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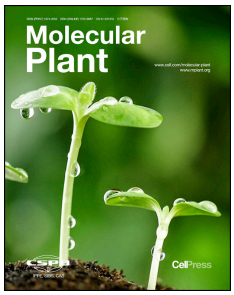

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Clp protease and OR directly control the proteostasis of phytoene synthase, the crucial enzyme for carotenoid biosynthesis in Arabidopsis

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Running title: Post-translational regulation of PSY by Clp protease and OR

Short Summary:

We reveal that PSY, the crucial enzyme in the carotenoid biosynthetic pathway, is a substrate of the Clp protease in chloroplasts. The Clp-mediated proteolysis is counteracted by the OR protein to maintain the PSY protein homeostasis and modulate carotenoid biosynthesis in plants. This study demonstrates a new post-translational control mechanism of carotenogenic enzymes.

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 networks, 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 enzymes over-accumulate in the *clpc1, clpp4* and *clpr1-2* mutants. The over-accumulated PSY was found to be partially enzymatically active. Loss of the Clp activity in *clpc1* results in 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 homeostasis are largely unknown.

Phytoene synthase (PSY) is the crucial enzyme for carotenogenesis 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 (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 *SlSGR1*) 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 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 elucidate the delicate mechanisms that control PSY homeostasis for carotenogenesis in plants.

Intraplastid proteolysis is a key process to maintain 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). Clp protease consists of multiple subunits, which include ClpS as the substrate recognition adaptor, two ClpC (ClpC1-2) and one ClpD subunits as chaperones for substrate unfolding, and five proteolytically active ClpP subunits (ClpP1 and ClpP3 to ClpP6) as well as four proteolytically inactive ClpR subunits (ClpR1 to ClpR4) as core protease components, along with accessory proteins (ClpT1-2) to assembly and stabilize the Clp core (Nishimura and van

Wijk, 2015). A plant-specific Clp component ClpF was recently hypothesized to form ClpF-ClpS1 adaptor complex for substrate recognition and delivery (Nishimura et al., 2015).

Various Clp subunits have been shown to contribute differently to the homeostasis of plastid proteins, such as ClpP1 in the degradation of thylakoid proteins, ClpR1 in the maturation of 23S and 4.5S chloroplast rRNA, and ClpC1 in the turnover of chlorophyllide a oxygenase (Majeran et al., 2000; Koussevitzky et al., 2007; Nakagawara et al., 2007). Recently, ClpC1 was also found to be required for the degradation of deoxyxylulose 5-phosphate synthase (DXS) in the methylerythritol 4-phosphate (MEP) pathway (Pulido et al., 2016). The different Clp subunits also exert distinct functions in affecting plant growth and development (Kim et al., 2009; Moreno et al., 2017). All of the Clp subunits have been identified from both green and non-green plastids in Arabidopsis (Peltier et al., 2004; Kim et al., 2009; Olinares et al., 2011). It is obvious that identification of Clp protease targets is critical to understand its involvement and contribution to plastid and plant development. Dozens of chloroplast-localized proteins involved in multiple processes were identified as potential targets of the Clp protease (Nakagawara et al., 2007; Stanne et al., 2009; Nishimura et al., 2013; Nishimura and van Wijk, 2015; Tapken et al., 2015). However, only few proteins were confirmed to be the specific substrates of the Clp protease (Apitz et al., 2016; Pulido et al., 2016). It remains a challenge to identify the specific targets that directly interact with the substrate recognition adaptor ClpS1 and/or the chaperones ClpC and ClpD for degradation (Nishimura and van Wijk, 2015).

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 (co-IP) in conjunction with analysis by mass spectrometry, 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, transgenic *Arabidopsis* expressing either *35S:PSY-GFP* (Supplemental Figure 1) or *35S:GFP* control were generated. Proteins from *35S:PSY-GFP* plants along with the *35S:GFP* control lines were extracted from four biological replicates and used for the co-IP experiments. The co-IP products were separated by SDS-PAGE gels and identified by LC-MS/MS analysis. A total of 202, 235, 238, and 163 proteins were identified from four biological replicates of co-IPs 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 viability of the co-IP 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 co-IP results, yeast two-hybrid (Y2H) analysis was carried out using a split-ubiquitin membrane Y2H system (Obrdlik et al., 2004). This system has been proved suitable for studying PSY protein interactions (Ruiz-Sola et al., 2016; Zhou et al., 2015). 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.

To further verify PSY and ClpC1 interaction *in vivo,* we performed 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 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 Directly 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 et al., 2015; Nishimura and van Wijk, 2015). To see 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*, ClpD and ClpS1 were selected 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 binary ClpF-ClpS1 adaptor complex (Nishimura et al., 2013; Nishimura et al., 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 co-IP experiments and known to be involved in folding of DXS (Pulido et al., 2013; Pulido et al., 2016). PSY was also found to directly interact with HSP70 both in Y2H and in BiFC assays (Figure 1C).

PSY Protein Over-accumulates in *clpc1, clpr1-2* and *clpp4*

The potential targets of Clp protease likely over-accumulate in various *clp* mutants (Nakagawara et al., 2007; Stanne et al., 2009; Nishimura and van Wijk, 2015). If PSY were a target of the chloroplast Clp protease, impairment of the Clp protease activity in the *clp* mutants would be expected to result in elevated levels of PSY protein. In order to explore this possibility, we obtained several *clp* mutants from *Arabidopsis* mutant collection (Pulido et al., 2016). As previously reported (Park and Rodermel, 2004; Sjögren et al., 2004; Kim et al., 2009; Nishimura et al., 2013), the *clpc1* and *clpr1-2* mutants displayed a pale-green phenotype with smaller leaves, whereas the *clpc2, clpd, clps1,* and *clpt1/t2* mutants had no visible phenotype compared with WT (Figure 2A). In addition, because *clpp4* knockout mutant is embryo-lethal and not viable (Kim et al., 2013), we generated *clpp4* antisense lines with reduced expression of *ClpP4* (Supplemental Figure 3B). The *clpp4* antisense lines showed a variegated chlorotic phenotype (Figure 2A) with small adult plants (Supplemental Figure 3A).

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 to 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 confirms 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), *xk-1* (Hemmerlin et al., 2006), *toc33* (Jarvis et al., 1998), *glk1/glk2* (Waters et al., 2008), and *fd2* (Voss et al., 2008). No highly increased PSY protein levels were 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*. Interestingly, 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 role of HSP70 in the Clp-mediated PSY homeostasis.

PSY Protein Turnover Rate is Reduced in the *clpc1* Mutant

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 is responsible for degradation of aggregated or misfolded PSY, PSY protein turnover rate following heat treatment was monitored 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 principle 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 maintained at around 70% in *clpc1*. 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 the *clpc1* and *clpr1-2* Mutants

To examine whether carotenoid biosynthesis and accumulation were affected by loss of Clp protease activity in Arabidopsis, we examined pigment formation and carotenoid pathway activity in the *clp* mutants. Consistent with the mutant plant phenotypes (Figure 2A), chlorophyll and total carotenoid contents of several *clp* mutants were not significantly different from WT except *clpc1*, *clpp4* and *clpr1-2*, in which the carotenoid contents were significantly reduced, especially in *clpp4* with white tissue (Figure 4A and 4B). Interestingly, the immediate product of PSY, phytoene, was found to accumulate in *clpd*, but absent in WT and all the other *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 *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; Lätari 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 HPLC (green leaves from *clpp4* were included). In comparison with the WT control, *clpc1* and *clpr1-2* showed significantly increased phytoene levels whereas the other *clp* mutants accumulated similar levels of phytoene following NFZ-treatment (Figure 4D). These results suggest that *clpc1* and *clpr1-2* exhibited increased PSY activity, and the accumulated phytoene in the *clpd* leaves was not caused by increased synthesis. We also measured the levels of the rest carotenoids and found similar carotenoid accumulation patterns following NFZ treatment as without NFZ treatment in the *clp* mutants (Supplemental Figure 4).

Over-accumulated 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; Fraser et al., 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, Supplemental Figure 5). In contrast, PSY protein was only detected in the membrane fractions (Figure 5A). Consistent with the observed PSY over-accumulation 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 similar PSY activity as 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 over-accumulated PSY were enzymatically inactive. Immunoblots of chloroplast subfractions confirmed that the inactive fractions of PSY remained membrane-associated and were not dislocated into the stroma in *clpc1* and *clpr1-2* (Figure 5A). Moreover, *in vitro* assays also confirmed that the phytoene accumulation in *clpd* (Figure 4B) was not caused by an increased PSY activity as *clpd* had similar total and specific PSY activity as WT (Figure 5B-C).

