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SHORT COMMUNICATION

UBC19 is a new interacting protein of ORANGE for its nuclear localization in *Arabidopsis thaliana*

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ABSTRACT

ORANGE (OR) is a member of the DnaJ-like zinc finger domain-containing protein family, of which all orthologs share a highly conserved quadruple repeat of the CxxCxxxG signatures at their C-termini. Dual subcellular localization and different interacting partner proteins have been reported for OR. In plastids, OR interacts with phytoene synthase, the entry enzyme for carotenoid biosynthesis, to promote chromoplast biogenesis and carotenoid accumulation in non-pigmented tissues. In the nucleus, OR interacts with the eukaryotic release factor eRF1-2 to regulate cell elongation in the petiole, and with the transcription factor TCP14 to repress the expression of *Early Light-Induced Proteins* (*ELIPs*) and chloroplast biogenesis in de-etiolating cotyledons. In this study, we demonstrated the E2 ubiquitin-conjugating enzyme UBC19 as a new interacting partner of OR. The lysine[58] of OR was found to be ubiquitinated, and OR lost its nuclear localization and the capability in repressing *ELIPs* when lysine[58] was substituted by alanine. Our findings raised the possibility that the ubiquitination by UBC19 is essential for the nuclear localization of OR.

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The DnaJ-like zinc finger domain-containing protein ORANGE (OR) was initially identified from a cauliflower mutant, which accumulated a large amount of carotenoids in its curd.[1,2] Subsequent studies demonstrated that OR and its orthologs from different higher plants are highly conserved, all sharing a C-terminal quadruple repeat of the CxxCxxxG signatures.[2–4] A distinct function of OR and its orthologs is to trigger the development of plastids from the non-pigmented forms, such as amyloplasts and leucoplasts, into chromoplasts, which facilitates and enhances the biosynthesis and sequestration of carotenoids [5–7]. Such a function largely relies on the protein–protein interaction between OR and phytoene synthase (PSY), the entry enzyme for carotenoid metabolic pathway, in plastids.[7,8] In addition to plastids, OR was also found in the nucleus.[9–12] OR was demonstrated to interact with the eukaryotic release factor eRF1-2 in the nucleus to regulate the elongation of petiole cells.[12] Recently, we further identified the physical and genetic interactions between OR and the transcription factor TCP14 in the nucleus, through which OR represses the expression of *Early Light-Induced Protein1/2* (*ELIP1/2*) and the development of chloroplasts in de-etiolating cotyledons.[10] However, the molecular mechanism underlying the nuclear localization of OR was unknown.

To address this question, we first performed yeast two-hybrid (Y2H) screening. The full-length OR (OR^{FL}) was used as a bait to screen a normalized library prepared from mixed Arabidopsis tissues. After screening, positive clones harboring the coding region for the E2 ubiquitin-conjugating enzyme UBC19 (*At3g20060*) were identified. Because OR possesses an N-terminal chloroplast transit peptide (cTP) and a C-terminal zinc finger domain (ZF), separated by two transmembrane domains (TMs), we individually tested OR^{FL} and its truncated peptides before and after the TMs (OR^{N} and OR^{C}, respectively) for their interactions with UBC19 by pairwise Y2H assays (Figure 1a). Our result revealed that both OR^{FL} and OR^{C} interacted with UBC19, whereas OR^{N} did not show a positive interaction (Figure 1b). In *A. thaliana*, OR and UBC19 share the highest sequence similarities with OR-Like (AT5G06130) and UBC20 (AT1G50490), respectively.[7,13] By pairwise Y2H test, we found that OR-Like and UBC19 did not interact, neither did OR and UBC20 (Figure S1). Therefore, OR-Like and UBC20 might not function redundantly in our study.

To verify the protein–protein interaction between OR and UBC19, we heterologously expressed UBC19 with a glutathione S-transferase (GST) fusion tag (GST-UBC19) and OR^{FL} with a maltose-binding protein (MBP) tag (MBP-OR^{FL}). A pull-down assay was performed using the affinity-purified fusion proteins. Our immunoblot analysis showed that OR^{FL} was captured by GST-UBC19, while no positive interactions were detected between OR^{N} and GST-UBC19, nor between OR^{FL} and GST (Figure 1c). These results demonstrated an *in vitro* interaction between OR and UBC19. We also performed a bimolecular fluorescence complementation (BiFC) assay to test the *in planta* interaction between OR and UBC19. We transiently expressed OR and UBC19 as fusion proteins with the N- and C-halves of enhanced yellow fluorescent protein (EYFP), respectively, in tobacco leaves by infiltration. After a 3-d growth in the dark, the reconstituted EYFP protein was mainly

