Ubiquitination of phytoene synthase 1 precursor modulates carotenoid biosynthesis in tomato

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Carotenoids are natural pigments that are indispensable to plants and humans, whereas the regulation of carotenoid biosynthesis by post-translational modification remains elusive. Here, we show that a tomato E3 ubiquitin ligase, Plastid Protein Sensing RING E3 ligase 1 (PPSR1), is responsible for the regulation of carotenoid biosynthesis. PPSR1 exhibits self-ubiquitination activity and loss of PPSR1 function leads to an increase in carotenoids in tomato fruit. PPSR1 affects the abundance of 288 proteins, including phytoene synthase 1 (PSY1), the key rate-limiting enzyme in the carotenoid biosynthetic pathway. PSY1 contains two ubiquitinated lysine residues (Lys380 and Lys406) as revealed by the global analysis and characterization of protein ubiquitination. We provide evidence that PPSR1 interacts with PSY1 precursor protein and mediates its degradation via ubiquitination, thereby affecting the steady-state level of PSY1 protein. Our findings not only uncover a regulatory mechanism for controlling carotenoid biosynthesis, but also provide a strategy for developing carotenoid-enriched horticultural crops.

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Carotenoids are a group of 40-carbon isoprenoid compounds that are synthesized by all photosynthetic organisms (bacteria, algae, and plants) and some non-photosynthetic bacteria and fungi. As natural pigments, carotenoids are indispensable to plants and humans. In plants, carotenoids act as structural components of photosynthetic machinery, protect plants from photooxidative damage, and serve as precursors for phytohormones (abscisic acid and strigolactones) and other signaling molecules. Carotenoids are also important contributors to fruit color and nutritional quality in horticultural crops such as tomato and citrus, which accumulate carotenoids during fruit ripening. In humans, carotenoids provide precursors for biosynthesis of vitamin A and serve as antioxidants. Epidemiological analyses demonstrate that dietary intake of carotenoid-rich foods can lower the risk of degenerative diseases.

Due to the pivotal role of carotenoids in nature, molecular dissection of carotenoid metabolism and its regulatory network has received considerable interest. The biosynthesis of carotenoids is a complicated process that involves a series of steps. The genes responsible for carotenoid biosynthesis in higher plants have been well-defined; however, the regulatory mechanisms that govern their action, especially the regulation at post-translational level, are poorly understood.

Plant carotenoids are synthesized in plastids, a type of plant-specific organelles, of which the typical members include chloroplasts and chromoplasts. Plastids are semi-autonomous organelles and more than 90% of the proteins in the plastid, including those in the carotenoid biosynthetic pathway, are nucleus-encoded. These plastid-targeted proteins are synthesized in the cytosol as preproteins (precursor proteins), which contain an N-terminal transit peptide that directs the precursor proteins into the plastid before being proteolytically removed. Recently, it was shown that protein ubiquitination, an essential process in the plastid, is involved in the degradation of precursor proteins (eGFP) and monomeric Cherry protein (mCherry), to generate a translational fusion with enhanced green fluorescent protein (eGFP) and HA-tagged SlUBC32, their coding sequences were introduced into a plasmid. The results showed that the protein ubiquitination, an essential process, plays a role in the regulation of carotenoid biosynthesis.

PPSR1 directly interacts with SIUBC32. To get insight into the molecular basis of SIUBC32 in regulating fruit ripening, we performed a yeast two-hybrid (Y2H) screen to identify proteins that interact with SIUBC32 using a tomato cDNA library. A putative new gene (RING) E3 ubiquitin ligase (Solyc01g006810), which we named PPSR1, was identified as the candidate SIUBC32-interacting protein. Y2H analysis confirmed the interactions between PPSR1 and SIUBC32 (Fig. 1a). We then carried out split luciferase complementation imaging (LCI) assay, in which eLCU-PPSR1 and SIUBC32-nLUC were transiently co-expressed in leaves of tobacco (Nicotiana benthamiana). An intense luciferase activity was detected in tobacco leaves co-expressing eLCU-PPSR1 and SIUBC32-nLUC, whereas the negative controls showed no luciferase activity (Fig. 1b), indicating that PPSR1 interacts with SIUBC32. We subsequently investigated whether PPSR1 interacts with SIUBC32 in vitro. The MBP-tagged PPSR1 (MBP-PPSR1) and HA-tagged SIUBC32 (SIUBC32-HA) recombinant proteins purified from Escherichia coli were mixed and incubated with anti-HA agarose, and then the precipitated products were examined by immunoblot analysis.

PPSR1 is a RING-type E3 ubiquitin ligase with self-ubiquitination activity. As a putative RING-type E3 ubiquitin ligase, the function of PPSR1 in tomato remains uncharacterized. PPSR1 is composed of 342 amino acids, containing a RING-type zinc finger domain (RING domain) in its N-terminal region. According to alignment of homologous protein sequences, the RING domain in PPSR1 is a C3H2C3-type zinc finger whose cysteine (C) and histidine (H) residues can chelate two zinc ions. PPSR1 gene was expressed in both vegetative and reproductive organs including roots, stems, leaves, flowers, and fruits, and declined gradually during fruit ripening (Supplementary Fig. 1a). In contrast, PPSR1 protein exhibited a relatively stable state in fruit (Supplementary Fig. 1b). RING domain-containing proteins generally have E3 ubiquitin ligase activity. To determine whether PPSR1 functions as an E3 ligase, the MBP-tagged recombinant PPSR1 protein (MBP-PPSR1) was purified from E. coli and subjected to in vitro ubiquitination assay by incubation with wheat E1, human E2, and Arabidopsis ubiquitin. The reaction products were detected by immunoblot analysis using anti-MBP and anti-ubiquitin antibodies, respectively. As shown in Fig. 2b and Supplementary Fig. 2, the signals of high...
molecular mass bands, which represent ubiquitinated proteins, were observed in the intact reaction system using both anti-MBP and anti-ubiquitin detection, but not in the absence of a single component. These data indicated that PPSR1 has E3 ubiquitin ligase activity in vitro and can catalyze its self-ubiquitination. When the intact PPSR1 was substituted by the mutated form (mtPPSR1) in which conserved cysteine (C49) and histidine (H51 and H54) residues in the RING domain were replaced by serine (S) and tyrosine (Y), respectively (Fig. 2a), the bands for the ubiquitinated proteins failed to appear (Fig. 2c), demonstrating the critical role of the RING domain for the E3 ligase activity.

In order to test whether PPSR1 acts in cooperation with ubiquitin E2 enzyme SlUBC32, the purified recombinant SIUBC32-HA, MBP-PPSR1, and MBP (negative control) were mixed as indicated, and incubated with anti-HA agarose. The eluted proteins were detected by immunoblot using anti-MBP and anti-HA antibodies, respectively. The red arrowhead indicates the predicted Flag-PPSR1. The black arrowhead refers to heavy chain of antibody (IgG). (Ub)n, polyubiquitin chain. Subcellular colocalization of PPSR1 and SIUBC32. The Agrobacteria carrying 35S::PPSR1-eGFP and 35S::SIUBC32-mCherry constructs were transiently co-transformed into tobacco leaves. The tobacco protoplasts co-expressing eGFP and mCherry were used as negative control. Scale bars, 20 μm.

