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Coordinating plant pigment production: A green role for ORANGE family proteins

Chlorophylls and carotenoids are essential pigments for photosynthesis and plastid/plant development. Chlorophylls are utilized for light harvesting and primary charge separation, whereas carotenoids are accessory light-harvesting pigments that also play major roles in photoprotection, in the assembly and stability of photosystems, and as precursors to phytohormones and other important signaling molecules. The biosynthesis of these pigments must be precisely regulated to ensure their balanced production for the synthesis, assembly, and maintenance of the photosynthetic apparatus.

ORANGE (OR) and OR-Like (ORL) proteins are members of the DNAJE1 family of molecular co-chaperones and are highly conserved in the plant kingdom and green algae, where they have multiple roles in plastid development, including the regulation of carotenogenesis (Lu et al., 2006). A functional link between the *Or* locus and carotenoid biosynthesis was reported nearly 50 years ago with the identification of a cauliflower (*Brassica oleracea* var. *botrytis*) mutant with bright orange curds. This atypical pigmentation was caused by a mutation in the *Or* gene, resulting in the formation of β-carotene-containing chromoplasts (Li et al., 2001; Lu et al., 2006). It has since been shown that OR proteins act as major posttranslational regulators of carotenogenesis in plants by interacting with and stabilizing phytoene synthase (PSY), the first enzyme in the carotenoid biosynthesis pathway (Zhou et al., 2015; Park et al., 2016; Welsch et al., 2018). A recent study by Sun et al. (2023) published in *Molecular Plant* provides evidence supporting a new role of OR family proteins. The authors show that OR is involved in posttranslational regulation of the magnesium chelatase (MgCh) enzyme that catalyzes the first committed step of chlorophyll biosynthesis and thus appears to coordinate the production of the two major classes of photosynthetic pigments in plants.

OR REGULATES PSY AND CHROMOPLAST FORMATION TO ENHANCE β-CAROTENE ACCUMULATION

In the first dedicated step of carotenoid biosynthesis, two molecules of geranylgeranyl pyrophosphate are condensed by PSY to produce phytoene (Figure 1A), the colorless precursor of all plant carotenoids. PSY is a key rate-limiting enzyme in carotenogenesis and is subjected to multifactorial regulation, in particular by OR (Zhou et al., 2015, 2022). An *Arabidopsis thaliana or orl* double mutant lacking both OR and ORL proteins contained only about 30% of the carotenoid and chlorophyll content of wild-type plants and had a dramatically reduced level of the PSY enzyme (Zhou et al., 2015). Consistent with impaired pigment synthesis, *or orl* plants displayed defects in growth, light-harvesting complex assembly and thylakoid membrane stacking, and were less tolerant to temperature stress (Zhou et al., 2015; Sun et al., 2023). Conversely, overproduction of OR, or a more active natural variant with an arginine to histidine substitution (OR^{His}), results in enhanced accumulation of carotenoids, in particular β-carotene (Yuan et al., 2015). From a biosynthetic viewpoint, it is not obvious how the interaction of OR with PSY would increase the levels of β-carotene relative to other carotenoids: downstream of PSY, the pathway for synthesis of all carotenoid species is shared up to the point of the intermediate lycopene, and β-carotene can be further modified to produce xanthophylls and phytohormones (Figure 1B). Instead, it appears that OR^{His} overproduction triggers the formation of chromoplasts, which act as sinks to sequester β-carotene (Sun et al., 2020).

A NEW ROLE FOR OR IN POSTTRANSLATIONAL REGULATION OF CHLOROPHYLL BIOSYNTHESIS

The C_{20} phytol tail of chlorophyll also originates from geranylgeranyl pyrophosphate, which is further required for the synthesis of other important molecules such as phylloquinone, tocopherols, and gibberellins. Chlorophyll *a* is synthesized from protoporphyrin IX (PPIX) in a pathway requiring seven enzymes (Figure 1A). The first dedicated step in the pathway is the insertion of a Mg^{2+} ion into PPIX by the multisubunit MgCh enzyme in an ATP-consuming reaction. CHLI is one of two AAA+ ATPase subunits of MgCh—the other being CHLD—and interacts with the large catalytic subunit CHLH and the porphyrin-binding protein GUN4. CHLI exists in two isoforms in Arabidopsis; CHLI1 is the predominant functional form and was shown by Sun and colleagues to interact with OR and ORL by yeast two-hybrid analysis, bimolecular fluorescence complementation assays and, in the case of the CHLI1–OR interaction, additionally by co-immunoprecipitations. The authors also found that OR interacts with the CHLI2 isoform, which is important for the assembly of MgCH, and CRD1, the Mg-PPIX monomethylester cyclase, which acts later in the chlorophyll biosynthesis pathway. Using an *or orl* double mutant and an *Or* overexpression line, Sun et al. further demonstrated that CHLI and PSY levels correlated positively with the level of OR, showing decreased stability of both enzymes in *or orl* plants and increased detection in overexpression lines, both under steady state and in response to heat stress. Thus, OR interacts with and stabilizes the first dedicated enzyme in the pathways for the two major classes of photosynthetic

