Running title: $OR^{His/Ala}$ promotes carotenoid accumulation

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Biochemistry and Metabolism

A Single Amino Acid Substitution in an ORANGE Protein Promotes Carotenoid Overaccumulation in Arabidopsis

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One-sentence Summary: A single amino acid substitution in an OR protein gains new function to promote carotenoid overaccumulation via regulating chromoplast biogenesis

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ABSTRACT

Carotenoids are crucial for plant growth and human health. The finding of ORANGE (OR) protein as a pivotal regulator of carotenogenesis offers a unique opportunity to comprehensively understand the regulatory mechanisms of carotenoid accumulation and develop crops with enhanced nutritional quality. Here we demonstrated that alteration of a single amino acid in wild type OR greatly enhanced its ability to promote carotenoid accumulation. While overexpression of *OR* from Arabidopsis (*AtOR*) or from an agronomically important crop sorghum (*SbOR*) increased carotenoid levels up to two-fold, expression of $AtOR^{His}$ (R90H) or $SbOR^{His}$ (R104H) variants dramatically enhanced carotenoid accumulation by up to seven-fold in the Arabidopsis calli. Moreover, we found that $AtOR^{Ala}$ (R90A) functioned similarly as $AtOR^{His}$ to promote carotenoid overproduction. Neither AtOR nor $AtOR^{His}$ greatly affected carotenogenic gene expression. $AtOR^{His}$ exhibited similar interactions with phytoene synthase (PSY) as AtOR in post-transcriptionally regulating PSY protein abundance, but was not accompanied/linked with enhanced PSY activity. $AtOR^{His}$ triggered biogenesis of membranous chromoplasts in the Arabidopsis calli, which shared similar structures as chromoplasts found in curd of orange cauliflower mutant. By contrast, AtOR did not cause plastid type change in comparison with wild type, but produced plastids containing larger electron dense plastoglobuli. The unique ability of $AtOR^{His}$ in mediating chromoplast biogenesis is responsible for its enhanced carotenoid overproduction. Our study demonstrates $OR^{His/Ala}$ as powerful tools for carotenoid enrichment in plants, and provides insights into the mechanisms underlying OR^{His} regulated carotenoid accumulation.

INTRODUCTION

Carotenoids are widely distributed in nature and give colors varying from light yellow, orange to dark red. As a group of the most abundant pigments, carotenoids are of great importance to both plants and animals (Fraser and Bramley, 2004; Cazzonelli and Pogson, 2010). In plants, carotenoids function as light harvesting pigments and photoprotectants in photosynthesis (Niyogi, 1999; Domonkos et al., 2013). They serve as precursors for the biosynthesis of phytohormones, abscisic acid (ABA) and strigolactones, and for the production of flower and fruit flavor and aroma (Auldridge et al., 2006; Walter and Strack, 2011). In animals like fish, birds and butterflies, the vivid colors produced by carotenoids affect reproduction and survival through courtship behavior and protective color (Cazzonelli, 2011). Most importantly, carotenoids are crucial for human nutrition and health by providing precursors for vitamin A biosynthesis and by reducing the risk of certain chronic diseases, such as cancers, cardiovascular diseases, and age-related eye diseases (Rao and Rao, 2007).

Carotenoids are synthesized *de novo* in plants and algae as well as in some bacteria and fungi (Moise et al., 2014; Nisar et al., 2015). The first committed step in carotenoid biosynthesis pathway is the condensation of two geranylgeranyl diphosphate molecules to phytoene catalyzed by phytoene synthase (PSY). Following four steps of desaturation and isomeration, phytoene is turned into lycopene. Lycopene can be cyclized to produce α- and β-carotene. Upon oxygenation and epoxidation, α-carotene is converted into lutein and β-carotene into zeaxanthin and other xanthophylls (Hirschberg, 2001; Zhu et al., 2010; Ruiz-Sola and Rodríguez-Concepción, 2012; Moise et al., 2014). Carotenoids can be synthesized in nearly all plastids, and stored at high concentration in chloroplasts of green tissues and in chromoplasts of non-green organs, like fruits, flowers and tubers (Lu and Li, 2008; Li and Yuan, 2013).

Significant efforts have been made to increase carotenoid content in food crops due to the importance of carotenoids to human nutrition and health (Giuliano et al., 2008; Farré et al., 2011). The common approach is to alter the catalytic activity of the pathway enzymes. For example, overexpression of a mini-pathway of β-carotene biosynthesis results in the production of Golden Rice, golden potato, and yellow corn (Paine et al., 2005; Diretto et al., 2007; Naqvi et al., 2009). Alteration of PSY and

endogenous gene expression leads to redirect metabolic flux into and between branches of carotenoid biosynthesis in several crop species (Fraser et al., 2002; Ducreux et al., 2005; Yan et al., 2010). A different strategy that builds a strong sink to enhance carotenoid accumulation has been arisen following the cloning of *OR* (*Orange*) from cauliflower orange curd mutant (Lu et al., 2006).

OR encodes a DnaJ cysteine-rich zinc finger domain containing protein, which is highly conserved among divergent plant species (Lu et al., 2006). The mutant allele of *OR* with a large retrotransposon insertion in cauliflower ($BoOR_{MUT}$) causes the normal white curd tissue to accumulate high levels of β-carotene. Expression of the $BoOR_{MUT}$ transgene in both white cauliflower and potato tubers leads to the production of orange tissues with enhanced carotenoid content (Lu et al., 2006; Lopez et al., 2008). The increased carotenoid accumulation in the $BoOR_{MUT}$ plants is found to be associated with the formation of carotenoid sequestration structures, which are suggested to serve as an effective sink to facilitate sequestration and storage of carotenoids (Lopez et al., 2008; Li et al., 2012).

While $BoOR_{MUT}$ has been demonstrated to be an effective molecular tool in enhancing carotenoid content in plants, $BoOR_{MUT}$ contains a large retrotransposon insertion and causes dwarf phenotype in the homozygous mutant in cauliflower (Li et al., 2001; Lu et al., 2006), which limits its potential application for crop nutritional improvement. In addition, the insertion of a large retrotransposon in $BoOR_{MUT}$ produces three alternatively spliced transcripts, and none of which by itself induces β-carotene accumulation at the level comparable with $BoOR_{MUT}$ (Lu et al., 2006), thus hindering the mechanistic study of the *OR* function.

