Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis

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Carotenoids are indispensable natural pigments to plants and humans. Phytoene synthase (PSY), the rate-limiting enzyme in the carotenoid biosynthetic pathway, and ORANGE (OR), a regulator of chromoplast differentiation and enhancer of carotenoid biosynthesis, represent two key proteins that control carotenoid biosynthesis and accumulation in plants. However, little is known about the mechanisms underlying their posttranscriptional regulation. Here we report that PSY and OR family proteins [*Arabidopsis thaliana* OR (AtOR) and AtOR-like] physically interacted with each other in plastids. We found that alteration of *OR* expression in *Arabidopsis* exerted minimal effect on *PSY* transcript abundance. However, overexpression of *AtOR* significantly increased the amount of enzymatically active PSY, whereas an *ator ator-like* double mutant exhibited a dramatically reduced PSY level. The results indicate that the OR proteins serve as the major posttranscriptional regulators of PSY. The *ator* or *ator-like* single mutant had little effect on PSY protein levels, which involves a compensatory mechanism and suggests partial functional redundancy. In addition, modification of *PSY* expression resulted in altered AtOR protein levels, corroborating a mutual regulation of PSY and OR. Carotenoid content showed a correlated change with OR-mediated PSY level, demonstrating the function of OR in controlling carotenoid biosynthesis by regulating PSY. Our findings reveal a novel mechanism by which carotenoid biosynthesis is controlled via posttranscriptional regulation of PSY in plants.

carotenoid | phytoene synthase | *Arabidopsis* | OR | posttranscriptional regulation

Carotenoids are a group of C40 isoprenoids synthesized in chloroplasts, chromoplasts, and other plastids in plants. Carotenoids serve as components of photosynthetic machinery, precursors for phytohormones, and important contributors to fruit nutritional quality and flower color (1, 2). The carotenoid biosynthetic pathway in higher plants has been well defined. However, identification of the regulatory mechanisms underlying carotenoid biosynthesis remains a challenge.

Phytoene synthase (PSY) catalyzes the first committed step in carotenoid biosynthesis and controls carbon flux into the carotenoid biosynthetic pathway (1–5). Alteration of *PSY* expression exerts profound effects on carotenoid content (6–11). A number of factors are known to affect *PSY* gene expression (12–18). *PSY* is found to be repressed by phytochrome-interacting factors in etiolated *Arabidopsis* seedlings (16). *PSY1* expression in tomato fruits is reported to be regulated by *cis*-carotenoids (14) and requires the MADS-Box transcription factor RIPENING INHIBITOR (18). Recently, it was discovered that PSY protein levels in carrot roots are modulated by a negative feedback emerging from carotenoids (19). The crucial role of PSY in carotenogenesis and the multiple factors affecting its expression suggest a complex regulatory system involved in controlling PSY. However, the factors involved in posttranscriptional regulation of PSY within plastids remain a mystery. No proteins have been reported to physically interact with PSY in plastids, the organelles where carotenoids are produced.

The *Orange* (*OR*) gene is involved in regulation of carotenoid biosynthesis and its mutation in cauliflower confers high levels of β-carotene accumulation (20). Previous studies of the *Brassica oleracea OR* gene mutation ($BoOR_{MUT}$) and its wild-type (WT) gene (*BoOR*) reveal that rather than affecting expression of carotenoid biosynthetic genes, $BoOR_{MUT}$ exerts its effect by triggering chromoplast differentiation, which enhances storage sink strength for carotenoid biosynthesis and accumulation (21–23). Interestingly, recent reports show that overexpression of a WT *OR* gene also promotes carotenoid accumulation in calli of rice (24) and sweet potato (25). However, the molecular basis for OR-mediated carotenoid increase is currently unknown.

OR is a plastid-localized protein and carries a cysteine-rich zinc finger domain, which is normally found in DnaJ-like molecular chaperones and essential for protein binding (23, 26). To investigate the molecular mechanisms underlying the OR action in controlling carotenoid biosynthesis, we conducted coimmunoprecipitation (co-IP) and mass spectrometry (MS) analyses and identified PSY as an OR-interacting protein. Both in vitro and in vivo interaction assays provided evidence for direct interaction between PSY and OR family proteins in plastids. Such interactions exerted no effect on *PSY* gene expression, but positively mediated PSY protein level and carotenoid content. These results demonstrate that the OR proteins are the major posttranscriptional regulators of PSY, representing an important regulatory mechanism underlying carotenoid biosynthesis in plants.

