with extraction buffer and eluting with 2 × SDS loading buffer.

**Proteomics Analysis**

The immunoprecipitated proteins were resolved on SDS–PAGE gels and then excised into 10 bands that were subjected to subsequent in-gel digestion as detailed previously (Yang et al., 2007). The digestes were analyzed using a nanoACQUITY UPLC system coupled with a Synapt HDMSTM (Waters) mass spectrometer equipped with a NanoLockSpray source (Wang et al., 2013). All of the raw data were output as PKL files by the ProteinLynx Global Server 2.4 (PLGS, Waters). Subsequent database searches were carried out by Mascot Daemon 2.3 (Matrix Science, Boston, MA) against *Arabidopsis* databases (Araport11_latest, https://www.araport.org). The search parameters used for the Mascot search were: one missed cleavage site by trypsin allowed with fixed carbamidomethyl modification of cysteine; and variable of oxidation on methionine and deamidation of Asn and Gln residues. The peptide and fragment mass tolerance values were 15 ppm and 0.1 Da, respectively. To reduce the probability of false identification, only peptides with
significance scores at the 99% confidence interval were counted as identified (Wang et al., 2013).

**Yeast Two-Hybrid Assay**

The split ubiquitin system was used as described previously (Zhou et al., 2015). The cDNA sequences of ClpC1 (At5g50920), ClpC2 (At3g48870), ClpD (At5g51070), and ClpS1 (At1g68660) along with plastidic Hsp70-2 (At5g49910) without the sequences encoding their transit peptides were cloned to make Nub plasmids. The cDNA sequence of an Arabidopsis K+ channel subunit KAT (At5g46240; Obrdlik et al., 2004) used as negative control was cloned into the Cub-expressing vector. The PSY-Cub and OR-Cub vectors were from the previous study (Zhou et al., 2015). Plasmids were transformed into yeast strain THY:AP4 (Nub) or THY:APS (Cub) and mated with each other. Interactions were examined by placing yeast strains with a series of dilutions on selection medium lacking leucine, tryptophan, adenine, and histidine (~LWAH) with 300 μM methionine supplementation after 2 days of growth at 28°C.

**Immunoblotting Analysis**

Total proteins were extracted from Arabidopsis leaves using the phenol method as described by Wang et al. (2013). Proteins were resolved on SDS–PAGE gels, transferred onto nitrocellulose membranes (BBS), and blocked with Tris-buffered saline (TBS) containing 5% milk for 1 hr at room temperature. Membranes were incubated with antibodies against GGPS11 (Eurogentec, Belgium), PSY (Abmart, Shanghai, China), DKS (Philippe Hugueney, INRA, France), PDS (Al-Babili et al., 1996), ZDS (Eurogentec, Belgium), BCh (Abmart, Shanghai, China), ZEP (Agrisera, OR (Lu et al., 2006), or actin (Sigma) in TBS containing 1% milk for 2 h. The ECL reagent (GE Healthcare, Munich, Germany) was used as the detection system. The relative protein levels were quantified using ImageJ (Schneider et al., 2012).

**Phytoene Synthase Degradation and Stability Assays**

To examine the effect of Clp protease on PSY degradation, we transferred 3-week-old Arabidopsis plants of WT and clpC1 grown at 23°C to 42°C for heat-shock treatment, and collected rosette leaves at different times after treatment. To measure the PSY stability, we treated 3-week-old rosette leaves with cycloheximide (CHX) for various times. Stromal proteins were incubated with antibodies against LUT5. This work was supported by Agriculture and Food Research Initiative competitive award no. 2016-67013-24612 from the USDA National Institute of Food and Agriculture and by the HarvestPlus research consortium (2014H6320.FRE). The authors declare no conflict of interest.

**SUPPLEMENTAL INFORMATION**

Supplemental Information is available at Molecular Plant Online.

**AUTHOR CONTRIBUTIONS**

R.W., X.Z., H.Y., D.A., T.S., D.S., and Y.Y. performed the experiments and data analysis. R.W., X.Z., H.Y., and L.L. designed the research. T.W.T. guided the proteomics experiments and data analysis. G.S., H.Z., and M.R.-C. provided research agents. M.R.-C. assisted in data analysis and interpretation. R.W., X.Z., and L.L. wrote the manuscript. All authors contributed to the final manuscript.

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**REFERENCES**


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