To further explore the function of *OR* and its utilization as an effective genetic tool either through traditional breeding or biotechnology approaches for developing crops with enhanced carotenoid content, we mutagenized wild type (WT) *OR* from Arabidopsis (*AtOR*) along with the agronomically important crop sorghum (*Sorghum bicolor*; *SbOR*). The mutations were based on the recent study of the melon OR protein, where a single arginine (Arg) to histidine (His) substitution is responsible for the orange-flesh melon fruit phenotype (Tzuri et al., 2015). Our data show that $AtOR^{His}$ (R90H) along with $SbOR^{His}$ (R104H) was able to greatly increase carotenoid content. In addition, $AtOR^{Ala}$ (R90A) also promoted high level of carotenoid accumulation. Both AtOR and $AtOR^{His}$ were found to post-transcriptionally regulate

PSY protein level. Transmission electron microscopy (TEM) analysis revealed that AtOR^{His} exhibited additional function and induced the formation of membranous chromoplasts in Arabidopsis calli, which was not observed in the calli of WT and *AtOR* overexpressing lines. Thus, the induction of chromoplast biogenesis with enhanced sink strength is responsible for the *AtOR*^{His} or *AtOR*^{Ala} induced carotenoid overaccumulation.

RESULTS

AtOR^{His} (R90H) Promotes Carotenoid Accumulation in Arabidopsis Callus

Cauliflower *BoOR$_{MUT}$* regulates high level of β-carotene accumulation (Lu et al., 2006). Our recent genetic and functional studies of a melon *OR* homologous gene (*CmOR*) reveal that *CmOR* controls the inheritance of fruit flesh color in melon (Tzuri et al., 2015). A single SNP in *CmOR* was found to be linked with the orange fruit phenotype. This SNP results in an Arg to His change in CmOR 108th amino acid. Alignment of the OR protein sequences showed that this Arg is highly conserved among various plant species (Fig. 1A; Supplemental Fig. S1).

To investigate whether alteration of this conserved Arg to His in other OR proteins induced carotenoid accumulation, site-directed mutagenesis (SDM) was carried out with AtOR to generate AtOR^{His} (R90H) mutation. The nucleotides 269 and 270 of *AtOR* were changed from GA to AT by SDM, resulting in the codon CGA to CAT alteration and changing Arg to His in AtOR (Supplemental Table S1). *AtOR^{His}* along with *AtOR* was overexpressed in Arabidopsis. Two T3 homozygous *AtOR* or *AtOR^{His}* transgenic lines with comparable expression levels were selected (Fig. 1B). They showed a correlated increase of the OR protein levels (Fig. 1C). The *AtOR^{His}* and *AtOR* transgenic lines grew normally as the WT plant (Fig. 1D and 1E), showing no growth inhibition.

Carotenoid composition and content are highly conserved in leaf tissues for optimal photosynthesis (Domonkos et al., 2013). Overexpression of *PSY*, the gene encoding the rate-limiting enzyme in carotenogenesis, exerts no effect on leaf carotenoid level in Arabidopsis (Maass et al., 2009). Similarly, the carotenoid content as well as chlorophyll level in the adult leaves of *AtOR* and *AtOR^{His}* transgenic Arabidopsis lines were similar to those of the WT control (Supplemental Fig. S2), like the case in leaves of WT and the *BoOR$_{MUT}$* cauliflower plants (Li et al., 2001).

In contrast to leaves, non-green tissues, such as callus, are frequently responsive to increased pathway flux with enhanced carotenoid accumulation (Maass et al., 2009; Cao et al., 2012). Indeed, callus has been established as an effective system for rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation (Bai et al., 2014). To examine whether *AtOR^{His}* caused carotenoid accumulation, calli were induced directly from the seeds of these T3