Significance

Carotenoids are indispensable to plants and humans. Despite significant achievements in carotenoid research, we still lack the fundamental knowledge of the regulatory mechanisms underlying carotenogenesis in plants. Phytoene synthase (PSY) and ORANGE (OR) are the two key proteins for carotenoid biosynthesis and accumulation in plastids. This study shows that OR family proteins interact directly with PSY and function as the major regulators of active PSY protein abundance in mediating carotenoid biosynthesis. The findings establish posttranscriptional regulation of PSY as a novel way to control carotenoid biosynthesis in plants and provide strategies for crop nutritional quality improvement.

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Results

Identification of OR-Interacting Proteins by Co-IP and MS. To examine the molecular network of OR and identify OR-interacting proteins, co-IP experiments were conducted with transgenic *Arabidopsis* expressing *35S:BoOR-GFP* and *35S:GFP*. A total of 130, 143, 109, and 71 proteins were identified by MS in the co-IP products from *Arabidopsis* expressing BoOR–GFP fusion protein, whereas 47, 47, 21, and 45 proteins were found from GFP-only controls in quadruplicates, respectively (Dataset S1). Five proteins were repeatedly identified from the quadruplicate BoOR–GFP samples but absent in the GFP controls (*SI Appendix*, Table S1). Among these proteins, PSY was the only one exclusively localized in chloroplasts as shown in a previous report (27) and the current study (*SI Appendix*, Fig. S1). The plastidial colocalization of PSY and OR, and their involvement in carotenoid biosynthesis, led us to propose that PSY was a potential OR-interacting protein.

OR Interacts Directly with PSY in Plastids. To verify the physical interaction between OR and PSY, we first performed yeast two-hybrid (Y2H) analysis using a split-ubiquitin membrane-based system (28) as OR is a transmembrane protein and PSY is considered to be membrane associated (23, 29). We found that BoOR specifically interacted with PSY in the Y2H assay (Fig. 1*A*). In addition, we investigated interaction between PSY and two *Arabidopsis* proteins that share 91% (AtOR; At5g61670) and 56% (AtOR-like; At5g06130) amino acid sequence identity with BoOR (*SI Appendix*, Fig. S2). Both *Arabidopsis* OR proteins interacted directly with PSY (Fig. 1*A*). The interactions were also confirmed by quantification of the reporter gene *lacZ* via *ortho*-nitrophenyl-β-galactoside (oNPG) activity measurements (*SI Appendix*, Fig. S3). Moreover, when PSY–GFP and AtOR–cMYC fusions were transiently coexpressed in *Nicotiana benthamiana* leaves and immunoprecipitated using anti-GFP beads, AtOR–cMYC was exclusively detected when PSY–GFP was coexpressed, but absent in all negative controls (Fig. 1*B*).

To confirm OR and PSY interaction in planta, we performed bimolecular fluorescence complementation (BiFC) assay in *N. benthamiana* leaves. When the N-terminal half of YFP fused to BoOR (BoOR–YN), AtOR (AtOR–YN), or AtOR-like (AtOR-like–YN) and the C-terminal half of YFP fused to PSY (PSY–YC) were coexpressed in tobacco leaf epidermal cells, YFP signals were observed between PSY and OR or AtOR-like (Fig. 1*C*). Such interactions occurred in chloroplasts, which was in good agreement with the plastid localization of these proteins (27) (*SI Appendix*, Fig. S1). In contrast, no YFP signals were detected when BoOR–YN, AtOR–YN, AtOR-like–YN, or PSY–YC were cotransformed with the respective controls (*SI Appendix*, Fig. S4). These results further confirmed interactions between PSY and OR.

OR Proteins Posttranscriptionally Regulate PSY Protein Level and Carotenoid Content. To investigate how OR affected PSY, two independent *Arabidopsis* transgenic lines with 40- to 50-fold increases in the *AtOR* expression were used for further study (*SI Appendix*, Fig. S5). Notably, *PSY* expression in these overexpressing lines was not significantly different from that in WT (Fig. 2*A*), indicating that increasing *AtOR* expression did not alter *PSY* expression. Considering the increasing evidence for a central role of posttranscriptional regulation of key enzymes for metabolite biosynthesis (19, 30, 31) and the possible involvement of OR in PSY regulation, we examined PSY protein levels in the *AtOR*-overexpressing lines by immunoblotting. Intriguingly, in comparing with WT, PSY protein levels in leaves were greatly increased following an enhanced AtOR expression in these overexpressing lines (Fig. 2*B*), emphasizing the posttranscriptional regulation of PSY by OR.

To examine whether the increase in PSY protein level resulted in an enhanced enzyme activity, PSY activity in chloroplast membranes isolated from WT and *AtOR*-overexpressing lines was measured by an in vitro assay containing recombinant mustard geranylgeranyl diphosphate synthase (GGPP synthase), dimethylallyl diphosphate (DMAPP), and ^{14}C-isopentenyl diphosphate (^{14}C-IPP) (29) (*SI Appendix*, Fig. S6). As shown in Fig. 2*C*, PSY activity was about 50% higher in the *AtOR*-overexpressing lines than in WT. Such enhanced activity correlated with increased PSY protein amounts in the plastid membranes (*SI Appendix*, Fig. S7).