Defect in Clp Protease Activity also Results in the Accumulation of Other Carotenogenic Enzyme Proteins

Recent reports show that *clpc1* and *clpr1-2* contain increased DXS protein levels (Pulido et al., 2013; Pulido et al., 2016). We analyzed the protein levels of a number of other pathway enzymes along with DXS in 3-week-old leaves of the *clp* lines. Consistent with recent reports, DXS protein was found to over-accumulate in *clpc1* and *clpr1-2*. In addition, we observed DXS over-accumulation in the *clpp4* antisense line (Figure 6). Interestingly, five additional carotenogenic enzymes, phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), β-carotene hydroxylases (BCH), carotenoid hydroxylase CYP97A3 (LUT5), and zeaxanthin epoxidase (ZEP), also over-accumulated in *clpc1, clpr1-2* and *clpp4* (Figure 6). The results suggest that several proteins in the carotenoid biosynthetic pathway could be the targets of Clp proteolysis and that proper Clp protease activity is required for maintaining proteostasis of these carotenogenic enzymes.

OR Delays PSY Degradation

To investigate the post-translational control of PSY that maintains the balance between its turnover by Clp protease and proper function, we examined the role of OR on PSY protein stability. Previously, we have shown that OR physically interacts with PSY to positively regulate PSY protein level and enzyme activity (Zhou et al., 2015). To independently corroborate the effect of OR on PSY, we constitutively expressed *PSY-GFP* chimeric gene in WT and in *OR*-overexpression background, and selected lines expressing similar levels of PSY-GFP fusion protein (Supplemental Figure 6).

Three-week-old leaf samples were treated with the protein synthesis inhibitor cycloheximide (CHX). The PSY fusion protein levels in the WT and *OR*-overexpression background were examined at 3, 6, and 9 h post-treatment by western blot analysis. As shown in Figure 7A, PSY protein level declined in the *PSY-GFP/*WT leaves, but remained unchanged in the *PSY-GFP*/*OR* line at 3 h post CHX treatment. PSY fusion protein levels were much higher in the *PSY-GFP*/*OR* samples than in *PSY-GFP/*WT after CHX treatment at all time points (Figure 7B), showing that OR was able to stabilize PSY to greatly reduce PSY protein turnover rate.

OR Enhances PSY Activity in *clpc1*

OR is known to physically interact with PSY and possess holdase activity (Zhou et al., 2015; Park et al., 2016). To investigate 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., 2015b; Zhou et al., 2015). The *clpc1 x 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 x AtOR* plants along with WT, *clpc1*, and the *AtOR* overexpressor.

Consistent with the result obtained above, more phytoene was observed in *clpc1* than 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 x AtOR* line than either in the *AtOR* overexpressor or in *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 to maintain 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 to be 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 function (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). While the selective PSY recognition and delivery mechanisms are currently unclear, data obtained here suggest a number of possible pathways. One is via the ClpS1-ClpF and ClpC1 pathway (Nishimura et al., 2013; Nishimura et al., 2015). In bacteria, adaptor ClpS selects and delivers substrates with an N-terminal 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 adaptor in recognizing and delivering PSY to Clp protease for degradation. However, as PSY protein did not over-accumulate in *clps1,* other adapter(s) or chaperones are likely more crucial and/or used to deliver PSY to the Clp protease.

A J-protein/Hsp70-dependent pathway for substrate recognition and delivery has been shown recently for DXS (Pulido et al., 2013; Pulido et al., 2016). A J-protein adaptor 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 (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 over-accumulates 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 Hsp70 (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 suggests that the ClpC1-direct pathway might play a key role in selecting and delivering PSY to the Clp core complex. ClpC is the principle 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 over-accumulation (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; Zybailov et al., 2009; Nishimura et al., 2013). Similarly, the MEP pathway enzyme hydroxymethylbutenyl-4-diphosphate synthase (HDS) over-accumulates in the *clp* mutants (Kim et al., 2009; Kim et al., 2013; Kim et al., 2015). Recently, Pulido et al. (2016) showed that the Clp protease plays a primary role for DXS proteolysis. We found that in addition to PSY, carotenogenic enzyme proteins PDS, ZDS, BCH, LUT5 and ZEP also over-accumulated 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 coexistence of both enzymatically active and inactive PSY forms in *clpc1* and *clpr1-2* (Figure 5). A large proportion of the over-accumulated 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 (Schledz 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 re-activation through membrane association during chloroplast formation. Similarly, a partial stromal relocalization 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 over-accumulated PSY protein in the *clp* mutants was also enzymatically active, similarly as shown for DXS in *clpc1* (Pulido et al., 2013; Pulido et al., 2016). However, in contrast to DXS in *clpc1* where the increased DXS correlates with equivalently higher enzymatic activity (Pulido et al., 2013; Pulido et al., 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 have indications for a contribution of HSP70-2 to PSY proteostasis (Figure 1C and Figure 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 help 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 like PDS and ZDS also accumulated in *clpc1* and *clpr1-2* (Figure 6). Desaturation intermediates like 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 WT (Figure 5B). Active PDS requires plastoquinones as electron acceptors, which are reoxidized by the cytochrome-b_6f-complex in the photosynthetic electron transport chain with a contribution of the plastid terminal oxidase (McDonald et al., 2011). Accordingly, mutants with impaired biosynthesis or reoxidation of plastoquinones accumulate phytoene in their leaves (Norris et al., 1995; Carol et al., 1999). Thus, it is possible that a defective ClpD impacted protein(s) associated with plastoquinone biosynthesis or regeneration to affect phytoene desaturation, resulting in the accumulation of phytoene in *clpd*.

OR and Clp Protease Counterbalance PSY Activity and Degradation

Our previous studies show that OR physically interacts with PSY to positively regulate PSY protein level and enzyme activity (Zhou et al., 2015; Chayut et al., 2017). OR appears to perform this function by maintaining PSY in a properly folded form and preventing PSY degradation by the Clp protease. This is supported by our experiments demonstrating an increased PSY protein stability in the *OR* overexpression background and enhanced PSY activity in the *clpc1 x AtOR* line (Figure 7). It is also supported by the recent discovery that OR possesses holdase activity to prevent PSY misfolding and aggregation (Park et al., 2016). Moreover, PSY is barely present in the *ator ator-like* double mutant and in the melon fruits of *lowβ* mutant (Zhou et al., 2015; Chayut et al., 2017), which results from lack of OR to protect PSY thus enhancing its degradation.

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 for membranes-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 posttranslational. The suggested regulatory mechanism shares similarities with the posttranslational regulation of tetrapyrrole and thus chlorophyll biosynthesis, catalyzed by GluTR (Apitz et al., 2016). The similar posttranslational 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.

METHODS

Plant Materials

Arabidopsis thaliana WT (ecotype Columbia-0) and mutant lines as well as *Nicotiana 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 *clpp4* antisense line was generated by introducing *ClpP4* antisense construct into Arabidopsis (ecotype Columbia-0). The *clpc1* was also crossed with an *AtOR* overexpressor (Zhou et al., 2015; Yuan et al., 2015b) to produce *clpcl x AtOR* homozygous line. The *PSY-GFP* transgenic lines in WT and *OR* expressing backgrounds were produced by introducing *35S:PSY-GFP* construct into Arabidopsis WT and the *OR*-overexpressing transgenic plants. The *rps5, xk-1, glk1/glk2, toc33, fd2,* and *hsp70-2* mutants were either ordered from ABRC or obtained from collaborators.

Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation was conducted with quadruplicate biological replicates as described previously (Zhou et al., 2015). Briefly, proteins were extracted from Arabidopsis plants expressing *35S:PSY-GFP* or *35S:GFP*, mixed with magnetic beads conjugated to anti-GFP antibodies (Miltenyi Biotec Inc. Auburn, CA), and incubated on ice for 30 min. Protein complexes containing PSY-GFP and GFP were purified in µ columns by washing 4 times with extraction buffer and eluting with 2 x 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 digests were analyzed using a nanoACQUITY UPLC^{TM} system coupled with a Synapt HDMSTM (Waters) mass spectrometer (MS) 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 analysis 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 plastidial 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.AP5 (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 29°C.