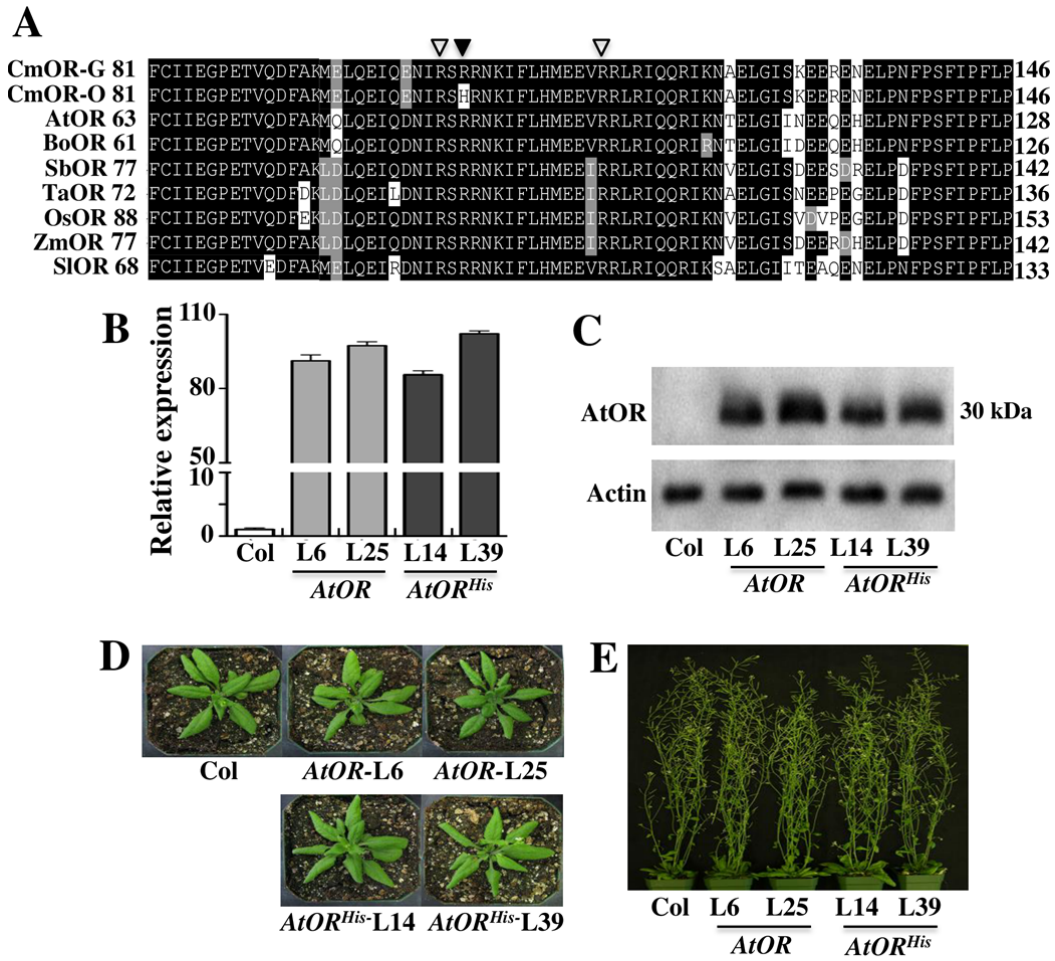

Figure 1. Characterization of *AtOR* and *AtOR*^{His} overexpressing transgenic lines. A, Alignment of partial OR protein sequences from melon CmOR (G and O with green- and orange-flesh fruit, respectively), Arabidopsis AtOR, cauliflower BoOR, sorghum SbOR, wheat TaOR, rice OsOR, maize ZmOR, and tomato SlOR. Protein accession numbers are given in Supplemental Figure S1 legend. The solid triangle shows the position of Arg to His mutation and the empty triangle indicates the 88th or 102th Arg (R) in AtOR. B, Real-time qRT-PCR analysis of relative expression level of *AtOR* in calli of Col, *AtOR* lines L6 and L25, and *AtOR*^{His} lines L14 and L39. C, Western blot analysis of AtOR protein levels in calli of Col, *AtOR* and *AtOR*^{His} lines. Actin protein levels were analyzed as loading control. D, Phenotype of 22 days old plants of Col, *AtOR* and *AtOR*^{His} lines grown in soil. E, Phenotype of 35 days old plants grown in soil.

homozygous *AtOR* and *AtOR*^{His} transgenic lines. The seed-derived Arabidopsis calli of WT were near white in color, the *AtOR* overexpressing calli had light yellow color and the *AtOR*^{His} lines exhibited dark orange hue (Fig. 2A).

To see whether the color difference was associated with carotenoid levels, carotenoid content in these calli was quantified by HPLC. The WT calli accumulated low levels of carotenoids with lutein and β-carotene as the main compounds (Fig. 2B). Consistent with the visible callus color difference, the two *AtOR* overexpressing lines showed slightly but significantly higher levels of total carotenoids than WT. The

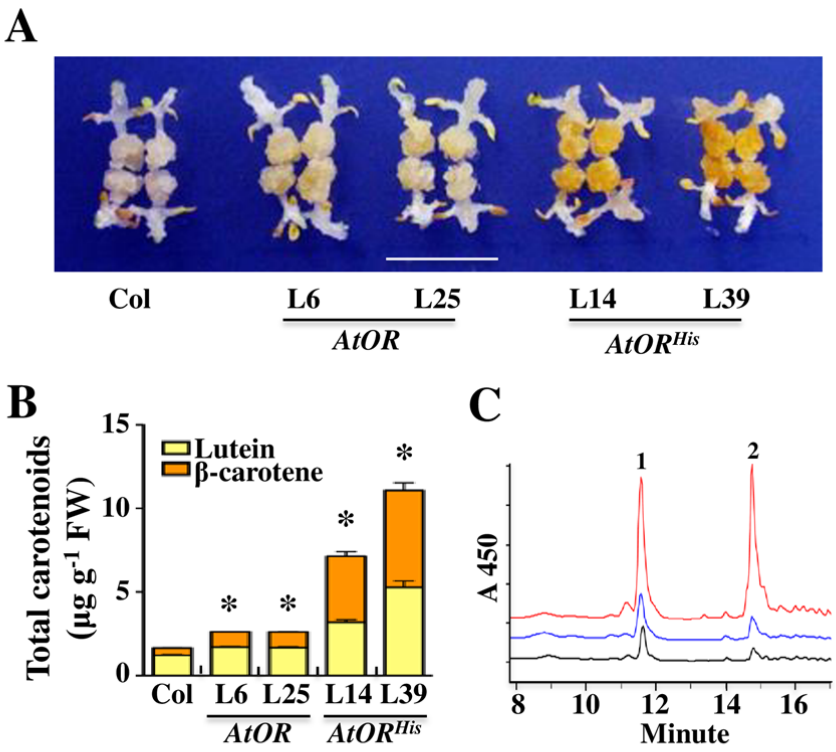

Figure 2. *AtOR*^{His} overexpressing transgenic lines accumulate high levels of total carotenoids in calli. A, Calli of Col, *AtOR* and *AtOR*^{His} lines. Bar =1 cm. B, Total carotenoid levels of Col, *AtOR* and *AtOR*^{His} lines measured by HPLC. Data are the average ± SE of three biological replicates. Asterisks indicate significant difference when compared to Col ($p \leq 0.05$, n=3). C, HPLC elute profiles of carotenoids from calli. Black line is for Col, blue line for *AtOR-L6* and red line for *AtOR*^{His}*-L39*. Peak 1 is lutein, and peak 2 is β-carotene.

AtOR^{His} overexpressing lines, however, accumulated much higher levels of carotenoids (Fig. 2B). The total carotenoid content in the *AtOR*^{His} overexpressing lines was up to 6.7 times of that in WT, whereas the level in *AtOR* overexpressing lines was up to 1.6 times. Noticeably, both *AtOR* and *AtOR*^{His} promoted β-carotene biosynthesis (Fig. 2B and 2C). In comparison to a ratio of 26% of β-carotene in the WT calli, *AtOR* increased the β-carotene ratio to 35%, whereas *AtOR*^{His} caused the calli to accumulate β-carotene at approximately 50% of the total carotenoids (Fig. 2B and 2C). The stimulation of β-carotene accumulation by *AtOR*^{His} was similar to that observed by *BoOR*$_{MUT}$ in the transgenic potato tubers (Li et al., 2012). The results suggest that *AtOR*^{His} confers carotenoid accumulation similarly as *BoOR*$_{MUT}$.

To further explore the implication of *OR*^{His} as a new genetic tool for crop nutritional quality improvement, a sorghum *OR* (*SbOR*) was mutagenized with R104H change (Supplemental Table S1) and overexpressed. Two pairs of *SbOR* and *SbOR*^{His} homozygous transgenic lines with comparable expression levels were

obtained (Supplemental Fig. S3B). While the calli from the *SbOR* lines had slight yellow color compared to the untransformed Arabidopsis calli, the *SbOR*^{His} lines showed intense orange calli (Supplemental Fig. S3A). Much higher levels of carotenoids were detected in the *SbOR*^{His} lines than the *SbOR* lines (Supplemental Fig. S3C). Like the cases found in the *AtOR*^{His} lines, *SbOR*^{His} induced carotenoid accumulation with β-carotene composing of approximately 50% of the total carotenoids (Supplemental Fig. S3C). These results indicate a conserved function of His substitution in an OR protein in inducing carotenoid accumulation among plant species.