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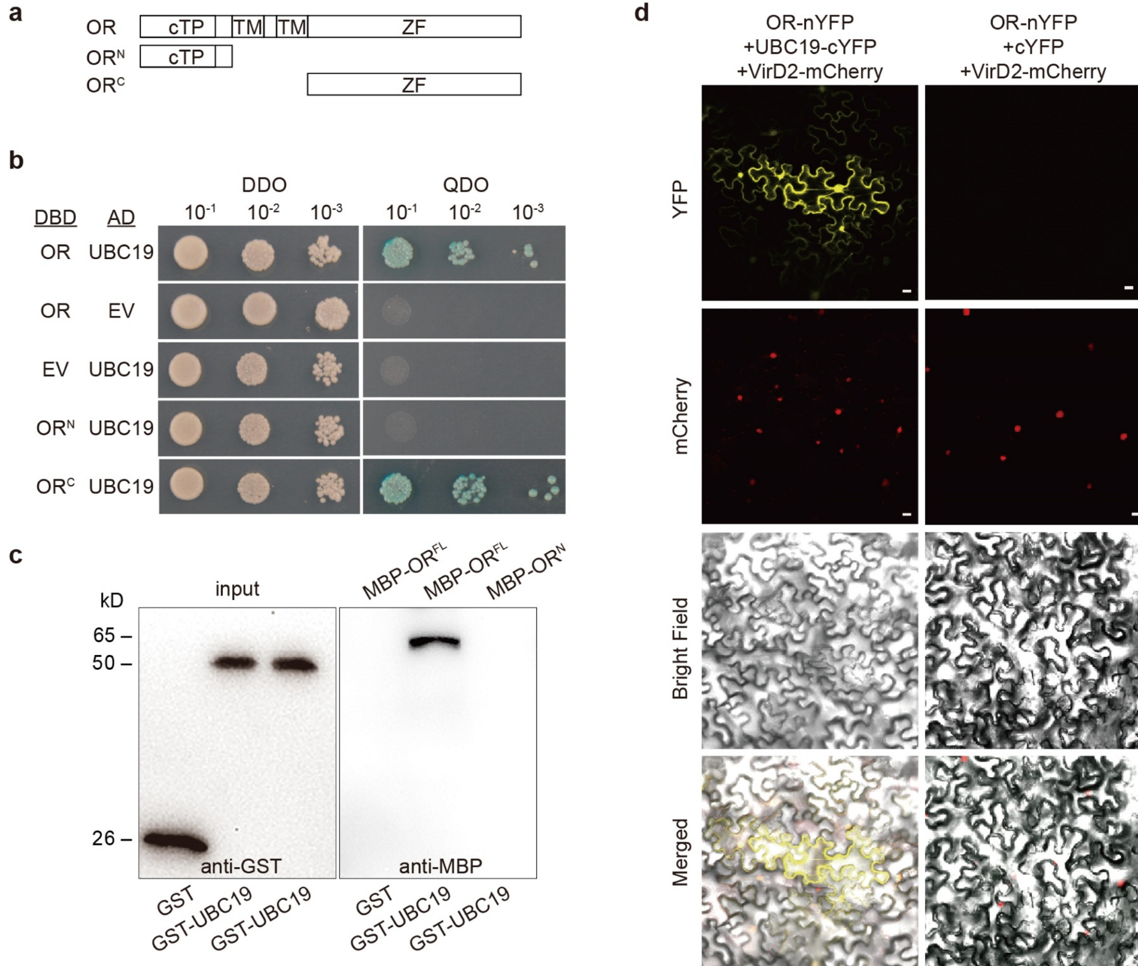