Bimolecular Fluorescence Complementation (BiFC) Assay

The *ClpC1*, *ClpD,* and *ClpS1* as well as *HSP70-2, TAC17* (At1g80480) and *ClpF* (At2g03390) coding regions without stop codons were individually cloned into pSPYNE173 vector (Waadt et al., 2008) between the appropriate restriction enzyme sites, and then transferred into *A. tumefaciens* strain GV3101. *Agrobacterium* cells carrying the *PSY* and individual *Clp* constructs were infiltrated into 4-week-old *N. benthamiana* leaves as previously described (Zhou et al., 2015). Two days after infiltration, YFP fluorescence was detected using a Leica TCS SP5 Laser Scanning Confocal Microscope (Leica Microsystems, Exon, PA USA) with excitation wavelength at 488 nm and emission filter at 520 nm. Chloroplasts were excited with the blue argon laser (488 nm), and emitted light was collected at 680 nm to 700 nm.

Immunoblotting Analysis

Total proteins were extracted from Arabidopsis leaves using the phenol method as described (Wang et al., 2013). Proteins were resolved on SDS-PAGE gels, transferred onto nitrocellulose membranes (B85), and blocked with TBS buffer containing 5% milk for 1 hr at room temperature. Membranes were incubated with antibodies against GGPS11 (Eurogentec,

584 Belgium), PSY (Abmart, Shanghai, China), DXS (Philippe Hugueney, INRA, France), PDS (Al-
585 Babili et al., 1996), ZDS (Eurogentec, Belgium), BCH (Abmart, Shanghai, China), ZEP
586 (Agrisera), OR (Lu et al., 2006), or actin (Sigma) in TBS buffer containing 1% milk for 2 h. The
587 ECL reagent (GE Healthcare, München, Germany) was used as the detection system. The
588 relative protein levels were quantified using ImageJ (Schneider et al., 2012).
589
590 **Phytoene Synthase Degradation and Stability Assays**
591 To examine the effect of Clp protease on PSY degradation, 3-week-old Arabidopsis plants of
592 WT and *clpc1* grown at 23 ^{0}C were transferred to 42 °C for heat shock treatment and rosette
593 leaves were collected at different times after treatment. To measure the PSY stability, 3-week-
594 old rosette leaf discs of PSY-GFP lines in the Arabidopsis WT and *OR* overexpressor
595 backgrounds were treated with 100 µM cycloheximide (CHX) for various times. Total proteins
596 from treated samples were extracted and immunoblot-analyzed (Zhou et al., 2015). Protein
597 concentrations were determined by the Bradford method. The PSY protein was immunoblotted
598 with anti-PSY antibody for PSY degradation assay and with anti-GFP for stability assay.
599
600 **Chloroplast Isolation and *In vitro* Enzyme Activity Assays**
601 *In vitro* PSY activity assay was carried with isolated chloroplast membranes as described (Zhou
602 et al., 2015). Briefly, chloroplasts were isolated from leaves of three-week old plants, and lysed
603 as described (van Wijk et al., 2007). Protein amounts were determined by Bradford assay (Bio-
604 Rad). Plastid membranes (100 µg protein) were incubated in a reaction mixture containing [^{14}C]-
605 isopentenyl diphosphate (IPP, 50 mCi mmol^{-1}; American Radiolabeled Chemicals), IPP,
606 dimethylallyl diphosphate (DMAPP), and 10 µg purified Arabidopsis GGPS11. Radioactive
607 labelled products were analyzed and quantified as described (Welsch et al., 2000). [^{14}C]-GGPP
608 and [^{14}C]-phytoene synthesis occurred linearly within the first 45 min after substrate addition.
609 PSY activities in different samples were determined after 15 min incubation. Stromal proteins
610 were concentrated by ultrafiltration (Microcon, MWCO 3 kD, Millipore).
611
612 **RNA Extraction, Reverse Transcription and Quantitative RT-PCR**
613 Total RNA was extracted from Arabidopsis leaves using TRIzol reagent according to the
614 manufacturer's instruction (Life Technologies, Carlsbad, CA). cDNA was synthesized using

SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) after RQ1 DNase treatment. Quantitative RT-PCR was performed using SYBR master mix (Bio-Rad, Hercules, CA) with gene specific primers (Supplemental Table 3) as described (Zhou et al., 2011a). The relative expression of selected genes was normalized to an Arabidopsis *actin* gene.

Pigment Analysis

Total carotenoid and chlorophyll extraction and HPLC analysis of carotenoids were performed as described (Welsch et al., 2008). Chlorophylls were extracted with 80% acetone and determined as previously described (Zhou et al., 2011b). Norflurazon (NFZ) treatments were done according to Zhou et al. (2015). Leaves from three-week old plants were detached, immediately transferred onto 70 mM NFZ and incubated for two hours in the dark. Leaves were transferred onto 10 mM NFZ and further incubated for four hours with 100 µmol photons $m^{-2}s^{-1}$. Leaves were incubated with the adaxial surface facing the air. Leaves were harvested immediately, frozen in liquid nitrogen, and lyophilized before carotenoid extraction.

AUTHOR CONTRIBUTIONS

RW, XZ, HY, DA, TS, DS, and YY performed the experiments and data analysis. RW, XZ, HY, and LL designed the research. TT guided the proteomics experiments and data analysis. GS, HZ, and MRC provided research agents. MRC assisted in data analysis and interpretation. RW, XZ, and LL wrote the manuscript. All authors contributed to the final manuscript.

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FIGURE LEGENTS

Figure 1. Interactions of PSY with Clp Subunits Involved in Substrate Selection.

(A) Interaction between PSY and ClpC1. Left: Y2H analysis. Interactions were examined by co-expressing pairs of proteins fused to either N-terminal or C-terminal ubiquitin moiety in yeast and spotting onto either nonselective (-LW) or fully selective medium plates with 300 µM Met (-LWAH+M) in a series of 10-fold dilutions. Empty vectors expressing Nub and Cub only and a K+ channel protein (KAT1) were used as negative and unspecific controls, respectively. Right: BiFC analysis. PSY as C-terminal YFP fusion (YC) and ClpC1 as N-terminal fusion (YN) were co-expressed in *N. benthamiana* leaves. Empty vector expressing YC only was included as control.

(B) Interactions between PSY and Clp subunits. Left: Y2H analysis. Right: BiFC analysis of PSY interactions with ClpD, ClpC and ClpS1, as well as ClpS1 with ClpF or PRLI-interacting factor L (TAC).

(C) Interaction between PSY and HSP70. Left: Y2H analysis. Right: BiFC analysis.

Direct interactions in chloroplasts were observed by confocal microscopy (Scale bars, 20 µm). CHL, chlorophyll autofluorescence.

Figure 2. PSY Protein and Transcript Levels in the *clp* Mutants.

(A) Representative images of 3-week-old *clp* mutants grown on soil.

(B) Immunoblot analysis of PSY protein levels. Actin was used as loading control. Analysis was performed with 40 µg leaf protein extracts from 3-week-old plants. Protein sizes (kD) are indicated.

(C) Quantification of PSY protein levels normalized with actin. Results are means ± SD from quantification of three biological replicates.

(D) *PSY* mRNA levels determined by real-time RT-PCR. *PSY* transcripts were normalized to *actin* levels and are expressed relative to one selected WT sample. Results are means ± SD from three biological replicates.

(E) PSY protein levels in Arabidopsis WT and unrelated chlorotic mutants (*rps5, xk1-1, toc33, glk1/2,* and *fd2*) as well as the *hsp70-2* mutant. Actin was used as loading control.

Figure 3. PSY Protein Turnover Following Heat Treatment in *clpc1*.

(A) Immunoblot analysis of PSY turnover. The 3-week-old Arabidopsis WT and *clpc1* plants were transferred to 42°C. PSY protein levels in rosette leaves were determined before 0 min and after 15, 30 and 45 min of heat treatment. Ponceau S staining shows protein loading.
(B) Relative PSY protein levels. PSY band intensities were normalized and expressed relative to the levels detected prior to heat treatment. Data represent the means ± SD from three biological replicates.