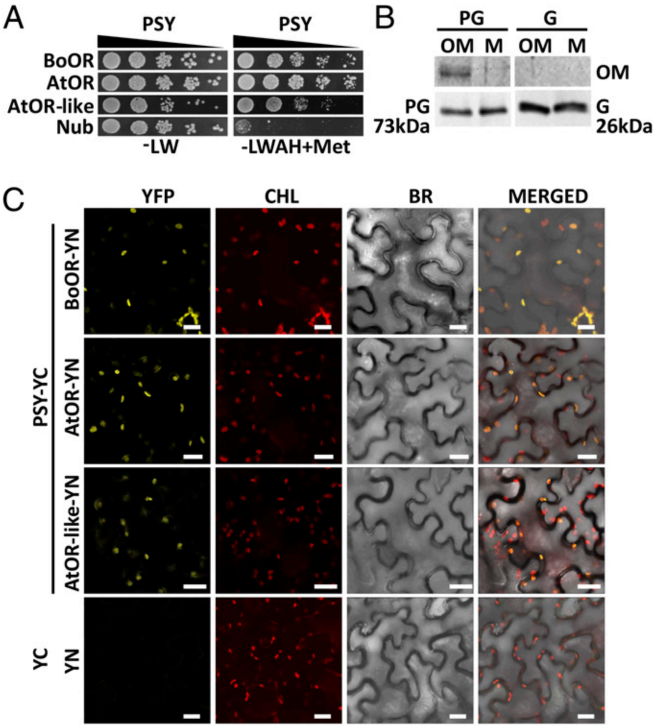

Fig. 1. PSY and OR interact with each other. (*A*) Y2H analysis. Interactions between *Arabidopsis* PSY and BoOR, AtOR, AtOR-like, or control (Nub) were examined by coexpression of pairs of proteins fused to either the N-terminal or C-terminal ubiquitin moiety in yeast and spotted onto either nonselective (–LW) or fully selective medium plates (–LWAH +50 μM Met) in a series of 10-fold dilutions. (*B*) PSY–GFP (PG) and AtOR–cMYC (OM) were coexpressed in *N. benthamiana* leaves. Proteins were immunoprecipitated with anti-GFP beads and immunoblotted with anti-cMYC antibody. GFP (G) and cMYC (M) were coexpressed with PSY–GFP and AtOR–cMYC, respectively, as negative controls. (*C*) BiFC analysis. PSY–YC or C-terminal YFP (YC) was coexpressed with BoOR–YN, AtOR–YN, AtOR-like–YN, or N-terminal YFP (YN) in *N. benthamiana* leaves. Direct interactions between PSY and BoOR, AtOR, or AtOR-like protein in chloroplasts were observed by confocal microscopy. (Scale bars, 20 μm.) BR, bright field; CHL, chlorophyll autofluorescence.

Leaf carotenoid levels in the *AtOR*-overexpressing lines were similar to controls (Fig. 2*D*). As constitutive overexpression of *AtPSY* in *Arabidopsis* results in similarly unchanged leaf carotenoids (7) (Fig. 2*D*), we considered that the enhanced phytoene synthesis might be compensated by its prompt conversion into downstream carotenoids/apocarotenoids. To monitor PSY activity in vivo, we treated leaves with norflurazone (NFZ), which inhibits the subsequent enzyme phytoene desaturase (32), and examined phytoene accumulation. The NFZ-treated leaves from the *AtOR*-overexpressing lines accumulated over 30% more phytoene than WT (Fig. 2*E*), reconfirming that the increased PSY amounts mediated by AtOR were enzymatically active in vivo.

In contrast to leaves, nongreen tissues frequently respond to increased pathway flux with increased carotenoid accumulation (7, 11). Immunoblotting showed that PSY protein levels were also increased in the *AtOR*-overexpressing roots compared with WT (Fig. 2*B*). Consequently, these roots contained more carotenoids than WT (Fig. 2*F*). Moreover, we analyzed phytoene levels in 4-d-old etiolated seedlings grown in the presence of NFZ, in which the phytoene amounts are previously shown to be proportional to PSY activity (31). PSY protein levels were higher in