Fig. 1 PPSR1 interacts with SIUBC32. a Y2H assay revealing the interactions between PPSR1 and SIUBC32. The PPSR1 fused with the binding domain (BD) of GAL4 (BD-PPSR1) and the SIUBC32 fused with the activation domain (AD) of GAL4 (AD-SIUBC32) were co-expressed in yeast. The transformants were selected on SD/-Leu/-Trp (-LW) and SD/-Leu/-Trp/-His/-Ade (-LWHA) with or without X-α-gal. b LCI assay revealing the interactions between PPSR1 and SIUBC32. The PPSR1 fused with the C-terminus of LUC (cLUC-PPSR1) was co-expressed with the SIUBC32 fused with the N-terminus of LUC (SIUBC32-nLUC) in tobacco (Nicotiana benthamiana) leaves. Scale bar, 1 cm. c Pull-down assay revealing the interactions between PPSR1 and SIUBC32. The recombinant SIUBC32-HA, MBP-PPSR1, and MBP (negative control) were mixed as indicated, and incubated with anti-HA agarose. The eluted proteins were detected by immunoblot using anti-MBP and anti-HA antibodies, respectively. The red arrowhead indicates the predicted Flag-PPSR1. The black arrowhead refers to heavy chain of antibody (IgG). (Ub)n, polyubiquitin chain. d Co-IP assay revealing the interactions between PPSR1 and SIUBC32. The Flag-PPSR1 and SIUBC32-HA fusion proteins were co-expressed in N. benthamiana leaves. The total proteins were extracted from the infected leaves treated with MG132 and immunoprecipitated by anti-HA agarose. The eluted proteins were then detected by immunoblot using anti-Flag and anti-HA antibodies, respectively. The red arrowhead indicates the predicted Flag-PPSR1. The black arrowhead refers to heavy chain of antibody (IgG). (Ub)n, polyubiquitin chain. e Subcellular colocalization of PPSR1 and SIUBC32. The Agrobacteria carrying 35S::PPSR1-eGFP and 35S::SIUBC32-mCherry constructs were transiently co-transformed into tobacco leaves. The tobacco protoplasts co-expressing eGFP and mCherry were used as negative control. Scale bars, 20 μm.

Given PPSR1 exhibits self-ubiquitination activity that may sharply reduce its stability, we measured the half-life of PPSR1 using a cell-free degradation assay. Protein degradation was observed for MBP-PPSR1 and its half-life arrived at about 2.5 h of incubation (Fig. 2g, h). By comparison, the protein levels of MBP-mtPPSR1 remained more than 70% of the initial content at that time, indicating that the degradation rate of MBP-PPSR1 is markedly faster than that of its mutant form (Fig. 2g, h). To assess the stability of PPSR1 in vivo, Flag-PPSR1 and Flag-mtPPSR1 were transiently expressed in tobacco leaves, respectively. Immunoblot analysis revealed that the levels of Flag-mtPPSR1 were higher than those of Flag-PPSR1, and both fusion proteins were accumulated after treatment with MG132, a 26S proteasome inhibitor (Fig. 2i). Collectively, these data pointed out that the RING domain is...
required for the E3 ligase activity of PPSR1, which may function as dimers and undergo degradation via self-ubiquitination.

**Loss of PPSR1 function increases carotenoid accumulation in tomato fruit.** To investigate the function of PPSR1 in tomato, we generated ppsr1 mutants using a CRISPR/Cas9 gene-editing system. Four single guide RNAs (sgRNAs) that contain different target sequences (T1, T2, T3, and T4) were designed to specifically target the exons of PPSR1 (Fig. 3a). Among transgenic plants in the second generation, three distinct homozygous mutant lines (ppsr1-4, ppsr1-10, and ppsr1-13) were isolated and confirmed by sequencing genomic regions flanking the target sites. These homozygous mutants carry 7-bp deletion (ppsr1-4), 1-bp insertion (ppsr1-10), and 1-bp deletion (ppsr1-13).
PPSR1 modulates carotenoid accumulation in tomato fruit during ripening. 

**a** Genotyping of mutations mediated by CRISPR/Cas9 gene-editing system in *ppsr1*-4, *ppsr1*-10, and *ppsr1*-13 mutants. Schematic illustration shows the single guide RNAs (sgRNAs) containing different target sequences (T1, T2, T3, and T4) that were designed to specifically target the exons of *PPSR1*. Red letters represent the protospacer adjacent motif (PAM). Red arrows indicate the editing sites that were verified by sequencing.

**b** Absence of PPSR1 protein in the *ppsr1* mutants. Total proteins were extracted from fruit of wild-type (WT) and *ppsr1* mutants at 38 days post-anthesis (DPA) and subjected to immunoblot using an anti-PPSR1 antibody. Equal loading was confirmed by an anti-actin antibody. IB, immunoblot.

**c** Phenotype analysis of *ppsr1* mutants. Fruit from WT and *ppsr1* mutants at 34, 38, 41, and 45 DPA are shown. Scale bars, 2 cm.

**d** Accumulation of carotenoids (phytoene, lycopene, and β-carotene) in fruit of WT and *ppsr1* mutants during ripening. Error bars represent the means ± standard deviation (SD) of three independent experiments. The circles indicate individual data points. Asterisks indicate significant differences (*P* < 0.05, **P** < 0.01, ***P*** < 0.001; two-tailed Student’s t-test).

**e** Venn diagram showing the overlap of proteins that exhibit differential expression in the *ppsr1* mutant fruit compared to the WT in two independent biological replicates of quantitative proteome analysis. Proteins isolated from WT and *ppsr1* mutant fruit at 38 DPA were subjected to iTRAQ (isobaric tags for relative and absolute quantification) labeling coupled with NanoLC-MS/MS.

(*ppsr1*-10), or 2-bp deletion (*ppsr1*-13) caused by target T4 in the sixth exon of *PPSR1* (Fig. 3a), and no editing events occur around the sequence of target T1/2/3. All mutants were predicted to cause premature termination of PPSR1 protein translation within the following 40-bp sequence of editing sites. Immunoblot analysis detected a band corresponding to the predicted size (~38-kDa) of the full-length PPSR1 only in the wild type (Fig. 3b). No bands were observed in the *ppsr1* mutants, indicating that the predicted truncated versions of PPSR1 (~19-kDa) did not generate and PPSR1 was successfully knocked out in three *ppsr1* mutants. The potential off-target sites in the tomato genome were predicted by CRISPR-P (version 2.0, http://crispr.hzau.edu.cn/CRISPR2/), and no mutagenesis was found in the six potential off-target sites (Supplementary Fig. 3), suggesting the specific mutation for PPSR1.

The *ppsr1* mutant lines (*ppsr1*-4, *ppsr1*-10, and *ppsr1*-13) showed obvious and similar ripening-accelerated phenotypes (Fig. 3c). A visible color change was observed at 34 days post-anthesis (DPA) in the *ppsr1* mutant fruit, while the wild-type tomato remained green at this stage (Fig. 3c). At 38 DPA, the *ppsr1* mutant fruit displayed a homogenous orange color, whereas the fruit from the wild type was only just starting to change color. This suggests that *PPSR1* is responsible for tomato fruit pigmentation. Detection of the content of three important carotenoids (phytoene, lycopene, and β-carotene) in tomato fruit indicated that the accelerated coloration in *ppsr1* fruit correlated with the significantly elevated carotenoid contents (Fig. 3d).