Figure 4. Pigment Levels and Pathway Activity in the *clp* Mutants.
(A) Chlorophyll levels in the *clp* mutants.
(B) Carotenoid levels in the *clp* mutants. Pigments were determined from rosette leaves of 3-week-old plants by HPLC.
(C) Carotenoid/chlorophyll ratio from A and B.
(D) Carotenoid pathway activity was determined by illuminating 3-week-old leaves incubated with norflurazon for 4 h. Phytoene was quantified by HPLC and expressed relative to that determined in WT. Bleached leaf areas of *clpp4* were used for A, B and C while green leaves were used for D due to lack of materials. Results are means ± SE from ≥ three biological replicates. Significant difference, *P<0.05 in comparison with WT.

Figure 5. *In vitro* PSY Activity and Distribution in Chloroplasts of the *clp* Mutants.
(A) Western blot analysis of DXS, GGPS11, and PSY in chloroplast membrane and stroma fractions. RuBisCO large subunit (RbcL, stroma) and light harvesting complex of PSII subunit (Lhcb, membranes) protein levels from Coomassie-stained gels were used for protein loading controls.
(B) Absolute PSY activity in WT and *clp* mutants. Chloroplast membranes were incubated with supplemental GGPP synthase. Reactions were started with DMAPP and [1-^{14}C]IPP. The synthesized product [1-^{14}C]phytoene was quantified after 15 min of reaction.
(C) Total and specific PSY activity normalized to WT. Specific activity was normalized to the corresponding PSY protein levels determined in the WT or mutants (A).
Results are means ± SD from three biological replicates. Significant difference, *P<0.05 in comparison with WT.

Figure 6. DXS and Other Carotenogenic Protein Levels in the *clp* Mutants.

Immunoblot analysis of DXS and five addition carotenoid biosynthetic pathway enzymes. PDS: phytoene desaturase, ZDS: ζ-carotene desaturase, BCH: β-carotene hydroxylases, LUT5: carotenoid hydroxylase CYP97A3, and ZEP: zeaxanthin epoxidase. Analyses were performed with 20 µg leaf protein extracts from 3-week-old plants of WT and the *clp* mutants. Membranes were stripped for reprobing 2-3 times. Actin signal from one representative blot is shown as loading control.

Figure 7. OR reduces PSY Turnover Rate and enhances PSY Activity in *clpc1*.

(A) Representative immunoblot analysis of PSY-GFP fusion protein. PSY-GFP was expressed in WT (*35S:PSY-GFP*/WT) and in a line constitutively overexpressing *OR* (*35S:PSY-GFP*/*OR*). PSY protein stability was monitored by immunoblotting using an anti-GFP antibody prior (0 h) and at 3, 6 and 9 h following treatment with cycloheximide (CHX) to inhibit cytoplasmic protein translation. Ponceau S staining shows protein loading.

(B) Relative PSY protein levels. PSY band intensities were quantified and expressed relative to the levels detected prior to CHX treatment. Results are means ± SD from quantification of three biological replicates.

(C) Immunoblot analysis of OR protein levels. Actin level is shown as loading control.

(D) Phytoene accumulation in leaves of 3-week-old plants following treatment with norflurazon for 4 h. Phytoene in *clpc1*, *AtOR*-overexpressing line (*OR*) and double homozygous crossing (*clpc1* x *OR*) was quantified by HPLC and expressed relative to that determined in WT. Results are means ± SD from three biological replicates. Phytoene amounts from all lines/WT were significantly different from each other (student's *t*-test, *P<0.05).

Figure 8. Model on Counterbalance of PSY Proteostasis by Clp Protease and OR.

Membrane-associated PSY requires interaction with the membrane-integral holdase OR in order to remain properly folded and enzymatically active. Misfolded PSY is recognized by Clp adaptor proteins and gets degraded by the Clp protease system through direct protein-protein interactions. OR maintains the population of properly folded PSY proteins and thus carotenogenesis, while absence of OR results in PSY degradation. Deficiency in Clp activity reduces PSY proteolysis and results in the accumulation of PSY populations, which contained enzymatically inactive as

well as active forms probably due to increased chaperone proteins to help folding or prevent aggregations.

SUPPLEMENTAL INFORMATION

The following materials are available in the online version of this article:

Supplemental Figure 1. Two independent transgenic lines expressing *35S:PSY-GFP*

Supplemental Figure 2. Negative controls for interaction studies

Supplemental Figure 3. Generation of *clpp4* Antisense lines

Supplemental Figure 4. Carotenoid levels in *clp* mutants after NFZ treatment

Supplemental Figure 5. GGPS activity in chloroplast membrane fractions

Supplemental Figure 6. PSY-GFP fusion protein expression in WT and *OR*-overexpression background

Supplemental Figure 7. Interaction between ClpS1 and mature PSY N-terminal sequence containing a putative N-degron by Y2H

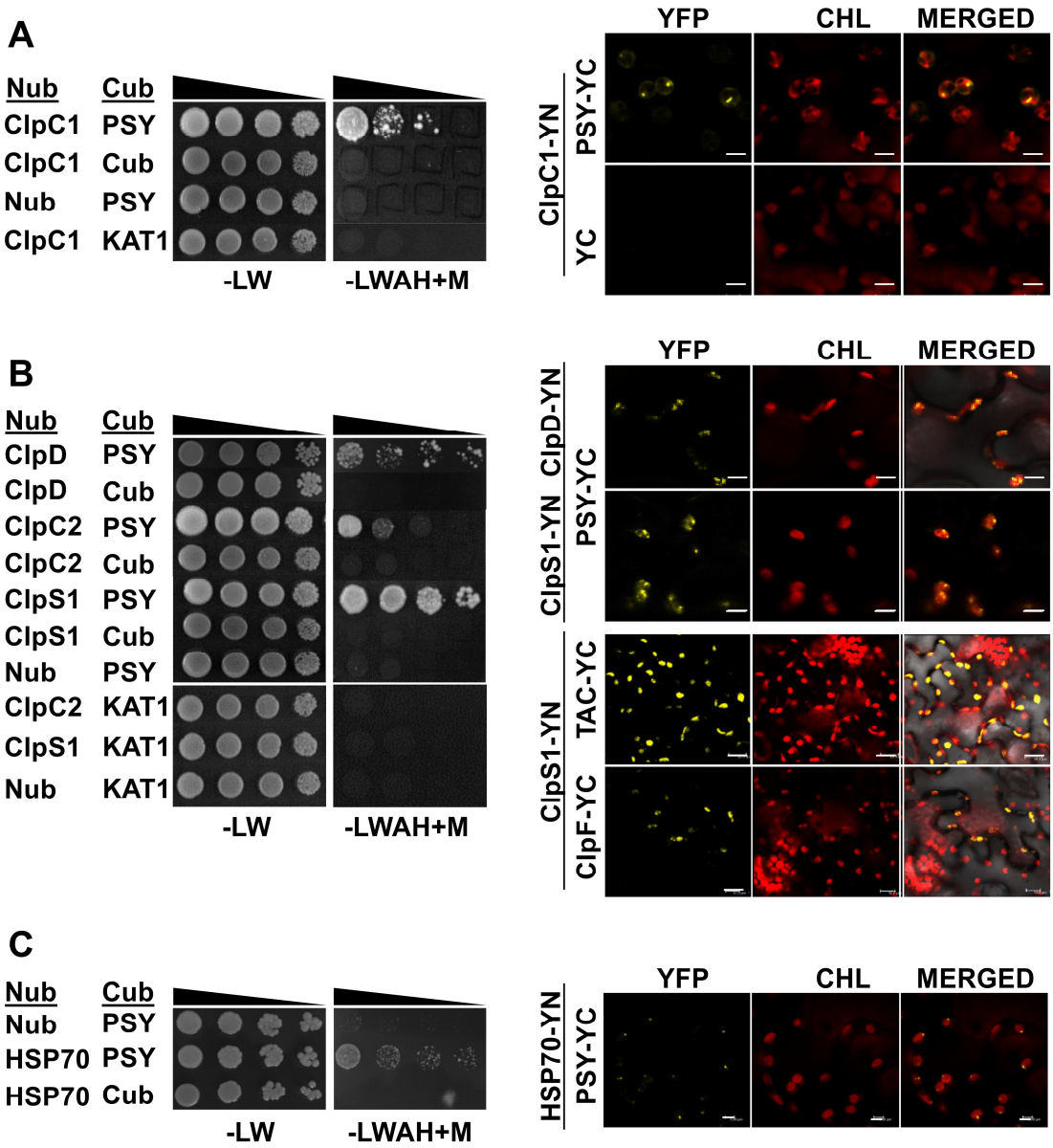
Supplemental Figure 8. Interaction between Hsp70, PSY and OR by Y2H assays