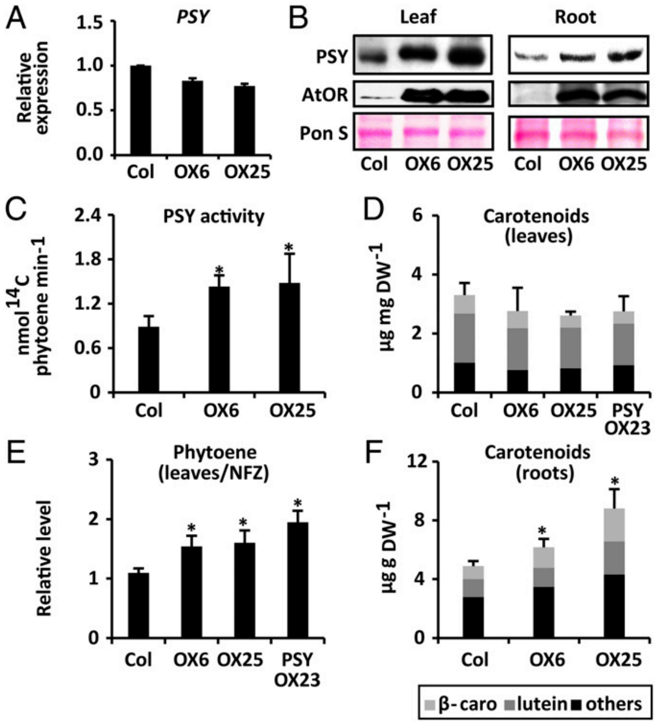

Fig. 2. PSY is positively regulated by AtOR at the posttranscriptional level in *Arabidopsis*. (*A*) qRT-PCR analysis of *PSY* gene expression in WT and *AtOR*-overexpressing plants. (*B*) Western blots of PSY and OR protein levels in leaves (60 µg proteins) and roots (30 µg proteins). Ponceau S (Pon S) staining shows equal loading. (*C*) PSY activity in WT and *AtOR*-overexpressing plants. (*D*) Total carotenoid levels in leaves. (*E*) Phytoene levels in leaves treated with NFZ. An *Arabidopsis* line constitutively overexpressing *AtPSY* (PSY OX23) (7) was used for comparison. (*F*) Total carotenoid levels in roots. *Arabidopsis* ecotype Columbia (Col) was used as WT. OX6 and OX25, *AtOR*-overexpressing lines. Results are means ± SD from three biological replicates. Significant difference, $^{*}P < 0.05$.

the *AtOR* transgenic seedlings than in WT (*SI Appendix*, Fig. S8*A*), with a correlated increase in phytoene levels (*SI Appendix*, Fig. S8*B*). Together, these results show that *AtOR* overexpression resulted in increased amounts of enzymatically active PSY, which produced enhanced carotenoids in roots as well as in NFZ-treated leaves and etiolated seedlings.

Both AtOR and AtOR-Like Proteins Are Required to Regulate PSY Protein Abundance. To investigate the consequence of reduced OR protein levels, T-DNA insertion lines for *ator* (GK-850E02-025840) and *ator-like* (SAIL_757_G09) were studied (Fig. 3*A*). The insertion at 105 nt upstream of the transcriptional start site in *ator* produced only 4% *AtOR* transcript of WT (Fig. 3 *A* and *B*). The insertion within the first intron in *ator-like* resulted in the complete absence of *AtOR-like* transcript (Fig. 3 *A* and *B*). Because both AtOR and AtOR-like proteins were able to interact with PSY, a double mutant line of *ator ator-like* was generated by crossing *ator* with *ator-like*. Transcripts of *AtOR* and *AtOR-like* were hardly detected in the double mutant (Fig. 3*B*). Interestingly, *AtOR* was expressed 1.8-fold higher in *ator-like* than WT, whereas a 4.6-fold increase of *AtOR-like* expression was observed in *ator* (Fig. 3*B*), showing that suppression of *AtOR-like* and especially *AtOR* resulted in increased expression of the other family gene probably by a compensatory mechanism. The single mutants grew normally as WT, whereas the double mutant was smaller with pale green phenotype (Fig. 3*C*).

As with the *AtOR*-overexpressing lines, similar levels of *PSY* transcript in leaves were observed among WT and the mutants (Fig. 3*B*). However, whereas PSY protein levels in leaves remained similar among WT and the single mutants, PSY amount was dramatically reduced in the *ator ator-like* double mutant, correlating with leaf AtOR protein levels among these plants (Fig. 3*D*). The results indicated that AtOR and AtOR-like were sufficient and required to regulate PSY protein levels in vivo, and that *AtOR* and *AtOR-like* were functionally redundant. Similar regulation of PSY protein by AtOR and AtOR-like was found in *Arabidopsis* roots and etiolated seedlings (Fig. 3*D* and *SI Appendix*, Fig. S9*A*). The findings also suggested that posttranscriptional regulation of PSY by AtOR and AtOR-like was independent of plastid type.