**E3 ubiquitin ligase mediates protein degradation, leading to the changes in protein abundance**. To identify the proteins that differentially accumulate in the *ppsr1* mutant, we performed a quantitative proteomic analysis using iTRAQ (isobaric tags for relative and absolute quantification) approach. Proteins isolated from *ppsr1* mutant fruit and wild-type fruit at 38 DPA were labeled with iTRAQ reagents and submitted to NanoLC-MS/MS analysis. A total of 5318 and 5375 proteins were identified in two independent biological replicates, respectively, with a global false discovery rate (FDR) below 1% in both. Quantitative analysis
indicated that 288 proteins were differentially expressed in the ppsr1 mutant fruit compared to the wild type in both biological replicates (Fig. 3e). Supplementary Data 1 shows these proteins along with the relevant identification information. We identified a number of ripening-related proteins, of which several are involved in carotenoid biosynthesis, including carotenoid isomerase (CRTISO), PSY1, and phytoene desaturase (PDS), and the levels of these proteins were higher in the ppsr1 mutant fruit (Supplementary Data 1), consistent with the carotenoid-enhanced phenotype.

**PSY1 is identified as a candidate substrate of PPSR1.** E3 ubiquitin ligases mediate the degradation of substrate proteins via ubiquitination, which involves the attachment of ubiquitin to lysine (K) residues on substrate proteins. We next sought to identify PPSR1 substrates and their ubiquitination sites by using a state-of-the-art technique, which couples a K-(GG) peptide immunoprecipitation with quantitative proteomic analysis (Fig. 4a). The proteins extracted from wild-type and ppsr1 mutant fruit at 38 DPA were trypsin digested and immunoprecipitated with an anti-K-(GG) antibody. The recovered ubiquitinated peptides were submitted to SWATH-MS (Sequential Window Acquisition of all Theoretical Mass Spectra) quantitative proteomic analysis. The ubiquitination sites, i.e., ubiquitinated lysine residues, were identified using MS/MS based on their retention time, mass-to-charge ratio (m/z) and charge states. Two independent biological replicates identified 265 and 236 diGly peptides, respectively. Seventy-four diGly peptides were overlapped in both biological replicates (Fig. 4b).

Quantitative analysis revealed that 27 of these diGly peptides changed abundance significantly (P < 0.05) in the ppsr1 mutant (Supplementary Data 2). We focused on diGly peptides with lower abundance in the ppsr1 mutant, because...
PPSR1 interacts with PSY1 precursor and mediates its ubiquitination. To test our hypothesis that PSY1 is a substrate of PPSR1, we first assessed the interactions between PPSR1 and PSY1 by Y2H analysis. As a plastid-localized protein, PSY1 contains a putative chloroplast transit peptide (cTP; 1–62 amino acids), which may interfere with the Y2H assay, in its N-terminal region according to the prediction of TargetP v1.1 (http://www.cbs.dtu.dk/services/TargetP-1.1/index.php) (Fig. 5a). It was shown that PSY1 without transit peptide (PSY1-63–412) interacted with PPSR1 (Fig. 5b). We then examined the region of PSY1 that is needed for the interactions. PSY1-63–412 was truncated into three fragments, namely PSY1-130–412, PSY1-239–412, and PSY1-130–238 (Fig. 5a). The result showed that PPSR1 interacted with PSY1-130–412 and PSY1-239–412, but not PSY1-239–412 (Fig. 5b), indicating that the 130–238 amino acids in the PSY1 sequence are required for the interactions between PSY1 and PPSR1. It should be noted that, while PPSR1 interacts with N-terminal end of PSY1, the ubiquitination sites of PSY1 occur at the C-terminus of the protein. This could be explained by the characteristics of E3 ligases, which specifically recruit substrate proteins and transfer the activated ubiquitin from E2 enzymes to the substrates. The E3 recognition regions and the ubiquitination sites in the substrates are likely to be different.

The LCI assay showed that the tobacco leaves co-expressing cLUC-PPSR1 and PSY1-nLUC generated luciferase activity, whereas the negative control exhibited no signal (Fig. 5c), confirming that PPSR1 interacts with PSY1. Semi-in vivo pull-down assay demonstrated that HA-tagged full-length PSY1 (PSY1-HA) can directly bind to MBP-tagged PPSR1 (MBP-PPSR1), but not MBP tag protein (Fig. 5d). To further verify the interactions between PPSR1 and PSY1, a Co-IP assay was carried out in tobacco leaves co-expressing Flag-PPSR1 and PSY1-HA. As shown in Fig. 5e, Flag-PPSR1 was immunoprecipitated with PSY1-HA by anti-HA agarose. Together, these data indicated that PPSR1 interacts with PSY1.

Fluorescence microscopy showed the green fluorescent signal from eGFP-tagged full-length PSY1 (PSY1-eGFP) merged the red fluorescent signal from mCherry-tagged PPSR1 (PPSR1-mCherry) in the cytosol (Fig. 5f), suggesting the subcellular colocalization of PSY1 and PPSR1. Since the mature PSY1 localized in the plastid, the fluorescent signal of PSY1-eGFP in the cytosol should be produced by the PSY1 precursor proteins.

We subsequently examined the ubiquitination of PSY1. The HA-tagged full-length PSY1 (PSY1-HA) was co-expressed with Flag-tagged ubiquitin (Flag-Ub) and PPSR1 in tobacco leaves, and then the total soluble proteins were extracted for ubiquitination assay. The formation of high molecular mass bands, which represent ubiquitinated PSY1-HA, was detected (Fig. 5g). This suggests that PPSR1 mediates the ubiquitination of PSY1. Notably, the ubiquitinated signal occurred over a band of the expected size (~48-kDa) for full-length PSY1 molecule (i.e., precursor) fused with HA tag. Due to the removal of the transit peptide (~7-kDa) from the PSY1 precursor during import into the plastid, the mature PSY1-HA protein, which could be detected using a robust protein extraction method (Supplementary Fig. 5), has a predicted molecular mass of ~41-kDa. Together, these data suggest that PPSR1 interacts with PSY1 precursor in the cytosol and mediates its ubiquitination.
ubiquitin enzymes cooperate to attach ubiquitin to the substrate proteins, which are then recognized and degraded by the 26S proteasome. The 26S proteasome in plant cells is present in both the cytoplasm and the nucleus, and therefore ubiquitination was initially thought to occur only in proteins of these cellular compartments. Later findings have indicated that ubiquitination and subsequent degradation also happens in proteins on cell surface membrane, or even in proteins from endoplasmic reticulum (ER) lumen and membrane. By contrast, the regulation of ubiquitination on plastid proteins remains elusive. Intriguingly, recent researches unveiled that chloroplast outer membrane protein degradation was regulated by the ubiquitin–proteasome system. However, whether proteins in plastid metabolic pathways are mediated by ubiquitination remains unclear. In this study, we found that the E3 ubiquitin ligase PPSR1 targets precursor of PSY1, a key enzyme in the carotenoid biosynthesis pathway, and mediates its ubiquitination and degradation. Such proteolytic regulation changes the steady-state level of PSY1 protein, thereby modulating carotenoid biosynthesis. These data uncover a specific regulatory role of E3 ubiquitin ligase on plastid metabolic processes, which was achieved by modulating precursors of plastid-destined proteins in the biosynthetic pathways.