Figure 1. UBC19 interacts with OR. (a) Structures of OR and its N- and C-terminal truncates (OR^N and OR^C, respectively). cTP, chloroplast transit peptide; TM, transmembrane domain; ZF, zinc finger domain. (b) Yeast two-hybrid assay. *OR* or its truncates was cloned into pGBK-T7, and *UBC19* was cloned into pGAD-T7. Yeast cells co-transformed with a combination of the indicated plasmids were plated in series dilution. EV, empty vector; DBD, DNA binding domain; AD, activation domain. DDO, double dropout (/-Leu/-Trp); QDO, quadruple dropout (/-Leu/-Trp/-His/-Ade). (c) Pull-down assay. Heterologously expressed and affinity-purified GST-UBC19 fusion protein was incubated with MBP-OR and glutathione particles. The bound protein was eluted, resolved by SDS-PAGE, blotted, and probed with the antibodies against the GST and MBP tags. GST and OR^N were used as negative controls. (d) BiFC observation showing that OR and UBC19 bind each other. A C-terminal fragment of yellow fluorescent protein (cYFP) was used as a negative control. The signal from the nuclear marker VirD2NLS-mCherry indicates the nucleus. Bar = 10 µm.

observed in the cytoplasm and the nucleus, agreeing with the localization of UBC19 as previously reported, and that of OR, which is translated in the cytoplasm before being targeted to either chloroplasts or the nucleus (Figure 1d).[11,13] Taken together, our results demonstrated UBC19 as a novel interacting partner of OR, while OR is also the first known interacting protein of UBC19.

To further resolve the nuclear localization of OR, we precipitated OR from 2-week-old *OR*-overexpression seedlings using the antibody against OR, and subjected to LC/MS-MS analysis after trypsin digestion. A ubiquitination modification at $lysine^{58}$ (K^{58}) of OR was identified (Table S1). We then generated a mutant of OR by substituting K^{58} with alanine (OR^{K58A}), and individually fused OR and OR^{K58A} with EYFP (OR-EYFP and OR^{K58A}-EYFP, respectively). When OR-EYFP or OR^{K58A}-EYFP was co-expressed with the cyan fluorescent protein (CFP) in tobacco leaves, both OR-EYFP and OR^{K58A}-EYFP were observed in chloroplasts (Figure 2a). However, when co-expressed with a UBC19-CFP fusion protein, OR-EYFP was observed in both cytoplasm and the nucleus but not in chloroplasts, whereas OR^{K58A}-EYFP showed only chloroplast localization (Figure 2a). This result suggested the possibility that OR might be ubiquitinated at K^{58} by UBC19 for its nuclear localization.

Because we recently reported that the nucleus-localized OR negatively regulates the expression of *ELIP1/2*, which are transiently induced during de-etiolation, we tested whether UBC19 was involved in such a regulation.[10,14] The transcript abundance of *UBC19* itself was about constant during de-etiolation (Figure S2). However, significantly higher expression levels of both *ELIP1* and *ELIP2* were found in the *ubc19* lines, especially at 3 and 6 h post illumination (Figure 2b, Figure S3). This result resembled the expression of *ELIPs* in the *OR*-silencing lines as previously reported,[10] and indicated a de-repression of *ELIPs* by silencing *UBC19*. Although the overexpression of *OR* severely repressed *ELIP1/2*,[10] the overexpression of *UBC19* did not affect the expression of *ELIP1/2* (Figure 2b). It is reasonable to postulate that a WT level of *UBC19* was sufficient to target OR to the nucleus in de-etiolating cotyledons.

Moreover, we overexpressed the OR^{K58A}-EYFP fusion protein in the *OR* knockout mutant (*OR-Cas*) to test whether the

Figure 2. Lys58 is essential for the nuclear localization of OR. (a) Confocal observation of tobacco leaves infiltrated to co-express OR or OR^{K58A} (as EYFP fusion proteins) with UBC19 (as CFP fusion protein). CFP was used as a negative control. (b, c) Transcript abundances of *ELIP1/2* in seedlings with different *UBC19* (b) or *OR* background (c) post illumination. Transcript abundance was normalized against *ACTIN2*. Data are means ± SE, * $P < .05$, **$P < .01$ or better (Student's *t* test, $n = 3$).

repression of *ELIPs* could be regained (Figure S4). Different from the OR-GFP protein, which we previously proved to localize in both chloroplasts and the nucleus,[11] OR^{K58A}-EYFP was only observed in chloroplasts (Figure S4e). No significant variation in the expression of *UBC19* was identified among the WT and *OR*-overexpressing, silencing, and complementation lines (Figure S4f). However, transcript abundances of *ELIP1/2* in the complementation lines were similar to their corresponding levels in *OR-Cas*, which were significantly higher than the WT levels during de-etiolation (Figure 2c). Protein abundances of ELIP1/2 also showed similar variations among these lines (Figure S5). These results demonstrated that OR^{K58A} is incapable of repressing *ELIPs* during de-etiolation, which is different from the function of OR.[10]