The single mutants had similar levels of leaf carotenoids (Fig. 3*E*) and chlorophylls (*SI Appendix*, Fig. S10) as WT. In contrast, the *ator ator-like* double mutant contained only about 30% carotenoids and chlorophylls compared with WT (Fig. 3*E* and *SI Appendix*, Fig. S10). Moreover, in NFZ-treated leaves, phytoene levels remained similar in *ator*, slightly reduced in *ator-like*, but drastically reduced in *ator ator-like* compared with WT (Fig. 3*F*).

Whereas carotenoid levels in roots were similar among WT and the single mutants, the *ator ator-like* double mutant contained significantly fewer carotenoids in roots than WT (Fig. 3*G*), correlating with reduced PSY and AtOR protein levels in root tissue (Fig. 3*D*). A similar change in phytoene levels in the NFZ-treated etiolated seedlings was also observed among WT and the mutant lines (*SI Appendix*, Fig. S9*B*). Collectively, the effect of OR proteins on PSY protein abundance and the consequent reductions of carotenoid levels in *ator ator-like* indicate an essential importance of the OR family proteins on PSY protein regulation.

PSY also Affects OR Protein Level. The results described above indicate a strong coregulation. To examine whether *PSY* expression affected OR level, transgenic *Arabidopsis* lines with altered expression of *PSY* were generated by constitutively overexpressing *PSY–GFP*. A *psy* cosuppressed line with white leaves and much reduced growth phenotype was obtained (Fig. 4*A*). Immunoblotting analysis showed that the *psy* cosuppressed line contained reduced PSY–GFP and endogenous PSY protein levels (Fig. 4*B*). Overexpression of *PSY–GFP* led to about a twofold increase of phytoene in NFZ-treated etiolated seedlings (Fig. 4*C*), indicating that *PSY–GFP* functioned properly in the transgenic lines.

AtOR transcript levels were found to be similar in the *PSY* overexpressing lines as WT, but reduced in the *psy* cosuppressed line, probably due to chloroplast impairment caused by the suppression of *PSY*, or due to transcriptional regulation of *AtOR* by retrograde signaling (Fig. 4*D*). Interestingly, increased AtOR protein amounts were clearly observed in the *PSY* overexpressing lines and reduced AtOR was found in the *psy* cosuppressed line, indicating that PSY also positively affected AtOR protein at the posttranscriptional level (Fig. 4*B*).

N-Terminal Region of OR Is Required for Interaction with PSY and C-Terminal Region Is Needed for OR Dimerization. OR contains two distinct regions linked by two membrane-spanning motifs: the N-terminal region with unknown function and the C-terminal cysteine-rich zinc finger domain (23) (Fig. 5*A*). To define the domain of OR and PSY interaction, *BoOR* gene fragments encoding the N-terminal region excluding the transit peptide (amino acids 54–124) and the C-terminal region containing the transmembrane motifs and cysteine-rich zinc finger domain (amino acids 125–307; Fig. 5*A*) were cloned to make fusions with the N-terminal moiety of ubiquitin (Nub). Interactions between the two BoOR domains with PSY were tested in the split-ubiquitin Y2H system. PSY was found to interact with BoOR via its N-terminal region (BoOR54–125; Fig. 5*B*). Interestingly, BoOR formed homodimers as well as heterodimers with AtOR or AtOR-like, which were mediated exclusively through the C-terminal moiety of BoOR (BoOR126–307), whereas the N-terminal moiety was not involved (Fig. 5*B*). These results support the specific roles of these two functionally distinct OR domains: an N-terminal PSY-interacting domain and a C-terminal domain required for OR dimerization.