**Fig. 5 PPSR1 binds to PSY1 and mediates its ubiquitination.** a, b Y2H assay revealing the region of PSY1 that interacts with PPSR1. a Schematic illustration for full-length PSY1 protein and the truncated forms used in Y2H analysis. Numbers indicate the positions of the first and last amino acid in the sequences. cTP, chloroplast transit peptide. b The PPSR1 fused with the binding domain (BD) of GAL4 (BD-PPSR1) and the truncated PSY1 fused with the activation domain (AD) of GAL4 were co-expressed in yeast. The transformants were selected on SD-/Leu-/Trp (-LW) and SD-/Leu-/Trp-/His-/Ade (-LWHA) with or without X-α-gal. c LCI assay revealing the interactions between PSY1 and PPSR1. The PPSR1 fused with the C-terminus of LUC (cLUC-PPSR1) was co-expressed with the PSY1 fused with the N-terminus of LUC (PSY1-nLUC) in tobacco (*Nicotiana benthamiana*) leaves. Scale bar, 1 cm. d Semi-in vivo pull-down assay revealing the interactions between PPSR1 and PSY1. The recombinant MBP-PPSR1 and MBP (negative control) were mixed with PSY1-HA expressed in tobacco leaves, and incubated with anti-HA agarose. The eluted proteins were detected by immunoblot using anti-MBP and anti-HA antibodies, respectively. IB, immunoblot. e Co-IP assay revealing the interactions between PSY1 and PPSR1. The Flag-PPSR1 and PSY1-HA fusion proteins were co-expressed in tobacco leaves. Subcellular colocalization of PSY1 and PPSR1. The PSY1-eGFP and PPSR1-mCherry fusion proteins were transiently co-expressed into tobacco leaves. The tobacco leaves expressing eGFP or mCherry were used as the negative control. Scale bars, 20 µm. g Ubiquitination assay of PSY1. The *Agrobacteria* carrying 35S::PSY1-HA, 35S::Flag-ubiquitin (Ub), and 35S::PPSR1 constructs were infiltrated into the tobacco leaves. For (e) and (g), the total proteins were extracted from the infected leaves treated with MG132 and incubated with anti-HA agarose to enrich PSY1-HA. The eluted proteins were subjected to immunoblot using anti-Flag and anti-HA antibodies, respectively. The red arrowhead indicates the predicted Flag-PPSR1. The black arrowhead refers to heavy chain of antibody (IgG). Blue asterisks refer to nonspecific bands. IB, immunoblot; (Ub)n, polyubiquitin chain.
Due to the importance of precursors on protein steady-state levels, we propose that E3 ligase-mediated protein ubiquitination and degradation may regulate various biological processes in plant cells by targeting the precursors of proteins involved in these biological processes.

The RING-type E3 ubiquitin ligases might mediate substrate degradation without the aid of chaperones, i.e., heat shock proteins (HSPs). We detected whether PPSR1 interacts with HSP70/HSP90, which have been reported to form a complex with Carboxy terminus of Hsc70-Interacting Protein (CHIP), an E3

**Fig. 6** PPSR1 modulates PSY1 protein level via ubiquitination. a Stability assay of PSY1. The PSY1-HA fusion protein was co-expressed with Flag-PPSR1 in tobacco (*Nicotiana benthamiana*) leaves. The total proteins were extracted and submitted to immunoblot using anti-HA and anti-Flag antibodies, respectively. The *N. benthamiana* actin was used as the loading control. IB, immunoblot. b Degradation rate assays of PSY1 under the action of PPSR1. The PSY1-HA and Flag-PPSR1 fusion proteins were co-expressed in tobacco leaves. The leaves were treated with translation inhibitor cycloheximide (CHX), and the total proteins were extracted for immunoblotting with anti-HA antibody at an indicated time point after treatment. c Quantification of protein levels in (b) by ImageJ. d Diagram showing PSY1 with ubiquitination site mutations. K, lysine; R, arginine. e Degradation rate assays of PSY1 and its mutated forms. The PSY1 and its mutated forms expressed in tobacco leaves were treated with CHX and submitted to immunoblot as described in (b). f Quantification of protein levels in (e) by ImageJ. g Expression of PSY1 protein in fruit of wild-type (WT) and *ppsr1* mutants. Total RNA was isolated and submitted to quantitative real-time PCR. The ACTIN gene was used as the internal control. For (c), (f), (g), and (h), error bars represent the means ± standard deviation (SD) of three independent experiments. The circles indicate individual data points. Asterisks indicate significant differences (**P < 0.01, ***P < 0.001; two-tailed Student’s t-test). i The working model for PPSR1-mediated post-translational regulation of PSY1. PPSR1 directly interacts with SlUBC32 and mediates degradation of PSY1 precursor, which is nucleus-encoded and synthesized in the cytosol, via 26S proteasome. In the absence of PPSR1, more PSY1 precursor was transported into the plastid, leading to the accumulation of PSY1 protein. The increased PSY1 protein accelerates the biosynthesis of carotenoid in the plastid.
ligase responsible for plastid-destined precursor degradation under stress conditions in Arabidopsis. No interactions were observed between PPSR1 and HSP70/HSP90 (Supplementary Fig. 6). In addition, there were no apparent differences in protein levels of HSP70/HSP90 between fruit of wild-type and ppsr1 mutants (Supplementary Fig. 7). These data suggest that PPSR1 mediates PSY1 precursor degradation in a HSP70/HSP90-independent manner. These characteristics of PPSR1 are similar to those of Misfolded Protein Sensing RING E3 ligase 1 (MPSR1), a RING-type E3 ligase that directly recognizes its substrates and mediates their degradation in the absence of chaperones or cofactors. It should be noted that our study does not eliminate the possibility of other chaperones to interact with PPSR1.

PSY1 is regulated at multiple levels. As the main rate-determining enzyme, PSY acts in the first committed step in carotenogenesis and directs metabolic flux into the carotenoid biosynthetic pathway. Constitutive overexpression of PSY has increased total carotenoid contents and substantially enhanced β-carotene synthesis in tissues of various crops, such as canola seeds, potato tubers, cassava roots, and tomato fruit. Due to its central role in carotenoid biosynthesis, PSY is regulated at multiple levels. Substantial insights have been made into the transcriptional regulation of PSY. In Arabidopsis seedlings, the expression of the PSY gene is under the control of two transcription factors, PHYTOCHROME INTERACTING FACTOR 1 (PIF1) and LONG HYPOCOTYL 5 (HY5), which act antagonistically in photomorphogenesis. PSY1 in tomato fruit is regulated by transcription factors of the MADS box family, such as RIPENING INHIBITOR (RIN) and FRUITFULL 1 (FUL1/PIF1), which are synthesized in mitochondria. Moreover, the proteostasis of PSY has been shown to be modulated by the cochaperone-like ORANGE (OR) protein and Clp protease. However, it is not clear whether PSY is regulated by protein post-translational modification.

In the present study, we identified two ubiquitination sites (Lys380 and Lys406) in PSY1, the only PSY protein associated with carotenoid biosynthesis during tomato fruit ripening, and demonstrated that these ubiquitination sites are responsible for PSY1 precursor degradation. We speculate that PPSR1 modulates PSY1 protein ubiquitination and degradation mainly in unripe fruit to restrict the biosynthesis of carotenoids. At the onset of fruit ripening, the PPSR1-mediated degradation of PSY1 protein could be relieved due to uncharacterized reasons and the expression of PSY1 gene is activated by various transcription factors. These findings provide insights into the regulation of carotenogenic enzymes and establish a link between protein ubiquitination and carotenoid biosynthesis.