Both Amino Acid Type and Position are Important and AtOR^{Ala} Functions Similarly as AtOR^{His}

To investigate whether His substitution at the conserved Arg position in an OR protein was essential for its role in promoting high levels of carotenoid accumulation, additional mutagenesis of *AtOR* was carried out. To see the amino acid type effect, a number of nucleotide substitutions were introduced into *AtOR* to replace the codon for the 90th Arg by SDM (Supplemental Table S1). Phenylalanine (Phe), lysine (Lys) and alanine (Ala) were chosen due to their amino acid properties. His is a polar aromatic amino acid with very unique chemical property, which makes it difficult to find a proper amino acid to replace it (Betts and Russell, 2003). Phe is also an aromatic acid and has some structural similarity with His. Lys was chosen for its similarity with Arg as normally these two amino acids could substitute each other without affecting the protein function. Ala was chosen for its small size and non-polar property (Pogliani, 1994).

The *AtOR*^{Phe}, *AtOR*^{Lys} and *AtOR*^{Ala} constructs generating R90F, R90K and R90A changes, respectively, were overexpressed in Arabidopsis. Approximately 20 independent transgenic lines for each construct were obtained. Calli from over 20 T2 transgenic seedlings for each line were induced and visually screened for the color phenotype. None of the *AtOR*^{Phe} lines showed the same intense orange calli as the *AtOR*^{His} lines (Supplemental Fig. S4A), indicating that Phe did not function as His in AtOR. *AtOR*^{Lys} containing Lys substitution also did not give the intensive orange calli (Supplemental Fig. S4A). By contrast, 7 out of 17 *AtOR*^{Ala} positive transgenic lines showed orange calli (Supplemental Fig. S4A). Analysis of these transgenic lines by

real-time qRT-PCR showed that *AtOR* in some of the $AtOR^{Ala}$, $AtOR^{Phe}$ and $AtOR^{Lys}$ transgenic lines were expressed at comparable levels (Supplemental Fig. S4B), indicating that the non-orange calli of $AtOR^{Phe}$ and $AtOR^{Lys}$ were not caused by low expression of the transgenes.

Two T3 homozygous $AtOR^{Ala}$ lines with high *AtOR* transcript and AtOR protein levels were generated (Fig. 3A and 3B). The calli of these two homozygous $AtOR^{Ala}$ lines exhibited dark orange color as those from $AtOR^{His}$ lines (Fig. 3C). They accumulated high levels of carotenoids with β-carotene accounting for approximately 55% of the total carotenoids (Fig. 3D). Both the total carotenoid level increase and the β-carotene to lutein ratio in the $AtOR^{Ala}$ lines were similar to those in the $AtOR^{His}$ transgenic lines. These findings suggest that the His substitution (R90H) in AtOR was not unique in inducing carotenoids accumulation. Ala with very different physiochemical properties to His was also able to change AtOR function.

In addition to the amino acid type, the amino acid position effect on the AtOR function was also tested. Both $AtOR^{His}$ and $BoOR_{MUT}$ mutations cause carotenoid overproduction phenotype. The sequence alterations were in the N-terminal region of OR proteins at amino acid position 90 in AtOR and 104 in BoOR. So two more His mutations of AtOR in this N-terminal region were produced to replace 88th Arg (R88H) and 102nd Arg (R102H) (Fig. 1A). None of the Arabidopsis transgenic lines overexpressing $AtOR^{His\text{-}88}$ and $AtOR^{His\text{-}102}$ showed orange calli as $AtOR^{His}$ and $AtOR^{Ala}$ calli despite similar transgene expression level in some lines (Supplemental Fig. S4A and S4B). This result indicates that His at 90th position is much more important than that at 88th or 102nd for $AtOR^{His}$ function. Together, these findings suggest that both the amino acid position and type are crucial for OR in conferring high levels of carotenoid accumulation.

Plant genomes contain another OR family protein named as OR-Like (Zhou et al., 2015). In Arabidopsis, AtOR-Like (At5g06130) shares 69% amino acid sequence identity with AtOR. AtOR-Like also has the conserved Arg at the 97th position (Supplemental Fig. S5). The Arg in AtOR-Like was mutated to His (R97H) and the $AtORL^{His}$ construct was overexpressed in Arabidopsis. T2 seeds of 30 independent transgenic lines were induced for callus formation. None of these $AtORL^{His}$ overexpressing lines showed orange calli as $AtOR^{His}$ and $AtOR^{Ala}$ (data not shown). It appears that the promotion of carotenoid overaccumulation by the His substitution