Supplemental Figure 9. Hsp70 and Hsp90 Levels in the *clp* mutants

Supplemental Table 1. Proteins identified in *35S:PSY-GFP* and *35S:GFP* Arabidopsis plants by co-IP and MS

Supplemental Table 2. Common proteins in quadruplicate PSY-GFP samples but absent in the GFP only controls

Supplemental Table 3. List of primers used in this study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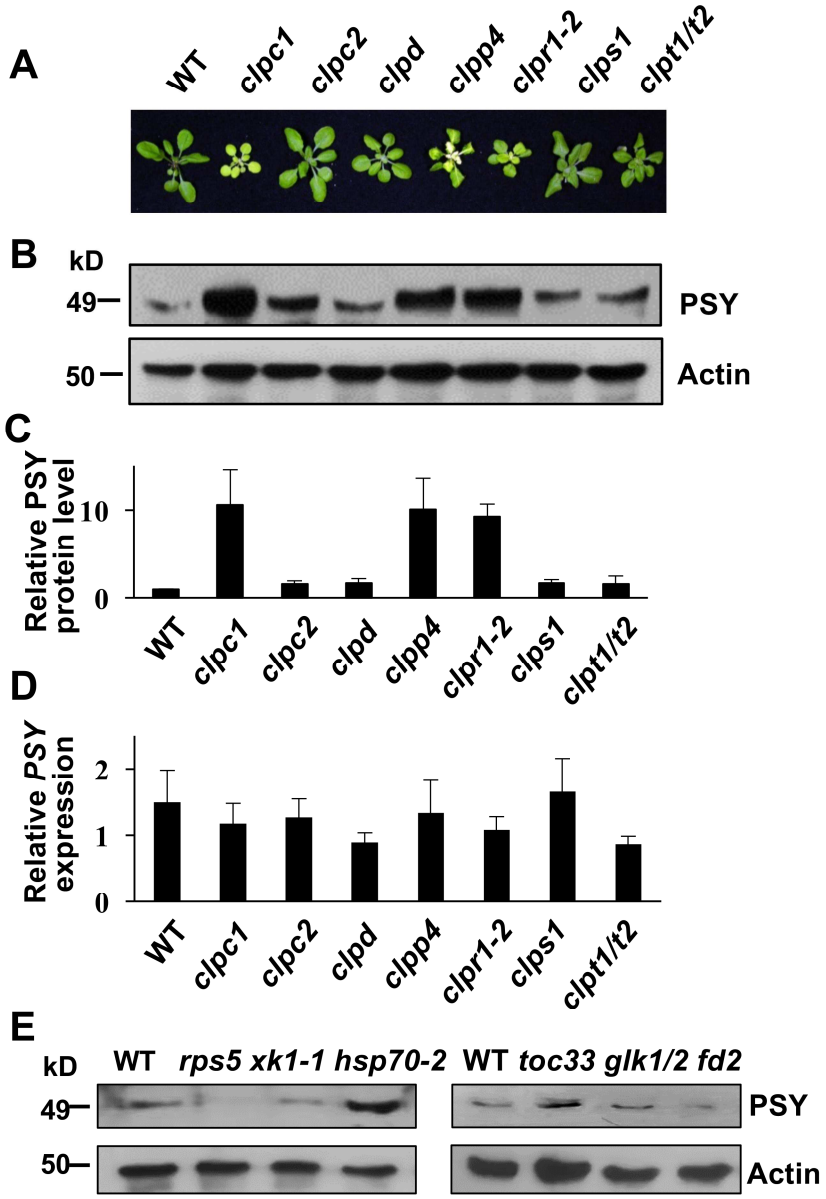
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50
Actin
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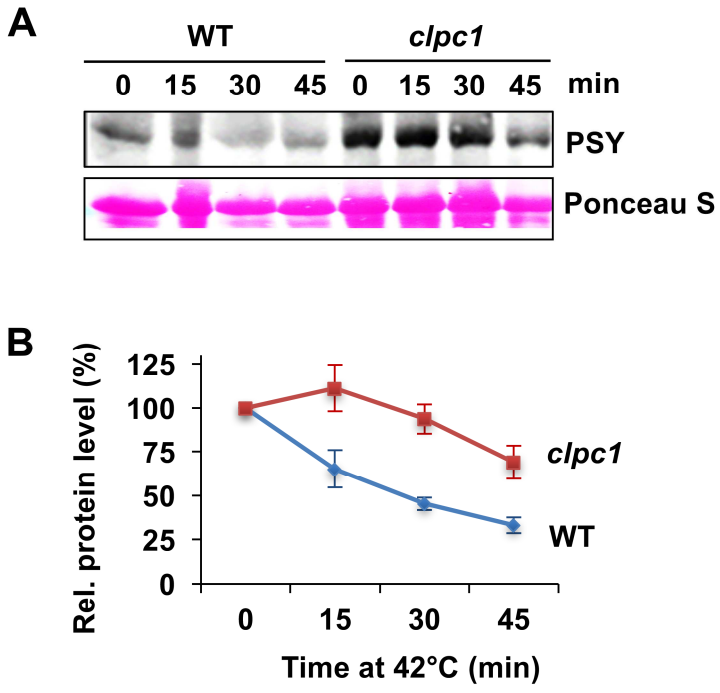