In the past decade, OR has been reported to regulate various developmental processes through its protein–protein interactions with different partner proteins. Here, we added UBC19 as its novel interacting partner. OR has a characteristic C-terminal DnaJ-like zinc finger domain with 8 conserved cysteine residues, which might facilitate such protein–protein interactions.[2,3] This postulation was first demonstrated in this study, as our Y2H assay showed the specific interaction between OR^{C} and UBC19 (Figure 1b). It is possible that OR interacts with various pattern proteins under specific spatiotemporal circumstances. It would be interesting to explore its other interacting proteins, which might help to decipher a full plethora regulation mechanisms of OR.

Ubiquitination is an essential post-translational modification in eukaryotes.[15,16] There have been numerous reports on its regulation of plant development and acclimation.[17–19] During de-etiolation, the E3 ubiquitin-ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) targets transcription factors such as HY5 and HYH for ubiquitination and degradation in the dark, and thus plays an essential role in the regulation of photomorphogenesis.[20–22] In this study, our results suggested that, during de-etiolation, the E2 enzyme UBC19 might be a new member of the regulatory machinery throught its interaction with OR, and also raised the possibility that the ubiquitination by UBC19 is essential for the nuclear localization of OR.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana plants used in this study were all in the Columbia-0 wild-type (WT) background. In general, seeds were stratified in the dark at 4°C for 3 d and then germinated on Murashige-Skoog (MS) plates at 22°C under a light intensity of 120 μmol photons m^{-2} s^{-1} with a 16 h/8 h light/dark photoperiod. One-week-old seedlings were transferred to grow in the soil (a mixture of peat moss, vermiculite, and perlite at 1:1:1) under the same conditions.

Antibodies for immunoblot assays

Antibodies against ACTIN, GFP, GST, and MBP were from Sangon (Shanghai, China). Polyclonal antibodies against OR, ELIP1, and ELIP2 were prepared in our previous study.[10] Horseradish peroxidase-conjugated secondary antibodies against rabbit/mouse IgG from Promega (Madison, WI, USA) and BeyoECL Star Western Blotting Substrate (Beyotime, Shanghai, China) were used for immunodetection. Standard protocols[23] and the manufacturer's manuals for SDS-PAGE, semi-dry blotting, and immunodetection were followed.

Molecular manipulation and gene expression quantification

Genomic DNA was extracted from leaves using the cetyltrimethylammonium bromide method.[24] Total RNA was isolated using the RNAiso reagent (TaKaRa, Shiga, Japan), and cDNA was synthesized with a PrimeScript Double Strand cDNA Synthesis Kit (TaKaRa) following the manufacturer's instructions. Gene expression levels were quantified by quantitative real-time PCR (qPCR) using TB Green Premix Ex Taq II (Tli RNaseH Plus) with a Thermal Cycler Dice Real-Time System TP800 (TaKaRa) following the manufacturer's instructions. *ACTIN2* (*At3g18780*) was used as a reference. The expression values were calculated according to the comparative C_T method.[25] For each sample, three biological replicates were analyzed, each with three repeats. All primers used in this study are listed in Supplemental Table 2.

Generation of transgenic plants

Total RNA was extracted from *A. thaliana* seedlings and reverse transcribed into a cDNA pool as previously reported for cloning *OR* and *UBC19*.[10] The full-length open reading frame (ORF) of *UBC19* was amplified from the cDNA pool. The amplicon was first cloned after the *35S* promoter in the pRTL2 vector, and then the entire cassette was digested from pRTL2 and cloned into the multiple cloning site of pCAMBIA1300 to form the pCAMBIA1300-35S:UBC19 construct, which was then used to generate *UBC19* overexpression (*UBC19-OE*) lines.[10]

For generating *UBC19* knockout lines (*ubc19*), a nucleotide fragment (GATGCTGCTTCCACCCCAATG) was designed to edit *UBC19* using the CRISPR/Cas9 strategy. The fragment was cloned into the BbsI site of the pCAMBIA1300-Cas9-AtU6 vector (kindly provided by Dr. Honggui La from Nanjing Agriculture University) to produce the pCAMBIA1300-Cas9-UBC19 construct.