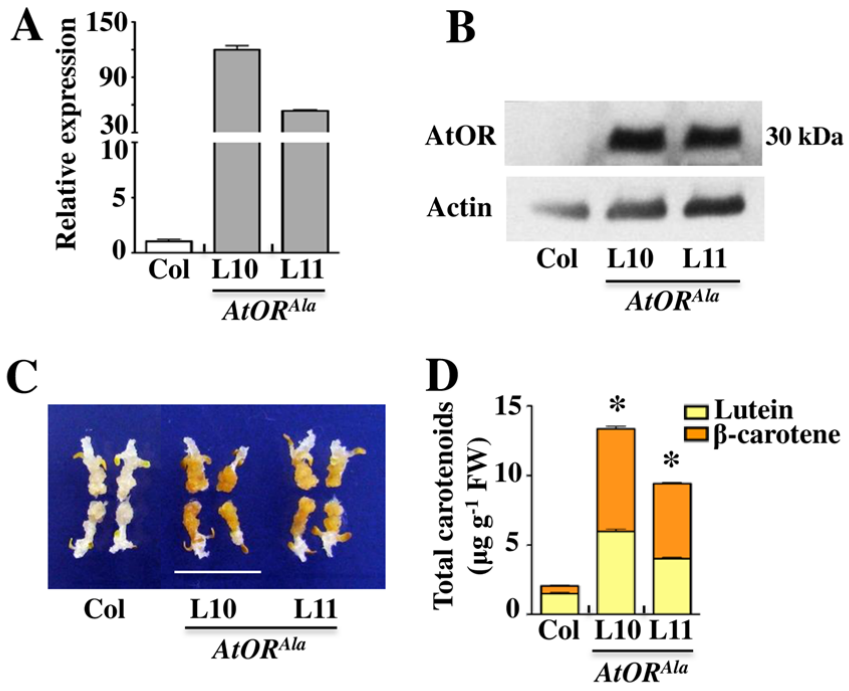

Figure 3. *AtOR*^{Ala} overexpressing transgenic lines accumulate high levels of total carotenoids in calli. A, Relative expression levels of *AtOR* tested by qRT-PCR in calli of Col and *AtOR*^{Ala} lines. B, AtOR protein levels in calli of Col and *AtOR*^{Ala} lines. C, Callus color of Col and *AtOR*^{Ala} lines. Bar = 1 cm. D, Total carotenoid levels in calli of Col and *AtOR*^{Ala} lines, measured by HPLC. Asterisks indicate significant difference when compared to Col ($p \leq 0.05$, n=3).

only works with *OR* but not with the *OR-Like* gene.

AtOR^{His} does not Dramatically Affect Carotenoid Metabolic Gene Expression or Alter Plastid Localization

Since both *AtOR*^{His} and *AtOR*^{Ala} shared similar effect on carotenoid accumulation, we focused our following studies on *AtOR*^{His} lines. To investigate whether *AtOR* and *AtOR*^{His} enhanced carotenoid accumulation by affecting carotenogenic gene expression, the transcript levels of carotenoid biosynthesis genes (Fig. 4A) as well as homologous genes of pepper plastid fusion and/or translocation factor (*Pfts;* Hugueney et al., 1995) in calli of these transgenic lines in comparison with WT were measured. RNA from the same callus samples used for carotenoids measurement was extracted. qRT-PCR analysis revealed that in general no dramatic difference in the expression of the core carotenoid biosynthetic genes was observed in both of the *AtOR* or *AtOR*^{His} overexpressing lines in comparison with the WT control although some genes in one line showed significant difference (Fig. 4B). The result was consistent with previous studies showing that the transcript levels of carotenoid

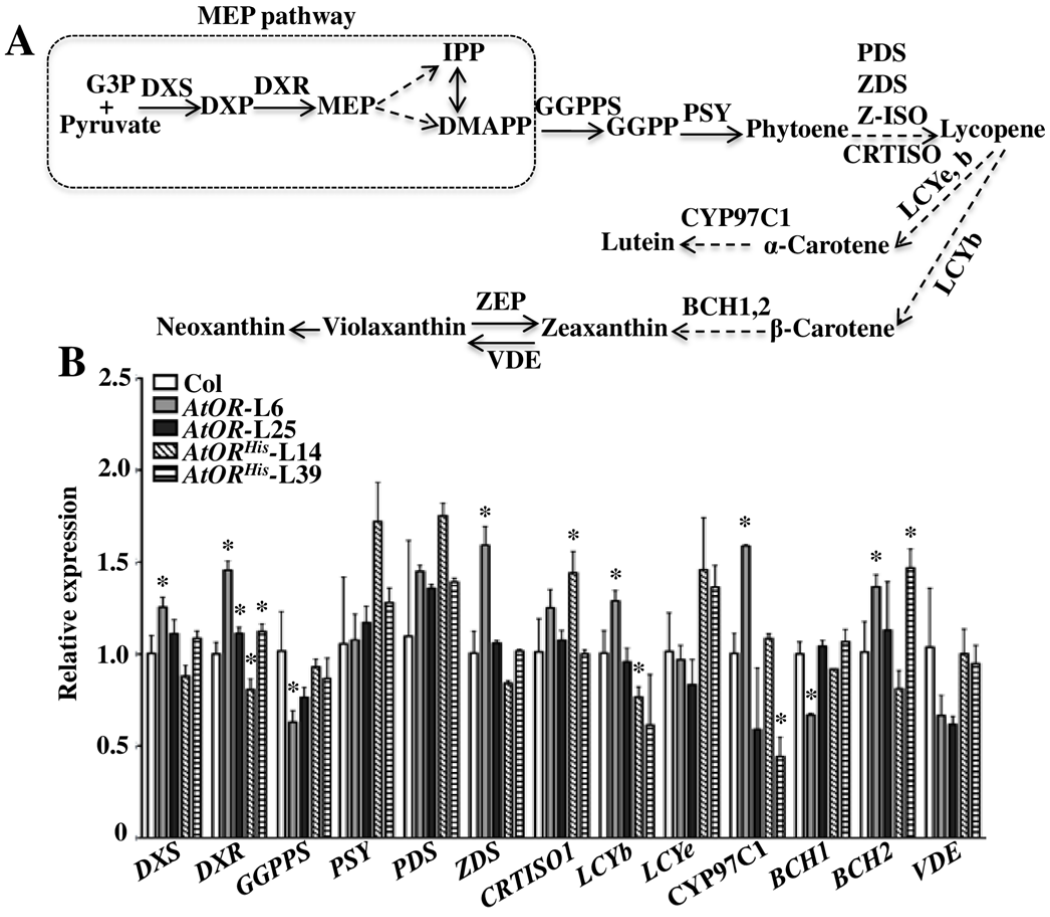

Figure 4. Expressions of carotenoid biosynthetic genes are not greatly affected in calli of *AtOR* and *AtOR*^{His} transgenic lines. A, Outline of carotenoid biosynthetic pathway. Solid arrows indicate one step, dashed arrows indicate multiple steps. G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate, GGPP, geranylgeranyl diphosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; Z-ISO, ζ-carotene isomerase; CRTISO, carotenoid isomerase; LCYe, lycopene ε-cyclase; LCYb, lycopene β-cyclase; CYP97C1, Cytchrome P450-type monooxygenase 97C; BCH1/2, β-carotenoid hydroxylase 1/2; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase. B, Relative expression level of carotenogenic genes in Col and *AtOR* and *AtOR*^{His} lines. Data are the average ± SE of three biological replicates with two technical trials. Asterisks indicate significant difference when compared to Col ($p \leq 0.05$, n=3).

biosynthetic genes were similar between white and orange cauliflower (Li et al., 2001). Thus, the *AtOR*^{His} induced carotenoid accumulation appeared not to be related to transcriptional regulation of carotenogenic genes. Moreover, examination of the homolog of pepper *Pfts* (*FtsH2)* along with its homologs in Arabidopsis revealed that the His substitution in *AtOR*^{His} did not significantly alter the *Pfts* homologous gene expression (Supplemental Fig. S6).