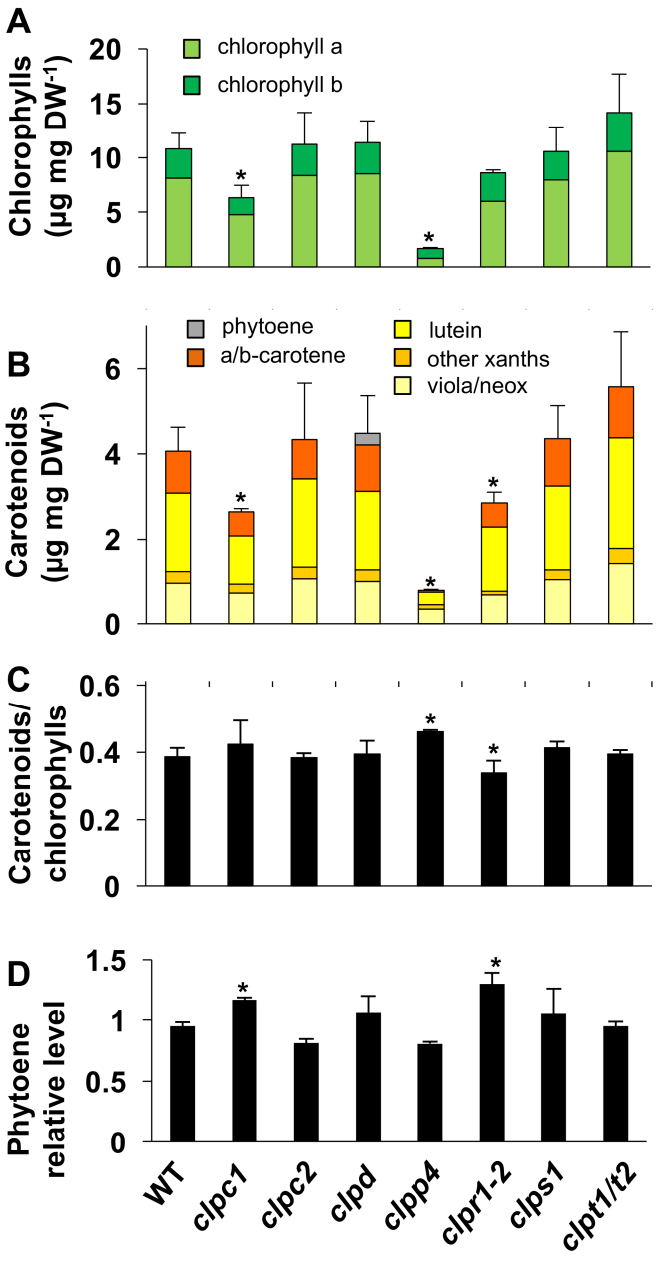
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viola/neox
C
Carotenoids/ chlorophylls
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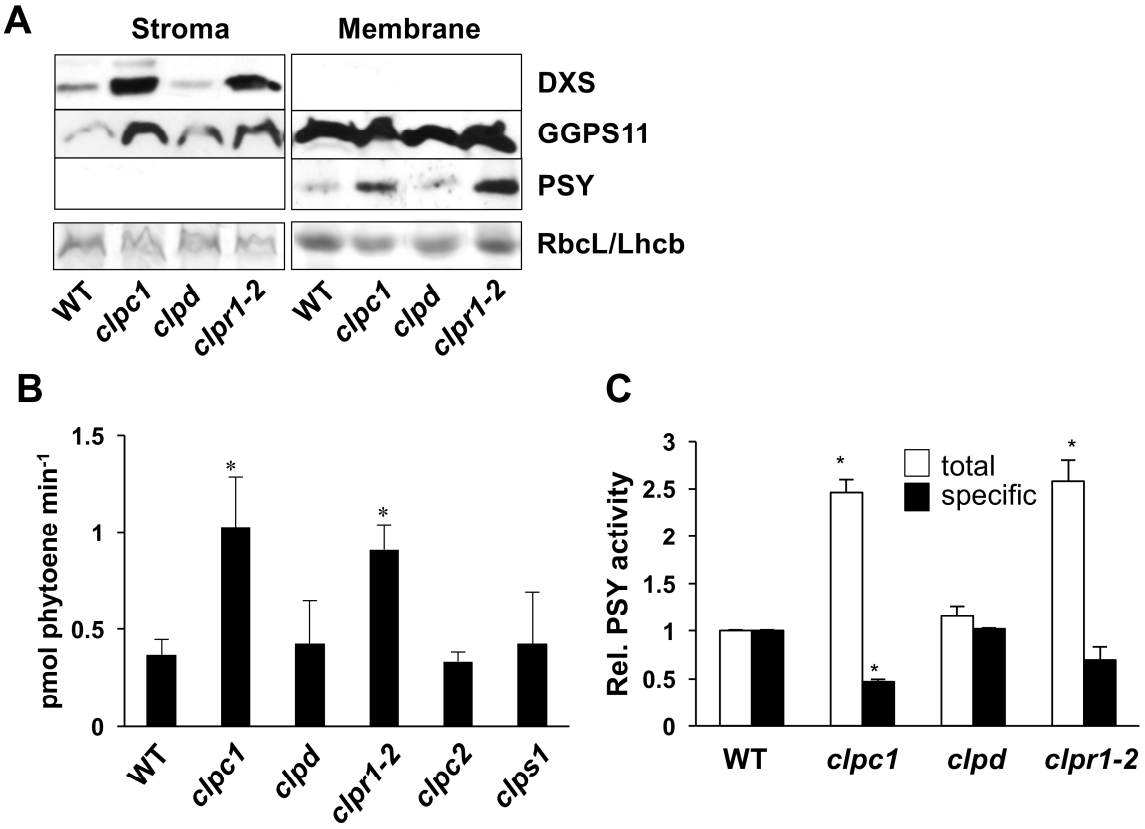
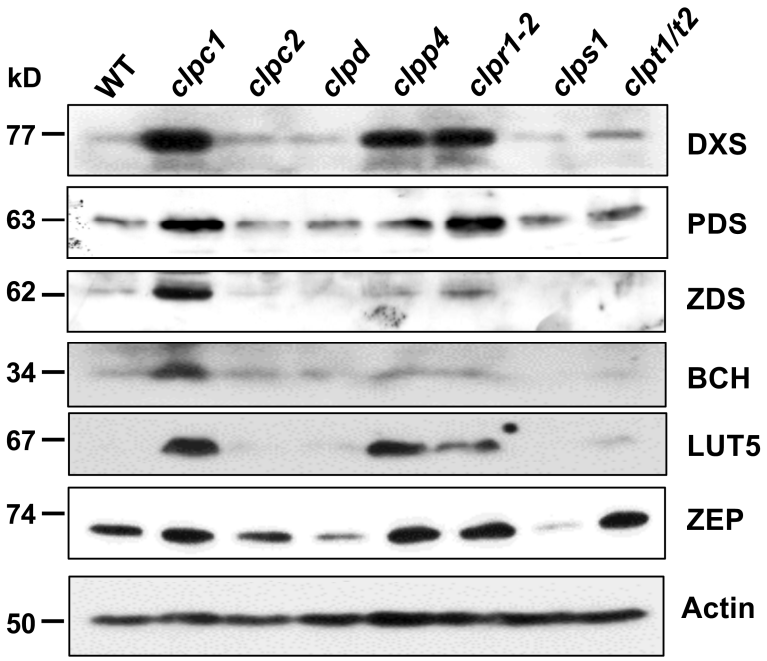
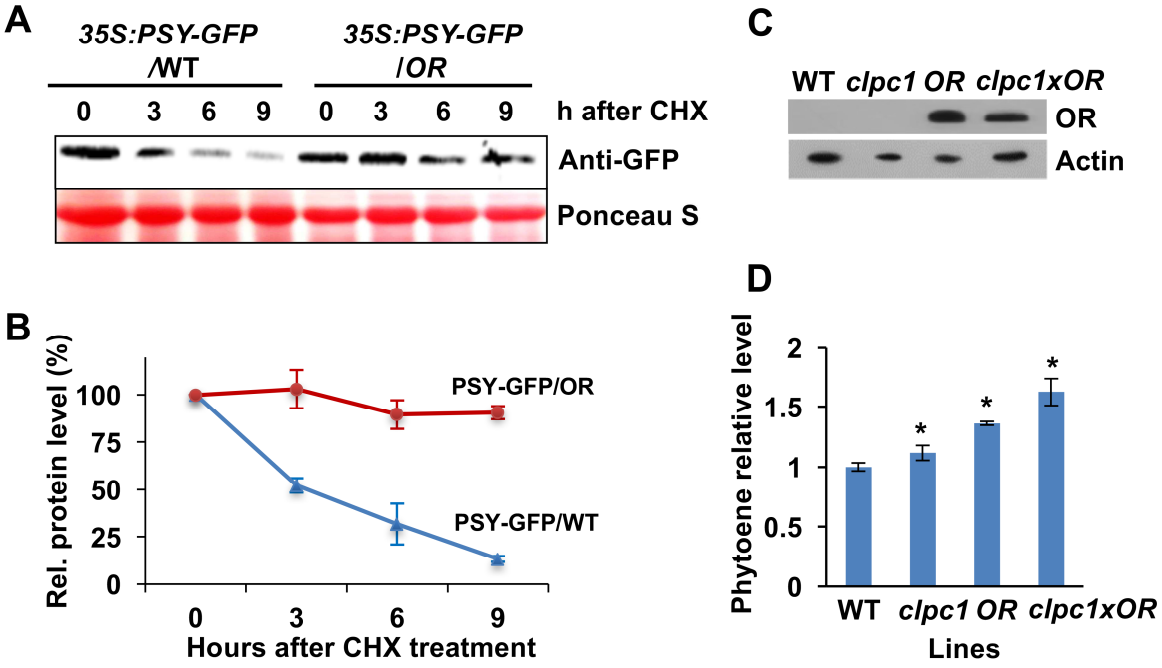
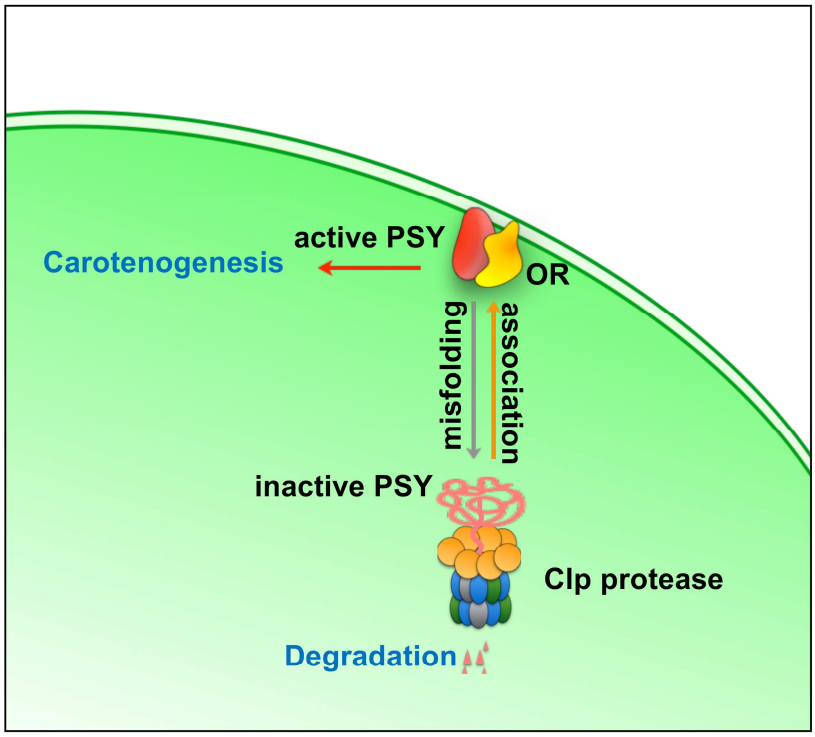
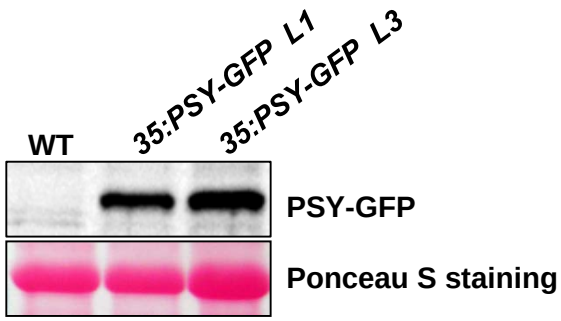