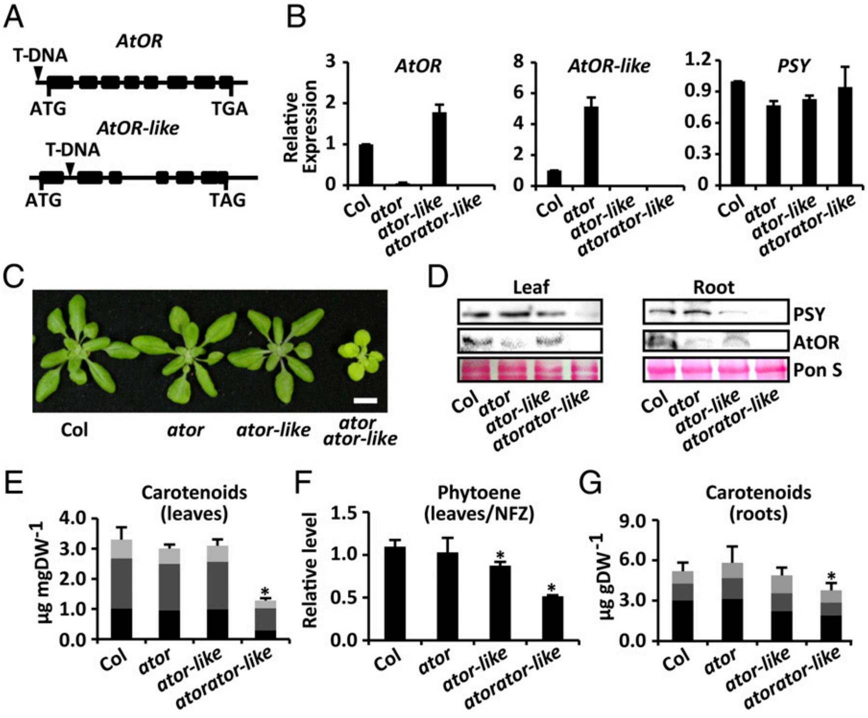

Fig. 3. Knockout of *AtOR* and *AtOR-like* leads to reduced PSY protein level and carotenoid content. (*A*) Structures of *AtOR* and *AtOR-like*. Exons, introns, and T-DNA insertion sites are shown as boxes, bars, and triangles, respectively. (*B*) Expression of *AtOR, AtOR-like*, and *PSY* in WT and the single and double mutants by qRT-PCR. (*C*) Three-week-old plants of WT, *ator*, *ator-like*, and *ator ator-like*. (Scale bar, 1 cm.) (*D*) PSY and OR protein levels in leaves and roots of the single and double mutants. Total proteins of 60 and 30 μg from leaves and roots, respectively, were used for immunoblotting. (*E*) Carotenoid levels in leaves. (*F*) Phytoene levels in leaves treated with NFZ. (*G*) Carotenoid levels in roots. Results are means ± SD from three biological replicates. Significant difference, $^*P < 0.05$.

Discussion

We demonstrate here that PSY and OR, two key proteins involved in carotenoid biosynthesis and accumulation, physically interacted with each other in plastids via the N-terminal region of the OR protein. Moreover, this work identifies the molecular basis underlying OR-regulated carotenoid biosynthesis by affecting PSY protein level and its catalytic activity. The findings provide strong evidence showing that PSY is posttranscriptionally regulated by OR, revealing a hitherto unidentified regulatory mechanism for carotenogenic enzymes in plants.

OR Proteins Function as the Major Regulators of PSY Protein Level and Activity. Our in vitro and in vivo interaction assays provided evidence for the direct interactions between PSY and OR family proteins in plastids. Although recently a tomato STAY-GREEN protein (SlSGR1) was identified as a SlPSY1 interacting protein in the nucleus, negatively regulating SlPSY1 activity by suppression of *SlPSY1* transcription (33), the regulators that posttranscriptionally control PSY in plastids remain largely unknown. The dramatically increased PSY protein level and activity in the *Arabidopsis AtOR*-overexpressing lines and strongly reduced PSY protein level in the *ator ator-like* double mutant indicated that the OR proteins are the major posttranscriptional regulators of PSY. Recently, the protein level of deoxyxylulose 5-phosphate synthase (DXS), the rate-limiting enzyme for upstream plastidial IPP biosynthesis, is reported to be controlled by J-protein J20 (34). Suppression of J20 results in high amounts of enzymatically inactive DXS (34), contrasting with our findings that OR abundance positively correlated with the enzymatically active PSY level.

OR and OR-like proteins are highly conserved among plant species, indicating their critical functions in plant growth and development (23). Although the OR proteins carry a DnaJ cysteine-rich zinc figure domain, they are not molecular chaperones due to the lack of the DnaJ-like defining J domain. A member of the specific OR group proteins is BUNDLE SHEATH DEFECTIVE2 (BSD2), which affects Rubisco accumulation and is believed to function via direct interaction with the polypeptide substrates for Rubisco assembly (35, 36). Accordingly, the physical association of OR with PSY might promote the proper folding of PSY to enhance its stability and activity. As a result, a correlated alteration of OR expression and PSY protein level was observed. Consistent with these observations, higher PSY levels are observed in $BoOR_{MUT}$ transgenic potato tubers (22). The ability of OR to alter PSY protein amounts and enzyme activity demonstrates a major regulatory role of OR in controlling PSY.