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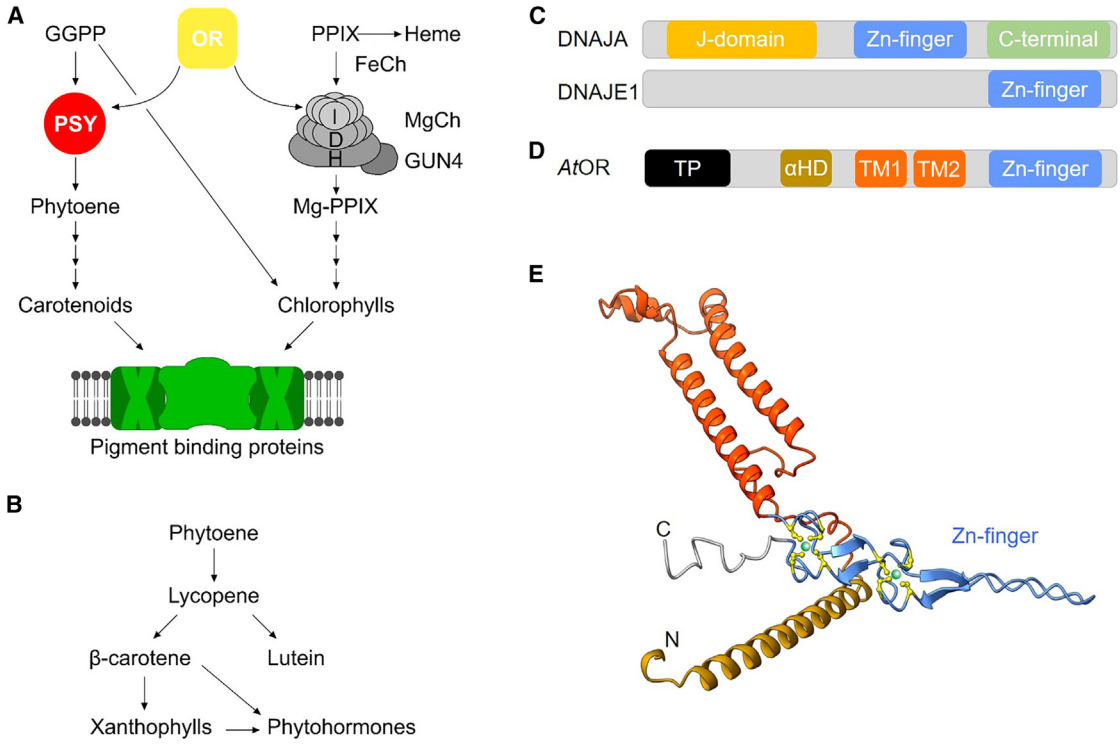

Figure 1. A possible role of OR proteins in co-regulation of carotenoid and chlorophyll biosynthesis and the structure of DNAJ and OR family proteins

(A) OR family proteins interact with the first dedicated enzymes in the carotenoid (phytoene synthase, PSY) and chlorophyll (CHLI subunit of magnesium chelatase) biosynthetic pathways to coordinate synthesis of these two crucial classes of photosynthetic pigments in plant chloroplasts. Figure based on the model proposed by Sun et al. (2023).

(B) A more detailed overview of the carotenoid biosynthesis pathway showing branching downstream of lycopene, leading to the production of β-carotene, lutein, other xanthophylls, and phytohormones. Note that a single arrow may represent multiple enzymatic steps.

(C) Schematic depiction of the characteristic domains of DNAJA (HSP70-dependent) and DNAJE1 (HSP70-independent) proteins. Figure based on the schematic in Pulido and Leister (2018).

(D) A more detailed schematic representation of *A. thaliana* OR showing the chloroplast transit peptide (TP), the alpha-helical domain (αHD), two transmembrane helices (TM1 and 2), and the zinc-finger domain.

(E) AlphaFold model of *A. thaliana* OR (https://alphafold.ebi.ac.uk/entry/Q9FKF4) lacking the predicted N-terminal chloroplast transit peptide and color coded according to the domain labels in panel **(D)**. Cysteine residues that coordinate zinc atoms are highlighted in yellow.

pigments in plants. It will be of interest to further explore how OR interacts with and stabilizes CHLI, which forms a hexameric ring structure that transfers the energy of ATP hydrolysis to CHLH via interaction with CHLD. Notably, cyanobacteria lack homologs of OR/DNAJE1 proteins. If and how the processes of carotenogenesis and chlorophyll biosynthesis are posttranslationally co-regulated in these relatively simpler prokaryotic phototrophs remain to be uncovered, although it is possible that the cyanobacterial CHLI and PSY enzymes are less heat sensitive than their plant counterparts, negating the need for dedicated chaperones.