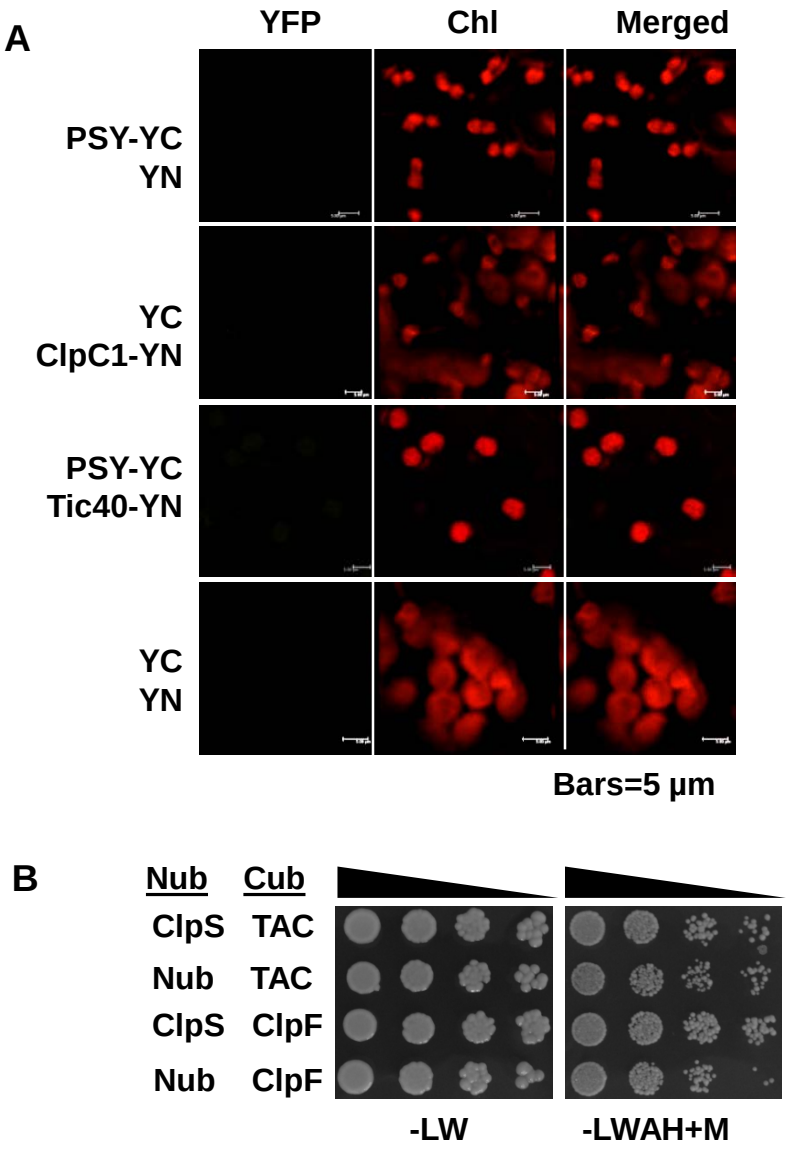




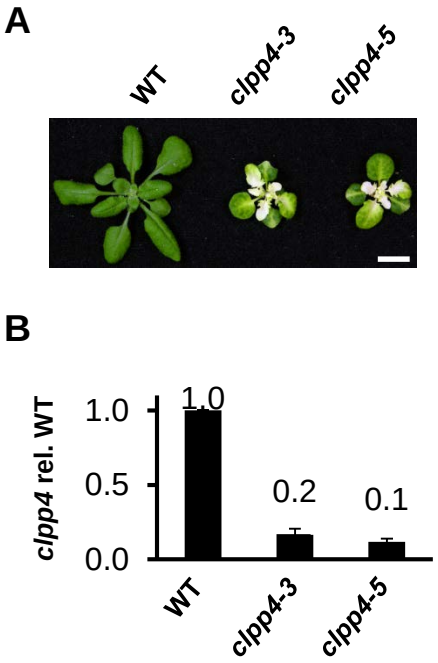




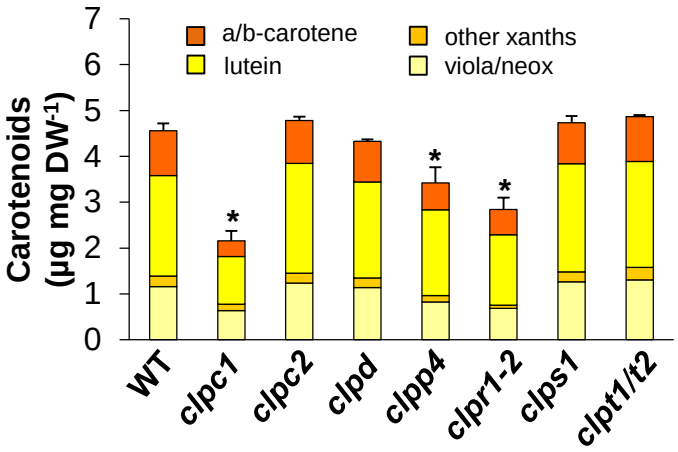
Supplemental Figure 1. Two independent transgenic lines expressing *35S:PSY-GFP.* Ponceau S staining shows protein loading.



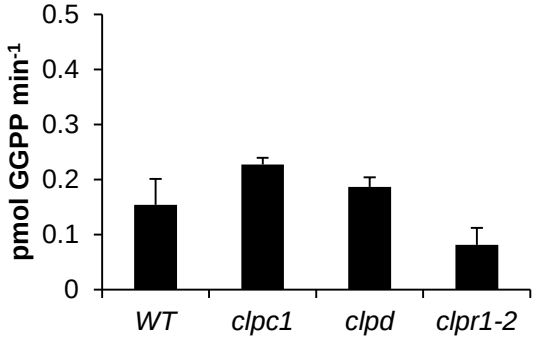
Supplemental Figure 2. Negative Controls for Interaction Studies.
(A) BiFC negative controls for PSY and ClpC1 interaction. PSY-YC, ClpC1-YN were co-expressed with the corresponding N- or C-terminal YFP (YN and YC, respectively) *in N. benthamiana* leaves. In addition, PSY-YN was coexpressed with Tic40-YN as unspecific negative control and empty vector control (YC+YC). No interactions were found in chloroplasts. Bars = 5 µm, CHL, chlorophyll autofluorescence.
(B) Y2H analysis with PRLI-interacting factor L (TAC) and ClpF. Interactions were examined by coexpression of pairs of proteins fused to either N-terminal or C-terminal ubiquitin moiety in yeast and spotted onto either nonselective (-LW) or fully selective medium plates (supplemented with 1 mM Met; -LWAH+M) in a series of 10-fold dilutions. Expression of TAC and ClpF showed strong reporter gene autoactivation, indicated by yeast growth in presence of only empty vectors expressing Nub and Cub. Therefore, specific interactions between ClpSa/TAC and ClpS1/ClpF could not be analyzed with this system.