For *OR*, its overexpression line *OE#2* from our previous study was used.[10] To generate its knockout mutant, a nucleotide fragment (GATGCTGCTTCCACCCCAATG) was designed to produce the pCAMBIA1300-Cas9-OR construct, which was then used to edit *OR* using the CRISPR/Cas9 strategy as mentioned above. For gene complementation, we re-designed the OR^{K58A} coding sequence, of which the gDNA region was re-coded to avoid editing but still encoded the same amino acids as the WT, and the codon for Lys^{58} (AAA) was substituted by that for Ala (GCA). The entire fragment was chemically synthesized (Convenience Biology, Changzhou, China) and fused to the 5′-end of *EYFP* in pA7-EYFP (ABRC, Columbus, OH, USA). The entire expression cassette was amplified, and subsequently

cloned to pCAMBIA1300/BAR, a vector in which we substituted the hygromycin-resistance gene of pCAMBIA1300 with the Basta-resistance gene from pFGC5941 (ABRC) through homologous recombination. This generated the pCAMBIA1300-ORK58A complementation construct.

For each of the constructs, *Agrobacterium tumefaciens* was used to transform *A. thaliana* using the floral dipping method.[26] In general, the WT seedlings were used for transformation. However, for *OR*, we used the previously reported *or-1* line, which contains a T-DNA insertion in its 5′-UTR,[10] for pCAMBIA1300-Cas9-OR to generate the gene edited *OR-Cas* lines, and used one of its progeny lines (*OR-Cas#1*) for pCAMBIA1300-ORK58A to generate the OR^{K58A} complementation lines ($OR\text{-}Cas/OR^{K58A}$). The transformants were screened on MS plates containing 50 mg L^{-1} hygromycin B, whereas those of the $OR\text{-}Cas/OR^{K58A}$ lines were further screened for their resistance to Basta, to homozygous for subsequent studies.

Protein–protein interaction assays

For yeast two-hybrid (Y2H) screening, full-length ORF of *OR* was amplified from the cDNA pool and cloned into pGBK-T7 vector (TaKaRa) to screen a Mate & Plate Library (Universal Arabidopsis Normalized, TaKaRa). Positive clones were screened on quadruple dropout (QDO, SD/-Leu/-Trp/-His/-Ade) plates according to the manufacturer's instructions.

For pairwise Y2H assay, ORF of *UBC19* was cloned into pGAD-T7 (TaKaRa) as a prey construct. The sequences corresponding to the N- and C-terminal fragments of OR (OR^N and OR^C, respectively) were separately amplified using the full-length *OR* as a template. *OR*, OR^N and OR^C were individually cloned into pGBK-T7 as the bait constructs. Yeast (*Saccharomyces cerevisiae* strain AH109) cells transformed with both bait and prey constructs were screened on QDO plates supplemented with X-α-Gal. Empty vectors were used as negative controls. For assessing the redundancies between homolog proteins, coding regions for OR-Like (*At5g06130*) and UBC20 (*At1g50490*) were similarly cloned and tested in parallel.

For pull-down assay, coding regions for OR and OR^N were individually cloned into pMAL-C5X (NEB, Ipswich, MA, USA) to express their corresponding fusion proteins with N-terminal MBP tags (MPB-OR and MBP-OR^N, respectively). The coding region of *UBC19* was cloned into pGEX-4T-1 (GE Healthcare, Pittsburgh, PA, USA) for the expression of a GST-UBC19 fusion protein. Recombinant proteins were heterologously expressed in *Escherichia coli* BL21(DE3) (Merck Millipore, Darmstadt, Germany) cells and affinity-purified following the manufacturer's instructions. Pull-down assay was carried out following our previous report using the MagneGST glutathione particles (Promega).[10] Proteins captured by the particles were separated by SDS-PAGE and examined by immunoblotting using antibodies against GST and MBP.

For BiFC assay, ORFs of *UBC19* and *OR* were cloned into pSAT1A-cEYFP-N1 and pSAT4A-nEYFP-N1 (ABRC), respectively, through homogenous recombination. Expression cassettes of both constructs, together with the cassette from the pSAT6-mCherry-VirD2NLS vector (for expressing VirD2NLS as a nuclear marker), were cloned into the I-SceI, AscI, and PI-PspI sites, respectively, of pPZP-RCS2-Bar (ABRC).[10] Tobacco leaves were infiltrated and observed using the FLUOVIEW FV1000 Laser Confocal Microscopy System (Olympus, Tokyo, Japan) as previously described.[10]