OR Modulates Carotenoid Biosynthesis by Means of Posttranscriptional Regulation of PSY. Given the rate-limiting role of PSY in carotenogenesis, increase of PSY following *AtOR* overexpression significantly enhanced carotenoid biosynthesis, whereas suppression of PSY in the *ator ator-like* double knockout resulted in

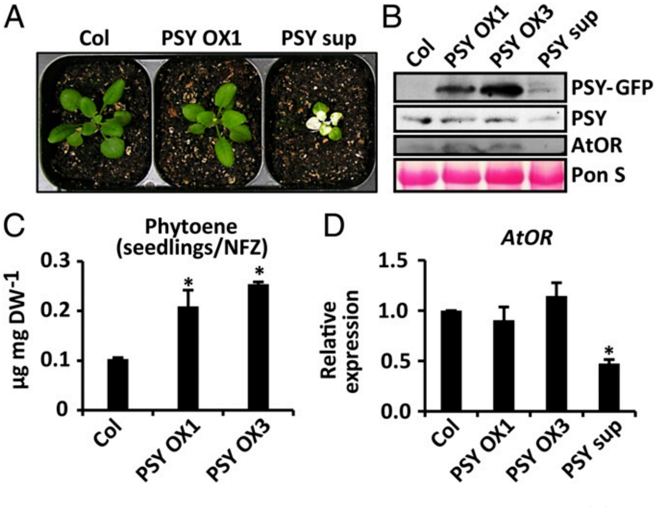

Fig. 4. PSY positively regulates AtOR protein level in *Arabidopsis*. (*A*) Phenotype of 3-wk-old *Arabidopsis* plants with altered expression of *PSY*. (*B*) PSY–GFP, PSY, and AtOR protein levels in leaves (45 μg proteins). (*C*) Phytoene levels in 4-d-old etiolated seedlings treated with NFZ. (*D*) Transcript levels of *AtOR* in *PSY*-overexpressing lines and cosuppressed line analyzed by qRT-PCR. Results are means ± SD from three biological replicates. Significant difference, $^*P < 0.05$.

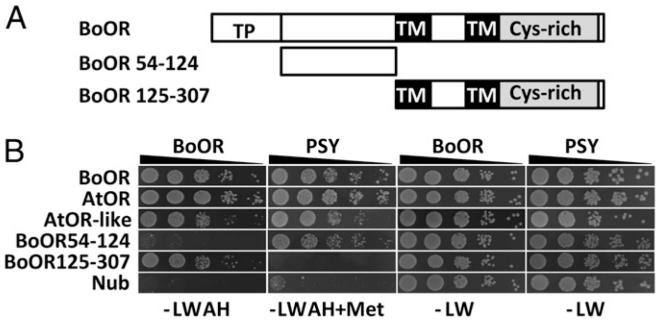

Fig. 5. N-terminal region of OR is required for PSY interaction and C-terminal for OR dimerization. (*A*) Schematic presentation of BoOR protein and variants. The positions of BoOR truncations are indicated. Cys-rich, cysteine rich zinc finger domain; TM, transmembrane domain; TP, transit peptide. (*B*) Growth of yeast coexpressing one Nub fusion protein containing different regions of BoOR with BoOR–Cub or PSY–Cub fusion protein, respectively. Interaction was examined on fully selective medium (−LWAH; +50 μM Met) in a series of 10-fold dilutions, whereas growth on nonselective medium (−LW) is shown as a control.

reduced carotenoid production in the NFZ-treated leaves and etiolated seedlings as well as in roots.

It is noted that in green leaves enhanced PSY protein levels in the *AtOR*-overexpressing lines did not alter carotenoid accumulation, in keeping with the fact that overexpression of *PSY* generally does not significantly perturb carotenoid content in leaves (7, 37). It is well known that carotenoid steady-state regulatory mechanisms are pronounced in photosynthetically active tissues for maintaining optimal photosynthesis (38, 39). This contrasts with nongreen tissues, such as tubers and roots that frequently respond to increased pathway flux with enhanced carotenoid accumulation (7, 8, 11). Accordingly, the effect of OR on PSY protein level and enzyme activity resulted in altered carotenoid levels in roots. Carotenoid levels were significantly increased in roots of the *AtOR*-overexpressing lines and reduced in the *ator ator-like* double mutant. Indeed, recent reports show that *OR* overexpression enhances carotenogenesis in calli of rice (24) and sweet potato (25). Although PSY protein levels were not examined in these studies, such enhanced carotenoid biosynthesis is likely due to increased PSY protein level. As demonstrated here, posttranscriptional regulation of PSY by OR represents an important mechanism by which carotenoid biosynthesis is controlled in plants.

It is interesting to note that $BoOR_{MUT}$ confers higher levels of carotenoid accumulation via triggering chromoplast formation (21, 23) and causes enhanced PSY protein level and stability in the $BoOR_{MUT}$ transgenic potato tubers (22). Thus, it is likely that $BoOR_{MUT}$ functions in regulating both chromoplast biogenesis and carotenoid biosynthesis. The capacity of $BoOR_{MUT}$ in triggering chromoplast differentiation with enhanced plastid sink strength likely accounts for the high level of $BoOR_{MUT}$-mediated carotenoid accumulation.