Methods

Generation of transgenic tomato plants. CRISPR/Cas9 mediated gene-editing was carried out as described by Ma et al. with minor modifications. In brief, four specific sgRNAs that targeted PPSR1 were designed by CRISPR-P (version 2.0, http://crispr.hzau.edu.cn/CRISPR2). The expression cassettes (Target_1-Target_2 and Target_3-Target_4) driven by AtU3b and AtU3d promoters, respectively, were amplified and cloned into the pYLCRISPR/Cas9Pubs H binary vector using the Golden Gate ligation method. The resulting constructs were transformed into Agrobacterium tumefaciens strain GV3101, which were subsequently infiltrated into the wild-type tomato cultivar Ailsa Craig. Mutation on transgenic lines was verified by sequencing genomic regions flanking the target sites. The potential off-targets were predicted by CRISPR-P. All primers used to generate these constructs are listed in Supplementary Data 3.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from tomato according to the method described by Moore et al. All tissues were ground into powder and mixed with extraction buffer containing 100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% SDS, and 50% water-saturated phenol. RNA was precipitated with 3 M LiCl and collected by centrifugation at 12,000 × g for 20 min at 4 °C. Genome DNA digestion and reverse transcription of the extracted RNA were performed using the PrimeScript® RT Reagent Kit (Takara) using the StepOne Plus Real-Time PCR System (Applied Biosystems). PCR primers listed in Supplementary Data 3 were designed by QuantPrime (http://quantprime.mpimp-golm.mpg.de/). PCR amplification was performed in a volume of 20 μl with the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The cycle threshold (Ct) was applied to the relative quantification method. ACTIN2 (Solyct11g00530) was used to normalize the expression values. Three independent biological replicates with three technical repeats each were conducted.
**Y2H analysis.** Y2H screening was performed as described by Wang et al.62. The tomato cDNA library constructed in the prey vector pGADT7 (AD) was screened with the S. erubescens cDNA fragment cloned into the pJG318 (BD) in *Saccharomyces cerevisiae* strain AH109 (Clontech). The yeast zygote was selected on SD/-Leu-Trp-His-Ade medium (-LWAH) supplemented with a *D*-galactoside (X-a-gal) according to the manufacturer’s instructions (Clontech). The positive clones carrying putative *S. erubescens* interacting proteins were identified by sequencing.

Y2H analysis was carried out using Matchmaker GAL4 Two-Hybrid System 3 following the manufacturer’s protocols (Clontech). The cDNA fragments of the proteins were cloned into the AD and BD vectors, respectively. For the primer sets used for these construction, please refer to Supplementary Data 3. The resulting constructs were co-transformed into *S. cerevisiae* strain and then plated on SD/-Leu-Trp medium (-LW) and SD/-Leu-Trp-His-Ade medium (-LWHA) containing X-a-gal. The transformants carrying empty vectors (BD or AD) were used as negative controls.

**LCI assay.** LCI assay was carried out as described by Chen et al.63. The coding sequence of *S. erubescens* PPSR1, and PPSR1 was amplified from tomato cDNA and separately ligated into the pCambia1300-MCS-HA or pCambia1300-MCS-eGFP vector to generate 35S::PPSR1-HA and 35S::PPSR1-eGFP, respectively. For colocalization analysis, the coding sequence of *S. erubescens* PPSR1, and PPSR1 was cloned into the pCambia1300-MCS-mCherry vector to generate 35S::PPSR1-mCherry. The resulting constructs were introduced into *A. tumefaciens* strain GV310158, which was subsequently infiltrated into *N. benthamiana* leaves. The *S. erubescens* plants were harvested 3 dpi, and the mesophyll protoplasts were isolated64 and observed using a Leica confocal microscope (Leica DMi6000C5). The primers used for vector construction are listed in Supplementary Data 3.

**Subcellular localization.** For colocalization analysis, the coding sequence of *S. erubescens* PPSR1 was amplified from tomato cDNA and inserted into the pCambia1300-MCS-mCherry vector to produce 35S::PPSR1-mCherry plasmid. The coding sequence of PPSR1 and PPSR1 was amplified and individually cloned into the pCambia1300-MCS-eGFP or pCambia1300-MCS-mCherry vector to generate 35S::PPSR1-GFP, 35S::PPSR1-mCherry, and 35S::PSSP1-eGFP constructs. The resulting plasmids were transfected into *A. tumefaciens* strain GV310158, which was subsequently infiltrated into *N. benthamiana* leaves64. The *N. benthamiana* plants co-expressing mCherry-tagged *S. erubescens* PPSR1 (S. erubescens-mCherry) and eGFP-tagged PPSR1 (S. erubescens-eGFP) were generated. The plants were cultured in the greenhouse for 36 h, and then the mesophyll protoplasts were isolated64 and observed using a Leica confocal microscope (Leica DMi6000C5). The primers used for vector construction are listed in Supplementary Data 3.

**Co-IP analysis.** Co-IP assay was carried out as described by Tan et al.65 with some modifications. The coding region of *S. erubescens* PSY1 was amplified from *S. erubescens* cDNA and cloned into the pCambia1300-MCS-HA vector (Beijing Protein Innovation) and anti-HA (Abmart) antibodies, respectively, as described by Wang et al.62. After centrifugation for 30 min at 4 °C, the supernatant containing the proteins was immunoprecipitated with 20 μl of anti-HA agarose (Cell Signaling Technology) at 4 °C for 3 h. The agarose beads were collected and washed once with extraction buffer. The proteins were eluted from the beads with 1× SDS loading buffer at 95 °C for 5 min, and then subjected to immunoblot using anti-HA (Abmart) and anti-Flag (MBL Life Science) antibodies, respectively, as described by Wang et al.62. The primers used for the generation of constructs are listed in Supplementary Data 3.

**Cell-free degradation assay.** The cell-free degradation assay was performed as described by Xie et al.73. Briefly, 500 ng of purified MBP-PPSR1 or MBP-PPSR1 recombinant protein was mixed with 100 ng of E1 (UBA1, M55604.1) from wheat, 200 ng of E2 (UBC21b, U39317.1) from human or S. erubescens-HA, and 2 μg of ubiquitin (UBQ14, A4102980) from Arabidopsis in 30 μl of reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM ATP, and 2 mM dihydrothreitol) at 30 °C for 2 h. The reaction was stopped with 1× SDS loading buffer at 95 °C for 5 min. The reaction products were submitted to immunoblot using anti-ubiquitin (P4D1, Santa Cruz Biotechnology) and anti-MBP (Beijing Protein Innovation) antibodies, respectively, as described by Wang et al.62. The band intensity was quantified using Image software (https://image.nl.gov/#index/index.html) as described by Girish and Vijayakishn74.