THE INTERACTION BETWEEN OR AND PSY IS CONSERVED IN GREEN ALGAE

OR family proteins are conserved in the green lineage, and OR overproduction in *Chlamydomonas reinhardtii* enhanced carotenoid and phytohormone accumulation and resulted in increased resistance to abiotic stress (Yazdani et al., 2021). More recently, OR was shown to promote carotenoid biosynthesis and regulate plastid development in the β-carotene-accumulating alga *Dunaliella salina* (Liang et al., 2023), and the authors demonstrated that the interaction of OR and PSY is conserved in this halotolerant species. Overexpression of *D. salina Or* increased cellular carotenoid levels, with β-carotene accounting for >80%. In addition to the effect on carotenoid accumulation, increased expression of *Or* also enhanced cellular chlorophyll content, and it will be interesting to see whether the interaction of OR with MgCh is also conserved in green algae. Furthermore, OR overproduction affected cell size and chloroplast morphology in *Chlamydomonas* and *Dunaliella,* consistent with the alteration in plastid size observed in *Or*-overexpressing plants (Sun et al., 2020).

Unlike the situation with the *D. salina* protein, OR from *Dunaliella* sp. FACHB-847, a non-β-carotene-accumulating algal species, did not interact with the PSY enzyme from this organism (Liang et al., 2023). These findings support a link between OR and the specific enhancement of β-carotene accumulation, as observed in plants. However, the biosynthesis of all carotenoid types was increased by *Or* overexpression in *C. reinhardtii* (Yazdani et al.,

2021), so the situation in different green algae warrants further investigation.

STRUCTURE AND FUNCTION OF OR/DNAJE1 PROTEINS

DNAJ proteins are co-chaperones required for the function of HSP70 chaperones, which have important roles in protein homeostasis and quality control in Arabidopsis (Pulido and Leister, 2018). Structurally, the archetypal DnaJ/DNAJA proteins consist of a ''J-domain'' responsible for binding of HSP70, a cysteine-rich ''zinc-finger domain'' involved in protein–protein interactions, and a ''C-terminal domain'' that facilitates dimerization and substrate binding (Figure 1C). Different groups of DNAJ-like proteins lack one or more of these canonical domains; OR and ORL belong to the DNAJE1 subgroup that contains only a ''DNAJ-like zinc-finger'' domain (Figures 1C and 1D) and are thus classified as HSP70-independent DNAJ-like proteins. Around 20 members of the DNAJE1 subgroup have been identified in *A. thaliana*, with multifunctional roles in plant growth, plastid development, and abiotic stress responses (Pulido and Leister, 2018). A structure of an OR family protein is currently unavailable, but an AlphaFold model of the Arabidopsis protein shows that the C-terminal zinc-finger domain is predicted to be preceded by an N-terminal alpha-helical region and two transmembrane helices (Figure 1E). While the precise mechanism of DNAJE1 proteins requires further study, they may operate as standalone chaperones to facilitate the folding and accumulation of their client proteins; indeed, the N-terminal domain of OR has been shown to possess holdase chaperone activity (Park et al., 2016; Sun et al., 2023).

CONCLUDING REMARKS

The interaction of OR family proteins with MgCh provides evidence of a new role for these proteins in co-chaperoning the biosynthesis of the two major classes of photosynthetic pigments in plants. Although the pathways for chlorophyll and carotenoid biosynthesis are well characterized and much is understood about their regulation, less was known regarding their concerted posttranslational regulation prior to the work of Sun and colleagues. It makes good sense to co-regulate these pathways to ensure the coordinated production of chlorophylls and carotenoids to feed light-harvesting complex and photosystem assembly and repair, and to allow rapid responses to environmental stresses, such as extremes of heat and light. It is also common to regulate the first dedicated step in biosynthetic pathways, preventing wasteful flux to unrequired products and, in the case of chlorophyll biosynthesis, the undesirable buildup of phototoxic intermediates. Posttranslational regulation of MgCh is conserved even in anoxygenic purple bacteria, albeit by a different mechanism, and carotenoids appear to protect enzymes in the chlorophyll biosynthesis pathway, further entwining the biosynthesis of these crucial pigment molecules.

The activity of OR is, however, far broader than pigment biosynthesis, extending to chromoplast formation, regulation of nuclear gene expression during photomorphogenesis, control of pre-protein import, and response to abiotic stresses. The observed thermotolerance and stress resistance imparted by OR make it a promising genetic target for the generation of climate-resilient crops, which will be aided by further molecular dissection of the diverse functions of OR family proteins in future studies.

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Andrew Hitchcock[1,], Matthew S. Proctor[1] and Roman Sobotka[2,3]*

[1]Plants, Photosynthesis and Soil, School of Biosciences, University of Sheffield, Sheffield S10 2TN, UK
[2]Institute of Microbiology of the Czech Academy of Sciences, Opatovický mlýn, Třeboň 379 01, Czech Republic
[3]Faculty of Science, University of South Bohemia, České Budějovice 370 05, Czech Republic
***Correspondence: Andrew Hitchcock (a.hitchcock@sheffield.ac.uk)**
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