OR has been shown to localize in plastids (Lu et al., 2006). In order to find out the basis of *AtOR*^{His} action in regulating carotenoid accumulation, we first checked

whether this Arg to His substitution affected its subcellular localization. *AtOR^{His}* fused with *GFP* was expressed in Arabidopsis. The *AtOR^{His}-GFP* transgenic lines also had orange color calli (Supplemental Fig. S7A), indicating that the AtOR^{His}-GFP was functional. Examination of the GFP signals in the *AtOR^{His}-GFP* and *AtOR-GFP* transgenic lines showed that AtOR^{His} exhibited the same subcellular location in chloroplast as AtOR (Supplemental Fig. S7B). This result revealed that the His substitution in the *AtOR^{His}* mutant did not affect the plastidial location of the OR protein.

AtOR^{His} has Similar Function as AtOR in Post-Transcriptional Regulation of PSY protein level

Our recent study discovers that AtOR physically interacts with PSY and post-transcriptionally regulates PSY protein level (Zhou et al., 2015). To find out whether AtOR^{His} enhanced carotenoid accumulation with strong regulation of PSY, we examined the interaction between AtOR^{His} and PSY, and compared the effect of AtOR and AtOR^{His} in regulating PSY protein level and activity.

To test the interaction between AtOR^{His} and PSY, bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana* leaves were performed to investigate whether AtOR^{His} and PSY interaction occurred *in vivo* and the subcellular localization of their interaction. YFP signals were observed when AtOR^{His}-nYFP and PSY-cYFP were co-expressed in tobacco leaf epidermal cells, similar to the interaction between AtOR-nYFP and PSY-cYFP (Fig. 5A). Such interactions occurred in chloroplasts, consistent with the plastidial localizations of these proteins (Supplemental Fig. S7B) (Shumskaya et al., 2012). In addition, a split-ubiquitin membrane-based yeast two-hybrid (Y2H) system was used to check their interaction in vitro as OR contains two transmembrane domains (Lu et al., 2006). Both AtOR^{His} and AtOR were found to interact directly with PSY in the Y2H assay and showed similar interactions by quantification of the reporter gene lacZ via ortho-nitrophenyl-β-galactoside (oNPG) activity measurements (Fig. 5B). These results demonstrate that AtOR^{His} was also physically associated with PSY, showing similar interaction with PSY as AtOR.

To see whether AtOR^{His} mediated the enhanced carotenoid production via promoting high accumulation or activity of PSY, PSY protein levels and activity in calli of the *AtOR* and *AtOR^{His}* as well as the *AtOR^{Ala}* transgenic lines were compared

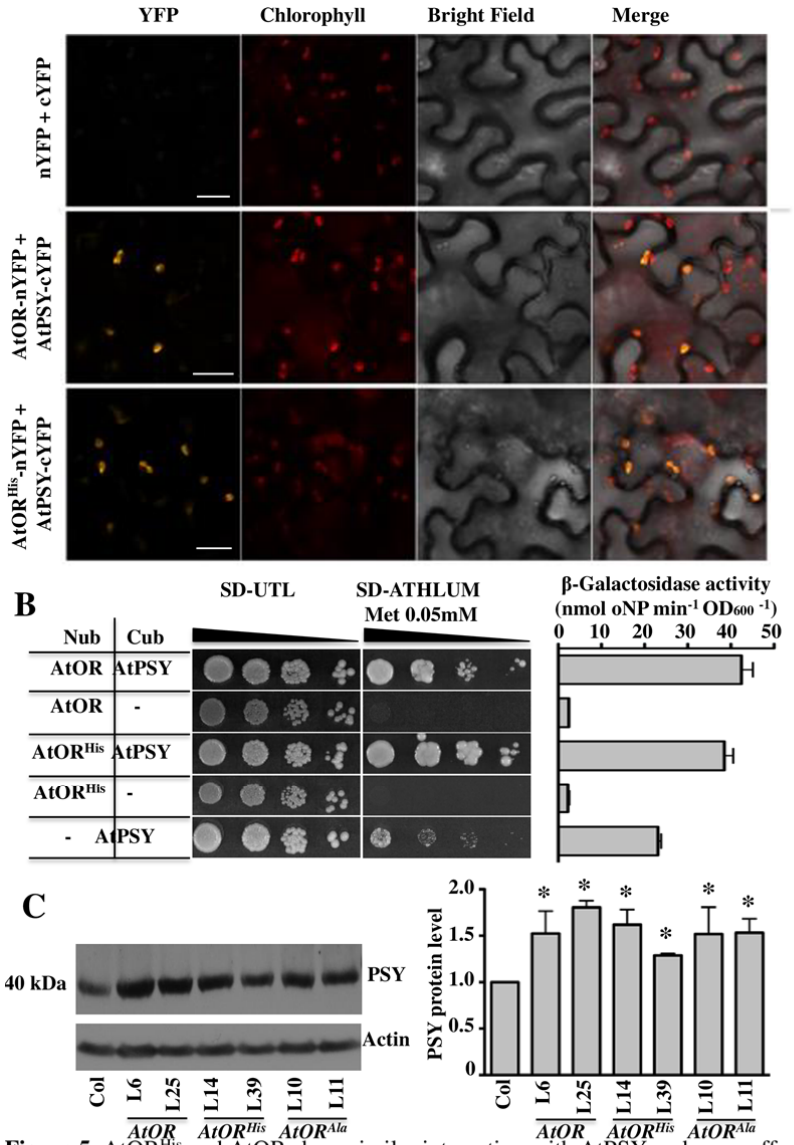

Figure 5. AtOR^{His} and AtOR share similar interaction with AtPSY and same effect on regulating AtPSY protein level. A, BiFC of AtOR or AtOR^{His} interaction with AtPSY. Vectors with nYFP (N terminal of YFP) and cYFP (C terminal of YFP), or AtOR-nYFP and AtPSY-cYFP, or AtOR^{His}-nYFP and AtPSY-cYFP were co-expressed in tobacco leaves. The fluorescence signals were detected by confocal microscope. Bar = 20 μm. B, Yeast-two-hybrid analysis of AtOR and AtOR^{His} interaction with AtPSY. Combinations of vectors with Nub (N terminal of ubiquitin), Cub (C terminal of ubiquitin), Nub-AtOR, Nub-AtOR^{His} or AtPSY-Cub were co-expressed in yeast cells. Yeast were grown on non-selective medium (SD-UTL) and selective medium (SD-ATHLUM, with 0.05 mM Met) with four series of 10 times dilution. For the β-galactosidase activity assay, yeast were cultured in liquid medium overnight and tested. C, Western of AtPSY in calli of Col, *AtOR, AtOR^{His}*, and *AtOR^{Ala}* lines. Actin protein level was used as loading control. Relative PSY protein levels were calculated from quantification of western band signals. Asterisks indicate significant difference when compared to Col ($p \leq 0.05$, n=3).