**iTRAQ-based quantitative proteomic analysis.** Proteins were extracted from wild-type and ppsr1 mutant fruit at 38 DPA using a phenol extraction method4. The isolated proteins were solubilized in lysis buffer (20 mM HEPES, pH 8.0, and 8 M urea), and the protein concentration was measured75. One hundred micrograms of proteins from each sample were reduced with 10 mM dihydrothreitol, alkylated with 50 mM iodoacetamide, and digested with 10 ng/ml trypsin overnight. The tryptic peptides were desalted on a Sep-Pak C18 column (Waters, Inc.), and then labeled with the iTRAQ Reagents 4-plex Kit (Applied Biosystems) according to the manufacturer’s protocol. Two independent biological replicates were applied for the iTRAQ analysis. The iTRAQ-labeled samples were then mixed, trypsinized, and fractionated with high-pH reversed-phase chromatography as described by Wang et al.62.

**Protein identification and relative quantification.** Protein identification and relative quantification were performed by the ProteinPilot™ 4.5 software (AB SCIENCE). The mass spectra data were used to

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[44x515](10 mM MES, pH 5.6, 10 mM MgCl2, and 100 µM acetosyringone) to a
expression and puri
tagged SlUBC32 (SlUBC32-HA), the coding sequence of
COMMUNICATIONS BIOLOGY|           (2020) 3:730 | https://doi.org/10.1038/s42003-020-01474-3 | www.nature.com/commsbio
leaves64. After in
Leu-Trp medium (-LW) and SD/-Leu-Trp-His-Ade medium (-LWHA) containing
sequencing.

After infiltration for 30 h, the tobacco leaves were treated with 50 μM MG132 for 6 h. Total proteins were extracted from the leaves with 1 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5% glycerol, 1 mM PMSF, 1× protease inhibitor cocktail, and 50 μM MG132). After centrifugation at 12,000 ×g for 20 min at 4 °C, the supernatant containing the proteins was immunoprecipitated with 20 μl of anti-HA agarose (Cell Signaling Technology) at 4 °C for 2 h. The agarose beads were collected and washed twice with extraction buffer. The proteins were eluted from the beads with 1× SDS loading buffer at 95 °C for 5 min, and then subjected to immunoblot using anti-HA (Abmart) and anti-Flag (MBL Life Science) antibodies, respectively, as described by Wang et al.62. The primers used for the generation of constructs are listed in Supplementary Data 3.

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**Protein identification and relative quantification.** Protein identification and relative quantification were performed by the ProteinPilot™ 4.5 software (AB SCIENCE). The mass spectra data were used to...
search the S. lycopersicum protein database (ITAG2.4_proteins_full_desc.fasta). By using the Pro Group™ algorithm (AB SCIEX), the peptide for quantification was automatically selected to calculate the reporter peak area. Then, the theoretical FDR for peptide identification, a reverse database search strategy was applied. Only identified proteins below the 1% global FDR were utilized to calculate the meaningful cutoff value using a population statistics method described in the biological replicates.

Ubiquitinated peptide enrichment and identification. Ubiquitinated peptide enrichment was performed using the PTMScan Ubiquitin Remnant Motif (K-e-G) kit according to the manufacturer’s protocol (Cell Signaling Technology). Briefly, proteins were extracted from wild-type and ppsr1 mutant at 38 DPA and solubilized in lysis buffer as described above. Approximately 10 mg of the isolated proteins were reduced with 10 mM dithiothreitol, alkylated with 50 mM iodoacetamide, and digested with 10 ng ml−1 trypsin overnight. After desalting on a Sep-Pak C18 column (Waters, Inc.), the tryptic peptides were lyophilized under vacuum and redisolved in immunoprecipitation buffer (IAP) containing 50 mM MOPS-NaOH buffer, pH 7.2, 10 mM Na2HPO4, and 50 mM NaCl, and then incubated with anti-K-e-G) antibody beads (Cell Signaling Technology) for 2 h at 4 °C. The beads were collected and washed twice with IAP buffer. The ubiquiti- nated peptides were eluted from the beads with 0.15% trifluoroacetic acid (TFA), desalted with C18 Stage Tips (Thermo Scientific), and analyzed by NanoLC-MS/MS.

Quantitative analysis of ubiquitinated peptides between wild-type and ppsr1 mutant was performed using the SWATH-MS method. Mass spectrometry was acquired on a TripleTOF 5600 Plus instrument (AB SCIEX) operating in the SWATH mode as described by Wang et al. The peptides and their ubiquitination sites were identified using ProteinPilot software (AB SCIEX) against the S. lycopersicum protein database (ITAG2.4_proteins_full_desc.fasta). The MS/MS spectra of the identified peptides were used to generate a library for SWATH processing and quantification by Peakview software (AB SCIEX). The library correlated both peptide identification and LC retention times to extract specific MS/MS transition data for each peptide. For each individual sample, the ion transitions from the ubiquitinated peptides were applied to retrieve quantitative data (a 0.05 Da extraction width over a ± 5 min LC time) and visualized with MarkerView (AB SCIEX). After normalizing using Total Area Sums, the extracted ions for selected peptides were analyzed using Student’s t-test within MarkerView (AB SCIEX) with three technical replicates for each sample. A P value < 0.05 was considered to be significant. The experiment was performed with two independent biological replicates.

Preparation of polyclonal antibodies. For PPSR1-specific antibody preparation, the coding region of PPSR1 lacking the conserved domain was amplified from tomato cdNA and cloned into the pET-30a vector (Merck KGAa), which was then transformed into E. coli BL21 (DE3). The recombinant protein expression was performed as described above. Then, the recombinant PPSR1 protein was purified by Ni-NTA resin according to the manufacturer’s instructions (Merck KGAa), followed by further purification using 12% SDS-PAGE. The recombinant protein was excised from the gel and used to immunize rabbits at the Abmart Shanghai Co., Ltd (http://www.ab-mart.com.cn). The PPSR1-specific polyclonal antibody was affinity-purified from antisera by the AminoLink Plus Coupling Resin following the purification manual (Thermo Scientific). To test the specificity of the PSY1 antibody, a synthetic peptide KSLVPYFTKASL was used to inject rabbits, followed by affinity-purification using the synthetic peptide. The primers used for vector construction are listed in Supplementary Data 3.

Immunoblot analysis. For immunoblot analysis, proteins were extracted from tobacco leaves as described above or from tomato fruit using a phenol extraction method. Protein samples were separated by 10% SDS-PAGE and then transferred to an Immobilon-P PVDF membrane (Millipore, IPVH00101) using a semi-dry transfer unit (Amersham, TE77). The membranes were blocked for 1 h at room temperature with 5% non-fat milk in TBST buffer. The immunoblotting was conducted with anti-HA, anti-Flag, anti-MBP, anti-actin, anti-ubiquitin, anti-PPSR1, or anti-PSY1 antibodies at room temperature for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG secondary antibody (1:5000) at room temperature for another 1 h. The membranes were then washed four times with TBST buffer, and the immunoreactive bands were visualized by using chemiluminescence detection kit (SuperSignal™, Pierce Biotechnology). Unprocessed original blot images are provided in Supplementary Figs. 9 and 10.