PSY and OR Mutual Regulation. In addition to being regulated by OR, PSY also controlled AtOR protein amounts as altered levels of OR were observed in the *PSY*-overexpressing and cosuppressed lines. Indeed, an earlier study shows that PSY does not only serve as rate-limiting enzyme in carotenoid biosynthesis, but also controls supplies of metabolic precursors for isoprenoid biosynthesis by regulating DXS protein abundance (31). However, as OR has no proven biosynthetic function, such a regulation suggests that it might have a structural function. Active PSY is membrane associated but coexists with an inactive stromal PSY population, as shown in daffodil chromoplasts (40) and during deetiolation (29). Plastid import studies showed that PSY is part of a soluble, chaperonin-containing complex but is released quickly to membranes (41). Membrane-bound OR might be required to associate soluble, inactive PSY populations to the membrane for activation. An overflow of this system by PSY overexpression might require higher OR levels to enable increased membrane association of PSY, ensuring a coordinated control of key proteins involved in carotenogenesis.

PSY has been reported by several independent groups to be a component of a "phytoene synthesizing complex" able to convert IPP into phytoene in vitro, suggesting its association with at least two upstream enzymes, IPP isomerase and GGPP synthase (42–44). This "phytoene synthesizing complex" was isolated exclusively from plastidial stromal fractions of pepper and tomato fruits, and it showed very low capacity to incorporate IPP into phytoene when isolated from leaf chloroplast stroma (42, 43). In other systems, this association appears rather weak and not readily amendable to biochemical investigations, such as in chloroplasts isolated from mustard seedlings (29) and in chloroplasts from *Arabidopsis* in the present study. Here PSY activity was exclusively found membrane bound and required addition of active GGPP synthase for in vitro assay. This weak association in leaf chloroplasts might explain why our co-IP experiments did not reveal IPP isomerase and GGPP synthase. Although we have conclusive evidence that OR physically interacted with PSY, at this point the function of OR may not extend on this complex organization.

OR transcript was greatly reduced in the *psy* cosuppressed plants, which could be an indication of retrograde signaling. Retrograde plastid-to-nucleus signaling regulates the expression of nuclear genes and a particular perturbance of plastid homeostasis affects distinctive sets of target genes (45–47). Suppression of *PSY* and a blockage of carotenogenesis cause profound metabolic changes in plastids. The metabolites might serve as signals to repress *AtOR* expression. Apocarotenoids produced in ζ-carotene desaturase mutants have been shown to signal nuclear gene expression in *Arabidopsis* (48).

Discrete Functions of OR Domains in Interaction with PSY and Formation of Dimers. OR contains two distinct domains (23). Evidence from this study reveals the important functional roles of the OR domains in controlling protein–protein interactions. The N-terminal region was required for interaction with PSY. Although no similarity to known functional domains was found, the region shares a high degree of amino acid identity and contains several segments of conserved sequences throughout different plant species (23), indicating that the N-terminal region could be a previously unidentified functional domain responsible for OR–PSY interaction.

The OR C terminus contains a cysteine-rich zinc finger domain known to be involved in protein–protein interaction (23, 26). We found that the C-terminal domain was also needed for OR dimerization. Protein dimerization is hypothesized to have several biological impacts, including regulation of gene expression, enzyme activities, and resistance to proteinases (28, 49, 50). Likely, OR dimerization may protect OR and PSY, and probably other interacting proteins from proteolysis to finely modulate carotenoid biosynthesis and accumulation in plastids.

Materials and Methods

Co-IP and Protein Identification by nLC-MS/MS. Co-IP was carried out using the μMACS GFP-tagged protein isolation kit (Miltenyi Biotec). Proteins were extracted from 4-wk-old *Arabidopsis* leaves expressing *35S:BoOR-GFP* or *35S:GFP* (23). The eluted co-IP proteins were separated on gradient (10–20%) SDS/PAGE gels. In-gel digestion, peptide extraction and separation, MS/MS analysis, and data analysis were performed as described previously (51). Experiments were performed with four biological replicates.

Y2H and BiFC Assay. The split ubiquitin system was used as described (28). *Agrobacterium* cells carrying pairs of BiFC constructs were infiltrated into *N. benthamiana*. The BiFC signals after 48-h infiltration were examined as described previously (26).

Carotenoid Analysis and qRT-PCR. Carotenoid extraction and HPLC analysis were performed as described (17). RNA extraction, cDNA synthesis, and qRT-PCR analysis were performed as described (26). Gene-specific primers are listed in *SI Appendix*, Table S2.

Western Blot Analysis and PSY Enzyme Activity Assay. Proteins were extracted with phenol as described (7). For immunoblots, monoclonal anti-PSY antibody or anti-OR antibody, and the ECL detection system were used (7, 23). In vitro PSY activity assay was carried out with isolated chloroplast membranes as described (29). An extended description of materials and methods used in this study is given in *SI Appendix, SI Materials and Methods*.

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