by western blot analysis and activity assay. Consistent with previous observation of enhanced PSY protein levels in leaves of the *AtOR* transgenic lines (Zhou et al., 2015), increased PSY protein abundance was observed in calli of the *AtOR* lines in comparison with WT (Fig. 5C). The *AtOR^{His}* and *AtOR^{Ala}* transgenic lines also

contained increased levels of PSY similarly as the *AtOR* lines (Fig. 5C). The result indicated that $AtOR^{His}$ and $AtOR^{Ala}$ post-transcriptionally regulated PSY protein abundance but did not promote high accumulation than *AtOR*. No enhanced PSY activities were observed in these transgenic lines (Supplemental Fig. S8). Thus, the dramatic differences in promoting carotenoid accumulation between *AtOR* and $AtOR^{His}$ suggest that the mutations might gain additional function to confer high levels of carotenoid production.

$AtOR^{His}$ Promotes Chromoplast Biogenesis

$BoOR_{MUT}$ is known to trigger chromoplast formation in cauliflower (Lu et al., 2006). To see whether $AtOR^{His}$ promoted chromoplast biogenesis, TEM analysis was carried out to examine plastids in calli of WT as well as the *AtOR* and $AtOR^{His}$ transgenic lines. In addition, plastids were compared with those from orange curd cauliflower mutant and calli of an Arabidopsis *PSY* overexpressing line (Maass et al., 2009). Plastids and plastoglobuli within these plastids were clearly seen in the calli of WT samples. Similar plastid structures but generally harboring larger plastoglobuli with higher electron density were observed in calli expressing *AtOR* than in WT calli (Fig. 6). Overexpression of *AtPSY* has been shown to result in carotenoid accumulation in calli of Arabidopsis transgenic lines (Maass et al., 2009). Like plastids in calli of *AtOR* lines, similar plastid structures but with much larger and higher electron density plastoglobuli were observed in calli of *AtPSY* lines (Fig. 6). By contrast, the $AtOR^{His}$ callus cells contained large plastids with dark sinuous membrane structures that generally lacked plastoglobuli (Fig. 6). The $AtOR^{His}$ induced plastids shared the typical membranous chromoplasts with those from the orange cauliflower mutant (Fig. 6) as shown previously (Paolillo et al., 2004). These findings suggest that $AtOR^{His}$ induced biogenesis of the same membranous chromoplasts as $BoOR_{MUT}$. Unlike $AtOR^{His}$, the similar plastid structures in cells of WT and *AtOR* calli indicate that *AtOR* did not confer chromoplast differentiation.

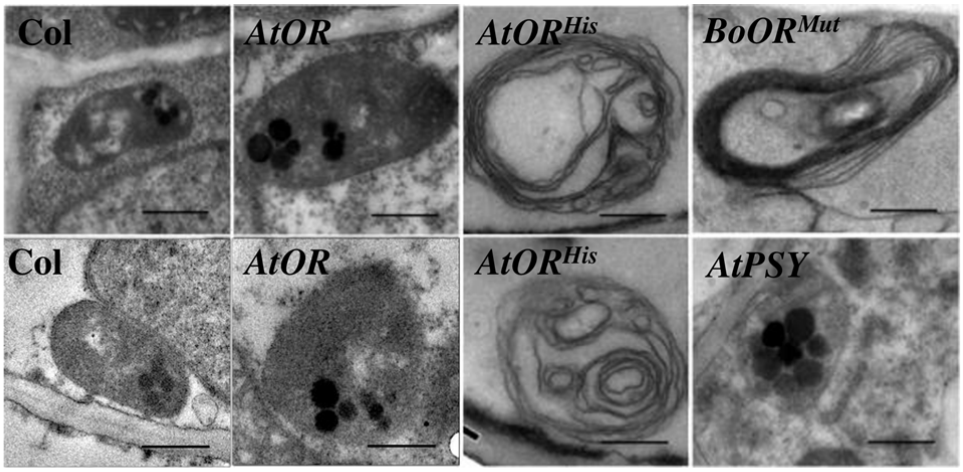

Figure 6. TEM images of plastids in Arabidopsis calli of Col, *AtOR*, $AtOR^{His}$ and *AtPSY* transgenic lines as well as in orange curd of $BoOR_{Mut}$ cauliflower mutant. Plastids with plastoglobuli were observed in calli of Col, *AtOR* and *AtPSY* transgenic lines. Characteristic membranous chromoplasts found in curd of $BoOR_{Mut}$ cauliflower mutant were observed in the $AtOR^{His}$ transgenic lines. Bar = 0.5 μm.

DISCUSSION

OR represents a novel class of regulatory genes in mediating carotenoid accumulation (Li and Yuan, 2013). Two natural mutations of *OR* are found to promote high levels of β-carotene accumulation in plants. The first one is $BoOR_{MUT}$ from orange curd cauliflower mutant. $BoOR_{MUT}$ contains a large retrotransposon insertion and produces three alternatively spliced transcripts, but none of them by itself induces massive carotenoid accumulation (Lu et al., 2006). The second one is the recently discovered *CmOR* in melon. The CmOR protein with a single amino acid difference of His or Arg distinguishes orange-flesh melon from white or green-flesh melon (Tzuri et al., 2015). In this study, we further demonstrated that this single Arg to His substitution was effective to change the functionality of a wild type OR in promoting carotenoid overaccumulation. In addition, we discovered that His was not the only amino acid that promoted carotenoid overproduction as Ala substitution also functioned similarly in enhancing carotenoid level. $AtOR^{His}$ was shown to posttranscriptionally regulate PSY protein abundance. However, its specific role in stimulating carotenoid overaccumulation laid on its unique ability to promote chromoplast biogenesis, which was documented in Arabidopsis calli. Our findings firmly imply the feasibility to create functional allele of *OR* in a crop species for nutritional quality improvement. In addition, since Arabidopsis normally does not contain a tissue with chromoplasts, the

demonstration of chromoplast formation establishes the Arabidopsis calli as a novel and effective system for functional study of genes involved in chromoplast biogenesis and carotenoid accumulation.