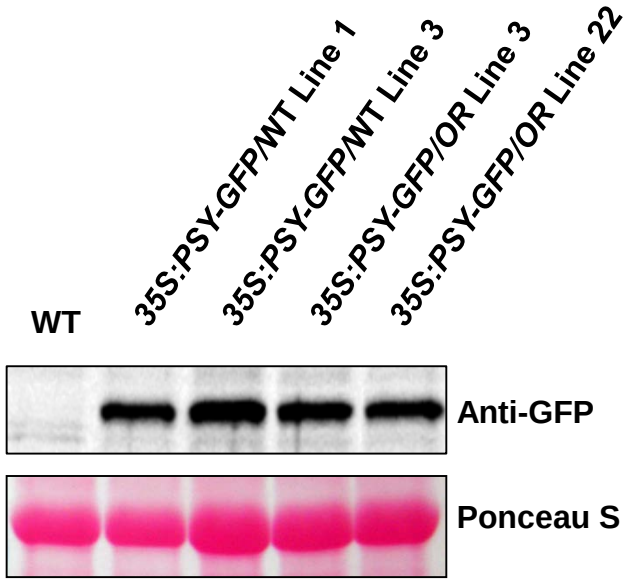
Supplemental Figure 3. Generation of *clpp4* Antisense Lines.
(A) *Clpp4* antisense construct was constitutively expressed in Arabidopsis WT (Col-0). Representative pictures from 4-week-old WT and two selected *clpp4* antisense lines. Bar = 1 cm.
(B) *ClpP4* expression levels were determined by real-time RT-PCR, normalized to actin levels and expressed relative to WT levels.



Supplemental Figure 4. Carotenoid Levels in the *clp* Mutants after NFZ Treatment. Carotenoid levels were determined in 3-week-old leaves incubated with norflurazon for 4 h. Phytoene levels are not shown. Results are means ± SE from ≥ three biological replicates. Significant difference, *P<0.05 in comparison with WT.

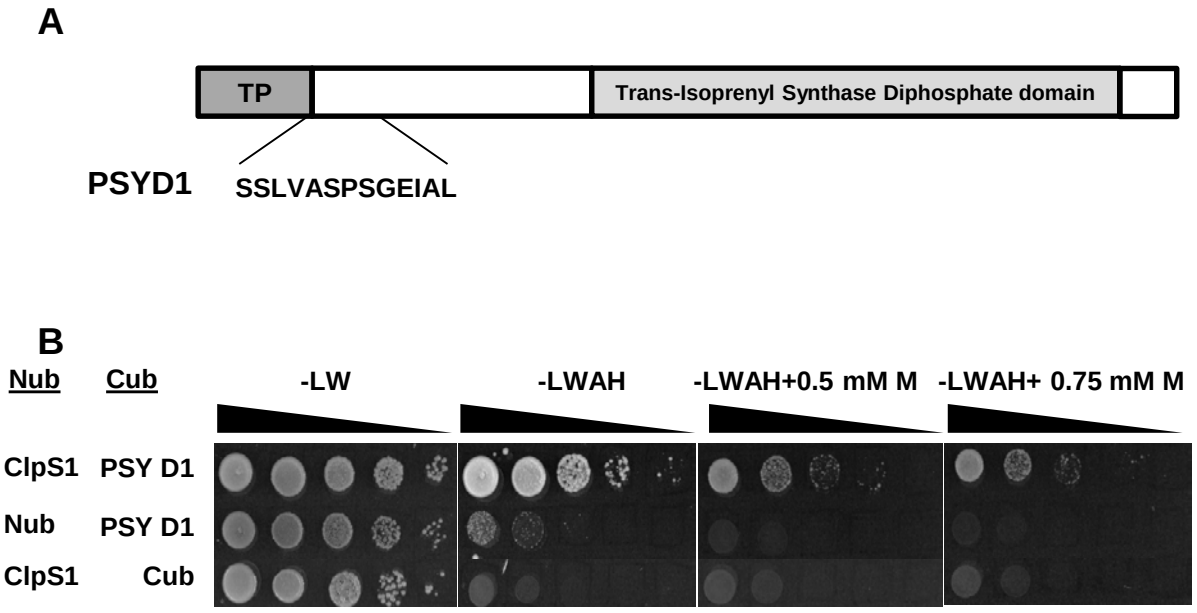


Supplemental Figure 5. GGPS Activity in Chloroplast Membrane Fractions.
Chloroplast membranes from 3-week-old plants were incubated with DMAPP and [1-^{14}C]IPP. The synthesized product [1-^{14}C]GGPP was quantified. No significant differences (student's *t*-test, P< 0.05) were determined for the mutant lines in comparison with wild-type control. Data are mean of two biological replicates.

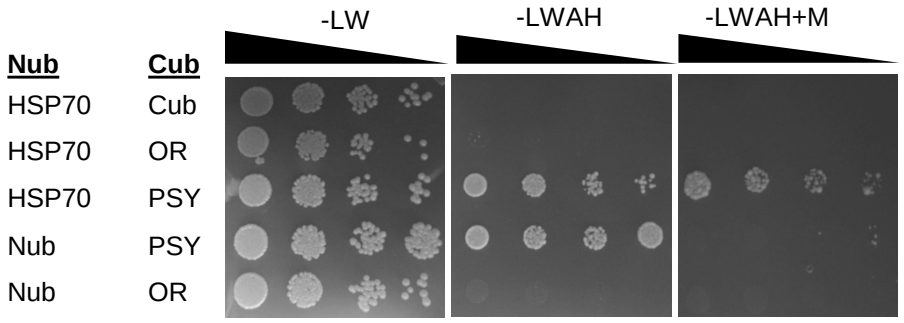


Supplemental Figure 6. PSY-GFP Fusion Protein Expression in WT and *OR* Overexpression Background.

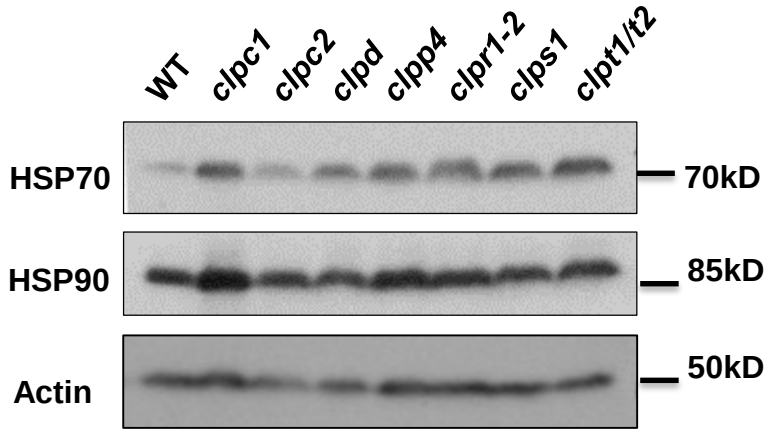
PSY-GFP was constitutively expressed in WT (35S:PSY-GFP/WT lines1 and 3) and in a transgenic line overexpressing *AtOR* (35S:PSY-GFP/OR lines 3 and 22). Immunoblot analysis of leaf protein extracts using anti-GFP antibodies. Two lines with similar level of PSY-GFP protein expression were used for PSY stability experiments in Figure 7.



Supplemental Figure 7. Interaction Between ClpS1 and Mature PSY N-terminal Sequence Containing a Putative N-Degron by Y2H.
(A) PSY protein structure. PSYD1 contains 13 amino acids (#71-83) as indicated.
(B) Y2H analysis. ClpS1 interacts with PSYD1 fragment in yeast.



Supplemental Figure 8. Interactions Between Hsp70, PSY and OR by Y2H Assay.
Interactions were examined by coexpression of pairs of proteins fused to either N-terminal or C-terminal ubiquitin moiety in yeast and spotted onto either nonselective (-LW) or fully selective medium plates (supplemented with 150 µM Met; -LWAH+M) in a series of 10-fold dilutions. Empty vectors expressing Nub and Cub only were used as negative or unspecific controls, respectively.



Supplemental Figure 9. Hsp70 and Hsp90 Levels in the *clp* Mutants.
Immunoblot analyses were performed with 80 µg leaf protein extracts from 3-week-old plants of WT and the *clp* mutants. Membranes were stripped for reprobing. Actin is shown as loading control.