Subcellular localization analysis

For detecting the co-localization of UBC19 and OR *in planta*, we cloned *UBC19* in pCNHP-CFP, which we constructed based on pCAMBIA1300 and harbored sequentially the enhanced Cauliflower mosaic virus (CaMV) *35S* promoter, synthetic 5′ and 3′ untranslated regions of Cowpea mosaic virus *RNA2* flanking the coding region fused in frame to the 5′-end of the gene for CFP, and the Heat Shock Protein (HSP) terminator from *A. thaliana*, to generate pCHNP-UBC19-CFP.[27] *OR* was cloned in pA7-EYFP as pA7-OR-EYFP. For generating the OR^{K58A} mutant, site-directed mutagenesis was introduced using the megaprimer strategy.[28] The PCR product was cloned into pA7-EYFP as pA7-ORK58A-EYFP. Different combinations of constructs for expressing CFP and EYFP fusion proteins were co-infiltrated to tobacco leaves. Fluorescent signals were observed after 3 d using the FLUOVIEW FV1000 Laser Confocal Microscopy System as previously described.[10]

Mass spectrometry

To determine the ubiquitination modification of OR, 2-week-old *OE#2* seedlings were collected, frozen in liquid nitrogen, ground into fine powder, and then suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF, 1 mM DTT). After a 10-min incubation on ice, the mixture was centrifuged at 4,000 *g* for 10 min. The supernatant was collected and further centrifuged at 12,000 *g* for 20 min. The supernatant was incubated with a mixture of Protein G beads (Promega) and the antibody against OR for 5 h. After incubation, the beads were washed with the lysis buffer for 5 times. OR was eluted from the beads by 100 mM glycine (pH 2.5) and subjected to SDS-PAGE. The gel bands were excised, in-gel digested, and analyzed by a Nexera UHPLC LC-30A system (Shimadzu, Kyoto, Japan) and a TripleTOF 4600 mass spectrometer (AB Sciex, Framingham, MA, USA) at the State Key Laboratory of Pharmaceutical Biotechnology of Nanjing University.

Statistical analysis

GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. To determine statistical significance, we employed Student's *t* test. Differences were considered significant at $P < .05$.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article