Carotenoid measurement. Pericarp carotenoids were extracted and quantified as described by Xiong et al. with some modifications. In brief, the pericarps of fruit (2 g) powdered in liquid nitrogen was added to 20 ml of extraction buffer (hexane: acetoneethanol at 2:1:1 by volume) containing 0.1% butylated hydroxytoluene (BHT). The mixture was vortexed for 30 min until the sample was decolored, followed by centrifugation at 4000 × g for 10 min. The supernatant was then col- lected and dried under a stream of nitrogen gas. The dried residue was resuspended in 2 ml of methyl tert-butyl ether (MTBE) containing 0.1% BHT. The resuspended sample was saponified with 10% KOH in methanol for 30 min and dried in a rotatory evaporator. The residue was redissolved with 1 ml MTBE containing 0.1% BHT and filtered through a 0.22 μm membrane to remove insoluble particles. All the procedures above were conducted in low light. The individual carotenoids (phytoene, lycopene, and β-carotene) were identified and quantified according to the retention time and dose-response curves. The HPLC grade standards (Supplementary Fig. 11) using ACQUITY UPLC™ System (Waters, Inc.) equipped with a C18 column according to the manufacturer’s recommendations. Each sample contained five fruits, and the experiment was performed with three independent biological replicates.

In vivo ubiquitination assay. For ubiquitination assay in vivo, the 35S::PSY1-HA construct, as described above. The protein was amplified and inserted into the pcMaiban1300-Flag-MCS vector to generate 35S:: Flag-ubiquitin construct. The full-length coding sequence of PPSR1 was ligated into the pcMaiban1300 vector to construct 35S::PPSR1. The resulting constructs were transformed into A. tumefaciens strain GV3101 and transiently expressed in N. benthamiana leaves. After 48 h of incubation, the agroinfiltrated leaves were treated with 50 µM MG132 for 6 h, and the total proteins were extracted from N. benthamiana leaves as described above. The total proteins were then subjected to immunoblot analysis using anti-Flag (MBL life science) and anti-HA (Abmart) antibodies, respectively. The primers used for vector construction are listed in Supplementary Data 3.

Protein stability and degradation rate assays. The protein stability assay was performed with a transient expression system in N. benthamiana. The 35S::PSY1-HA construct (35S::Flag-PPSR1 construct was generated as described above). The resulting constructs were individually introduced into A. tumefaciens strain GV3101. After cultivation, the Agrobacteria harboring the respective plasmids were infiltrated into N. benthamiana leaves. Forty-two hours after infiltration, the leaves were treated with 50 µM MG132 or DMSO (negative control) for 6 h. The total proteins were then extracted from the N. benthamiana leaves as described above and subjected to immunoblot analysis using anti-Flag (MBL life science) and anti-HA (Abmart) antibodies, respectively. Equal loading was confirmed with an anti-actin antibody (Abmart).

For degradation rate analysis, the agroinfiltrated N. benthamiana leaves were treated with 250 µM cycloheximide (CHX) after 36 h of incubation. The mutated form of PSY1 (PSY1K380R, PSY1K406R, and PSY1K380/406R) was generated by site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) following the manufacturer’s instructions. The band intensity was quantified using ImageJ software (https://image.nih.gov/ij/index.html). Values represent the average of three independent replicates. The primers used for vector construction are listed in Supplementary Data 3.

Statistics and reproducibility. Statistical analyses of data were performed using Microsoft Excel and GraphPad Prism 8.0 software. The band intensity of western blot was quantified by ImageJ software. Results are shown as the means ± standard deviation (SD) of three independent biological experiments. Statistical significance was analyzed by two-tailed Student’s t-test. P-values of 0.05 or less were considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001) and shown in figures. Regression analyses were conducted by linear or exponential regression model. Source data underlying the graphs are provided in Supplementary Data 4.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data that support the findings of this study are available from the corresponding author upon request. The mass spectrometry proteomics data for iTRAQ and ubiquitinated peptide identification have been deposited in the PRIDE archive (NOS. PXD018731 and PXD018707, respectively; https://www.ebi.ac.uk/pride/archive/).

Received: 12 May 2020; Accepted: 10 November 2020; Published online: 03 December 2020

References

1. Nisar, N., Li, L., Lu, S., Khin, N. C. & Pogson, B. J. Carotenoid metabolism in plants. Mol. Plant. 8, 68–82 (2015).

2. Yazdani, M. et al. Ectopic expression of ORANGE promotes carotenoid accumulation and fruit development in tomato. Plant Biotechnol. J. 17, 33–49 (2019).
3. Cazzonelli, C. I. & Pogson, B. J. Source to sink: regulation of carotenoid biosynthesis in plants. Trends Plant Sci. 15, 266–274 (2010).

4. Lhotta, R., Maree, J. F., Stange, C. & Rodriguez-Concepcion, M. Illuminating colors: regulation of carotenoid biosynthesis and accumulation by light. Curr. Opin. Plant Biol. 37, 49–55 (2017).

5. Liu, Y. et al. Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. Proc. Natl Acad. Sci. USA 101, 9897–9902 (2004).

6. Kato, M. et al. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiol. 134, 824–837 (2004).

7. Llorente, B. et al. Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. Plant J. 85, 167–179 (2016).

8. Murtsaha, M. A. et al. Antioxidants, carotenoids, and risk of rectal cancer. Am. J. Epidemiol. 159, 32–41 (2004).

9. Park, H., Kreunen, S. S., Cuttress, A. J., DellaPenna, D. & Pogson, B. J. Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis. Plant Cell 14, 321–332 (2002).

10. Sun, T. et al. Carotenoid metabolism in plants: the role of plastids. Mol. Plant 11, 58–74 (2018).

11. Ling, Q., Huang, W., Baldwin, A. & Jarvis, P. Chloroplast biogenesis is regulated by direct action of the ubiquitin-proteasome system. Science 338, 659–662 (2012).

12. Lee, S. et al. Heat shock protein cognate 70-4 and an E3 ubiquitin ligase, Hsp70-4-associated ubiquitin ligase, function in brassinosteroid-mediated salt stress tolerance. Plant Cell 14, 20, 401–412 (1999).

13. Duceux, L. J. et al. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. J. Exp. Bot. 56, 81–89 (2005).

14. Fraser, P. D. et al. Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. Plant Cell 19, 3194–3211 (2007).

15. Welsch, R. et al. Provitamin A accumulation in cassava (Manihot esculenta) roots driven by a single nucleotide polymorphism in a phytoene synthase gene. Plant Physiol. 122, 3348–3356 (2000).

16. Toledo-Ortiz, G., Huq, E. & Rodriguez-Concepcion, M. Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. Proc. Natl Acad. Sci. USA 107, 11626–11631 (2010).

17. Fujiwara, M. et al. The HY5-PIF regulatory module coordinates light and temperature control of photosynthetic gene transcription. Plant Cell Physiol. 50, 1806–1817 (2009).

18. Fujisawa, M. et al. Transcriptional regulation of fruit ripening by tomato FRUITFULL homologs and associated MADS box proteins. Plant Cell 26, 89–101 (2014).

19. Kanachovskaya, D. E., Fuller, S., Isaacson, T. & Hirschi, K. Epistasis in tomato color mutations involves regulation of phytoene synthase 1 expression by cis-carotenoids. Proc. Natl Acad. Sci. USA 109, 19021–19026 (2012).

20. Arango, J., Jourdan, M., Geoffrau, E., Beyer, P. & Welsch, R. Carotene hydroxylase activity determines the levels of both α-carotene and total carotenoids in orange carrots. Plant Cell 26, 2223–2233 (2014).

21. Álvarez, D. et al. Carotenogenesis is regulated by 5’UTR-mediated translation of phytoene synthase splice variants. Plant Physiol. 172, 2314–2326 (2016).

22. Welsch, R. et al. Clp protease and OR directly control the protein stability of phytoene synthase, the crucial enzyme for carotenoid biosynthesis in Arabidopsis. Mol. Biol. Cell. 19, 149–162 (2018).