References

1. Li L, Paolillo DJ, Parthasarathy MV, DiMuzio EM, Garvin DF. A novel gene mutation that confers abnormal patterns of β-carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). Plant J. 2001;26(1):59–67. doi:10.1046/j.1365-313x.2001.01008.x.
2. Lu S, Van Eck J, Zhou X, Lopez AB, O'Halloran DM, Cosman KM, Conlin BJ, Paolillo DJ, Garvin DF, Vrebalov J, et al. The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. Plant Cell. 2006;18(12):3594–3605. doi:10.1105/tpc.106.046417.
3. Lu Y, Hall DA, Last RL. A small zinc finger thylakoid protein plays a role in maintenance of photosystem II in *Arabidopsis thaliana*. Plant Cell. 2011;23(5):1861–1875. doi:10.1105/tpc.111.085456.
4. Shimada H, Mochizuki M, Ogura K, Froehlich JE, Osteryoung KW, Shirano Y, Shibata D, Masuda S, Mori K, Takamiya K. *Arabidopsis* cotyledon-specific chloroplast biogenesis factor CYO1 is a protein disulfide isomerase. Plant Cell. 2007;10(10):3157–3169. doi:10.1105/tpc.107.051714.
5. Egea I, Barsan C, Bian W, Purgatto E, Latché A, Chervin C, Bouzayen M, Pech J-C. Chromoplast differentiation: current status and perspectives. Plant Cell Physiol. 2010;51(10):1601–1611. doi:10.1093/pcp/pcq136.
6. Sun T, Yuan H, Cao H, Yazdani M, Tadmor Y, Li L. Carotenoid metabolism in plants: the role of plastids. Mol Plant. 2018;11 (1):58–74. doi:10.1016/j.molp.2017.09.010.
7. Zhou X, Welsch R, Yang Y, Alvarez D, Riediger M, Yuan H, Fish T, Liu J, Thannhauser TW, Li L. Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis. Proc Natl Acad Sci USA. 2015;112 (11):3558–3563. doi:10.1073/pnas.1420831112.
8. Ruiz-Sola MÁ, Rodríguez-Concepción M. Carotenoid biosynthesis in Arabidopsis: a colorful pathway. Arabidopsis Book. 2012;10: e0158. doi:10.1199/tab.0158.
9. Kim SH, Ahn YO, Ahn M-J, Jeong JC, Lee H-S, Kwak -S-S. Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. Plant Physiol Biochem. 2013;70:445–454. doi:10.1016/j.plaphy.2013.06.011.
10. Sun T-H, Zhou F, Huang X-Q, Chen W-C, Kong M-J, Zhou C-F, Zhuang Z, Li L, Lu S. ORANGE represses chloroplast biogenesis in etiolated Arabidopsis cotyledons via interaction with TCP14. Plant Cell. 2019;31(12):2996–3014. doi:10.1105/tpc.18.00290.
11. Sun T-H, Zhou F, Liu C-J, Zhuang Z, Lu S. The DnaJ-like zinc finger domain protein ORANGE localizes to the nucleus in etiolated cotyledons of *Arabidopsis thaliana*. Protoplasma. 2016;253 (6):1599–1604. doi:10.1007/s00709-015-0919-x.
12. Zhou X, Sun T-H, Wang N, Ling H-Q, Lu S, Li L. The cauliflower *Orange* gene enhances petiole elongation by suppressing expression of *eukaryotic release factor 1*. New Phytol. 2011;190(1):89–100. doi:10.1111/j.1469-8137.2010.03578.x.
13. Criqui MC, de Almeida Engler J, Camasses A, Capron A, Parmentier Y, Inze D, Genschik P. Molecular characterization of plant ubiquitin-conjugating enzymes belonging to the UbcP4/E2-C/UBCx/UbcH10 gene family. Plant Physiol. 2002;130 (3):1230–1240. doi:10.1104/pp.011353.
14. Hutin C, Nussaume L, Moise N, Moya I, Kloppstech K, Havaux M. Early light-induced proteins protect *Arabidopsis* from photooxidative stress. Proc Natl Acad Sci USA. 2003;100(8):4921–4926. doi:10.1073/pnas.0736939100.
15. Mukhopadhyay D, Riezman H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science. 2007;315 (5809):201–205. doi:10.1126/science.1127085.
16. Zheng N, Shabek N. Ubiquitin ligases: structure, function, and regulation. Annu Rev Biochem. 2017;86(1):129–157. doi:10.1146/annurev-biochem-060815-014922.
17. Callis J. The ubiquitination machinery of the ubiquitin system. Arabidopsis Book. 2014;12:e0174. doi:10.1199/tab.0174.
18. Nezames CD, Deng XW. The COP9 signalosome: its regulation of cullin-based E3 ubiquitin ligases and role in photomorphogenesis. Plant Physiol. 2012;160(1):38–46. doi:10.1104/pp.112.198879.
19. Vierstra RD. The ubiquitin–26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol. 2009;10(6):385–397. doi:10.1038/nrm2688.
20. Lau OS, Deng XW. Effect of Arabidopsis COP10 ubiquitin E2 enhancement activity across E2 families and functional conservation among its canonical homologues. Biochem. J. 2009;418 (3):683–690. doi:10.1042/BJ20081943.
21. Lau OS, Deng XW. The photomorphogenic repressors COP1 and DET1: 20 years later. Trends Plant Sci. 2012;17(10):584–593. doi:10.1016/j.tplants.2012.05.004.
22. Qin N, Xu D, Li J, Deng XW. COP9 signalosome: discovery, conservation, activity, and function. J Integr Plant Biol. 2020;62 (1):90–103. doi:10.1111/jipb.12903.
23. Green MR, Sambrook J. Molecular Cloning: a Laboratory Manual. Fourth. Cold Springer Harbor (NY): Cold Spring Harbor Laboratory Press; 2012.
24. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980;8(19):4321–4325. doi:10.1093/nar/8.19.4321.
25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. Nat Protoc. 2008;3(6):1101–1108. doi:10.1038/nprot.2008.73.
26. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998;16(6):735–743. doi:10.1046/j.1365-313x.1998.00343.x.
27. Wang Q, Cao T-J, Zheng H, Zhou C-F, Wang Z, Wang R, Lu S. Manipulation of carotenoid metabolic flux by lycopene cyclization in ripening red pepper (*Capsicum annuum* var. *conoides*) fruits. J Agric Food Chem. 2019;67(15):4300–4310. doi:10.1021/acs.jafc.9b00756.
28. Ke S-H, Madison EL, Madison EL. Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method. Nucleic Acids Res. 1997;25(16):3371–3372. doi:10.1093/nar/25.16.3371.