23. Fantini, E., Falcone, G., Frusciante, S., Giliberto, L. & Giuliano, G. Dissection of tomato lycopene biosynthesis through virus-induced gene silencing. Plant Physiol. 163, 986–998 (2015).

24. Deikman, J., Kline, R. & Fischer, R. L. Organization of ripening and ethylene regulatory regions in a COLORLESS NONRIPENING-BOX transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. Plant Physiol. 157, 1568–1579 (2011).

25. Fujiwara, M., Nakano, T., Shima, Y. & Ito, Y. A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. Plant Cell 25, 371–386 (2013).

26. Shima, Y. et al. Tomato FRUITFULL homologues act in fruit ripening via forming MADS-box transcription factor complexes with RIN. Plant Mol. Biol. 82, 427–438 (2013).

27. Morreale, F. E. & Walden, H. Types of ubiquitin ligases. Cell 165, 248–248 (2016).

28. Kim, J. H. et al. MPSIR is a cytoplasmic PQC E3 ligase for eliminating emergent misfolded proteins in Arabidopsis thaliana. Proc. Natl Acad. Sci. USA 114, E10009–E10017 (2017).

29. Moon, J., Parry, G. & Estelle, M. The ubiquitin-proteasome pathway and plant development. Plant Cell 16, 3181–3193 (2004).

30. Xu, C., Paige, J. S. & Jaffrey, S. R. Global analysis of lysine ubiquitination by ubiquitin receptor mass spectrometry profiling. Nat. Biotechnol. 28, 868–873 (2010).

31. Gillet, L. C. et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Mol. Cell. Proteom. 11, 016717 (2012). O111.

32. Liu, Y. et al. Glycine degradation analysis of prostate cancer tissues by SWATH mass spectrometry discovers N-acetylcarnosine adducin and protein tyrosine kinase 7 as signatures for tumor aggressiveness. Mol. Cell. Proteom. 13, 1753–1768 (2014).

33. Shen, G. et al. The chloroplast protease subunit ClpP4 is a substrate of the E3 ligase AtCHIP and plays an important role in chloroplast function. Plant J. 49, 228–237 (2007).

34. Shen, G., Adam, Z. & Zhang, H. The E3 ligase AtCHIP ubiquitylates FtsH1, a component of the chloroplast FtsH protease, and affects protein degradation in chloroplasts. Plant J. 52, 309–321 (2007).

35. Smalle, J. & Vierstra, R. D. The ubiquitin-26S proteasome system at the nexus of plant development. Advances in plant biology. Plant Cell Physiol. 55, 555–590 (2004).

36. Xu, L. et al. The E2 ubiquitin-conjugating enzymes, AtUBC1 and AtUBC2, play redundant roles and are involved in activation of FLC expression and repression of flowering in Arabidopsis thaliana. Plant J. 57, 279–288 (2009).

37. Santer, A. & Estelle, M. The ubiquitin-proteasome system regulates plant hormone signaling. Plant J. 61, 1029–1040 (2010).

38. Cai, F. et al. Arabidopsis ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance. Plant Cell 24, 233–244 (2012).

39. Park, C. H. et al. The Magnaporthe oryzae effector AvpRz1 targets the RING E3 ubiquitin ligase AIP6 to suppress pathogen-associated molecular patterns (PAMP) recognition. Plant Cell 24, 4748–4762 (2012).

40. Ling, Q. et al. Ubiquitin-dependent chloroplast-associated protein degradation in plants. Science 363, eaav4467 (2019).
60. Moore, S., Payton, P., Wright, M., Tanksley, S. & Giovannoni, J. Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. *J. Exp. Bot.* 56, 2885–2895 (2005).

61. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C_{\text{\small T}} method. *Nat. Protoc.* 3, 1101–1108 (2008).

62. Wang, W., Cai, J., Wang, P., Tian, S. & Qin, G. Post-transcriptional regulation of fruit ripening and disease resistance in tomato by the vacuolar protease SlVPE3. *Genome Biol.* 18, 47 (2017).

63. Chen, H. et al. Firefly luciferase complementation imaging assay for protein-protein interactions in plants. *Plant Physiol.* 146, 368–376 (2008).

64. Sparkes, I. A., Runions, J., Kears, A. & Hawes, C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* 1, 2019–2025 (2006).

65. Pryor, K. D. & Letting, K. High-level expression of soluble protein in *Escherichia coli* using a His_{6}-tag and maltose-binding-protein double-affinity fusion system. *Protein Expr. Purif.* 10, 309–319 (1997).

66. Zhou, L., Tian, S. & Qin, G. RNA methylomes reveal the m6A-mediated regulation of DNA demethylase gene SlDML2 in tomato fruit ripening. *Genome Biol.* 20, 156 (2019).

67. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram amounts of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 2, 248–254 (1976).

68. Hu, B. et al. Nitrate-NRT1.1B-SPX4 cascade integrates nitrogen and phosphorus signalling networks in plants. *Nat. Plants* 5, 401–413 (2019).

69. Tang, X. et al. Ubiquitin-conjugated degradation of golden 2-like transcription factor is mediated by CUL4-DDB1-based E3 ligase complex in tomato. *Phytol.* 209, 1028–1039 (2016).

70. Lei, R. et al. A simple and effective method to encapsulate tobacco mesophyll protoplasts to maintain cell viability. *MethodsX* 2, 24–32 (2015).

71. Xie, Q. et al. SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. *Nature* 419, 167–170 (2002).

72. Wang, F. et al. Biochemical insights on degradation of *Arabidopsis* DELLA proteins gained from a cell-free assay system. *Plant Cell* 21, 2378–2390 (2009).

73. Girish, V. & Vijayalakshmi, A. Affordable image analysis using NIH Image/J ImageJ. *Indian J. Cancer* 41, 47 (2004).

74. Saravanan, R. S. & Rose, J. K. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. *Proteomics* 4, 2522–2532 (2004).

75. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* 4, 207–214 (2007).

76. Gan, C. S., Chong, P. K., Pham, T. K. & Wright, P. C. Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ). *J. Proteome Res.* 6, 821–827 (2007).

77. Xiong, C. et al. A tomato B-box protein SIBBX20 modulates carotenoid biosynthesis by directly activating *PHYTOENE SYNTHASE 1*, and is targeted for 26S proteasome-mediated degradation. *N. Phytol.* 221, 279–294 (2019).

Acknowledgements

We would like to thank Z. Lu for analysis of M6U/MS and J. Li for assistance with confocal microscopy. We thank J. Zhou (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for providing the pCambia1300-cLUC/nLUC vectors, Y. Liu (South China Agriculture University) for providing the pYLCRISPR/Cas9PuBI-H binary vector, and J. Jin (Institute of Botany, Chinese Academy of Sciences) for providing E. coli strains expressing E1, E2, and ubiquitin. We also thank the PRIDE team for the deposition of our mass spectrometry proteomics data to the ProteomeXchange Consortium. This work was supported by the National Natural Science Foundation of China (grant Nos. 31925035, 31930086, and 31572174).

Author contributions

G.Q. designed the research. P.W., Y.W., and W.W. performed the experiments. T.C. and S.T. provided discussions. G.Q., P.W., and Y.W. analyzed the data. G.Q. and P.W. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s42003-020-01474-3.

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