His/Ala Substitution of the Conserved Arg in an OR Protein Promotes High Levels of Carotenoid Accumulation

Both *AtOR*^{His} and *AtOR*^{Ala} were found to mediate high levels of carotenoid accumulation in Arabidopsis callus system, especially to promote the overproduction of β-carotene, the most potent provitamin A carotenoid. Clearly, the conserved Arg position in an OR protein is crucial for the OR^{His} or OR^{Ala} function in enhancing carotenoid accumulation. The His substitutions of Arg at nearby sites, such as at 88th or 102nd position of AtOR, were unable to induce carotenoid accumulation. While His substitution at the conserved Arg site of OR was important, His was not the only amino acid that altered carotenoid levels in the OR mutants. AtOR^{Ala} had a similar functional role as AtOR^{His}.

Histidine is one of the most common amino acids found in active or binding site of proteins (Betts and Russell, 2003). His mutation in an OR may change or create a new active center or give new interaction ability with some unknown proteins to this region, which is under investigation. Although Ala has quite different chemical properties from His, Ala may cause the same change in the active center or structure of AtOR as His. Unfortunately, there are presently no related 3-D structures available to allow for protein modeling that could shed light on these structural changes. Future protein 3-D structure analysis of the OR protein could be helpful in prediction of active center and important amino acid sites. The information could help explain the basis of the His and Ala substitutions, and produce new candidates for OR mutations to mediate carotenoid metabolism. Indeed, the *BoOR*$_{MUT}$ mutation does not affect the conserved Arg site.

Recent reports show that the WT OR proteins also have the capacity to induce carotenoid biosynthesis. Overexpression of a sweetpotato *OR* gene produces light yellow calli and increases carotenoid content in the transgenic sweetpotato roots (Kim et al., 2013; Park et al., 2014). Similarly, expression of *AtOR* produces clear color difference with approximately 2-fold increased carotenoid levels in the *AtOR-ZmPSY*

418 transgenic rice calli in comparison with the *ZmPSY* only transgenic lines (Bai et al.,
419 2014). Consistent with these reports, significantly increased carotenoid levels with
420 clearly visible color difference were also observed in this study when *AtOR* and *SbOR*
421 were overexpressed. However, the $AtOR^{His}$, $AtOR^{Ala}$ and $SbOR^{His}$ transgenic lines
422 produced much large quantities of carotenoids with intense orange calli in comparison
423 with the WT *AtOR* lines.
424
425 **Dual Function of $AtOR^{His}$ in Regulating PSY Protein Abundance and**
426 **Chromoplast Biogenesis**
427 Phytoene synthase catalyzes the rate-limiting step in the carotenoid
428 biosynthetic pathway. The transcript levels of *PSY* along with other carotenogenesis
429 genes was not dramatically affected by the overexpression of either *AtOR* or $AtOR^{His}$,
430 as shown in the other studies (Li et al., 2001; Lopez et al., 2008; Kim et al., 2013).
431 OR is recently discovered to serve as a major post-transcriptional regulator of PSY
432 protein level and activity (Zhou et al., 2015), providing an explanation for a WT
433 OR-mediated carotenoid biosynthesis. Here we found that $AtOR^{His}$ also directly
434 interacted with PSY in plastids, the place where carotenoids are synthesized. Similar
435 interactions of AtOR or $AtOR^{His}$ with PSY were observed in Y2H and BiFC assays,
436 showing that the His substitution in OR did not alter its interaction with PSY. As a
437 similar up-regulation of PSY protein levels was observed in the *AtOR* and $AtOR^{His}$
438 transgenic lines, it suggests that $AtOR^{His}$ does not promote higher accumulation of
439 PSY protein abundance than *AtOR*. Interestingly, no increased PSY enzyme activity
440 was detected between the callus samples of WT and transgenic lines, which might be
441 due to low sensitivity of activity assay for the *OR* callus samples. The fact that there
442 was no dramatic difference in PSY protein level and activity between the $AtOR^{His}$ and
443 *AtOR* lines indicates that $AtOR^{His}$ gains additional function to mediate carotenoid
444 overproduction.
445 Our previous studies show that $BoOR_{MUT}$ confers carotenoid accumulation in
446 chromoplasts (Li et al., 2001; Lu et al., 2006). Expression of the $BoOR_{MUT}$ transgene
447 in both white cauliflower and potato tubers leads to the biogenesis of chromoplasts
448 with enhanced carotenoid content (Lu et al., 2006; Lopez et al., 2008). Consistent
449 with these studies, here we demonstrated that $AtOR^{His}$ also functioned in inducing
450 chromoplast biogenesis. $BoOR_{MUT}$ is known to induce the formation of membranous
451 chromoplasts containing sinuous or scattered membranes in orange cauliflower

mutant (Paolillo et al., 2004). The typical and similar membranous chromoplasts were only observed in the *AtOR*^{His} samples, but not seen in the *AtOR* transgenic lines. In the *AtOR* samples, different type of plastids containing plastoglobuli was observed, which shared the same structures as those found in the WT controls. These findings support the specific function of AtOR^{His} in conferring chromoplast biogenesis.

Chromoplasts are the plastids that possess unique mechanisms to synthesize and store large quantities of carotenoids (Lu and Li, 2008; Egea et al., 2010; Li and Yuan, 2013). Biogenesis of chromoplasts enhances sink strength for carotenoid accumulation by effectively sequestering the newly synthesized carotenoids to stimulate continuous biosynthesis and stable storage within the plastids (Rabbani et al., 1998; Li et al., 2012; Kilambi et al., 2013). Increases of either chromoplast size or number have been found to contribute to enhanced carotenoid content in tomato pigment mutants (Liu et al., 2004; Kolotilin et al., 2007; Galpaz et al., 2008). As both *AtOR* and *AtOR*^{His} lines shared similar levels of OR expression and *AtOR*^{His} did not promote higher PSY activity than *AtOR* to control carotenoid biosynthesis, the much higher level of carotenoid accumulation in the *AtOR*^{His} transgenic lines was likely due to the specific capacity of *AtOR*^{His} in triggering chromoplast biogenesis, which provides enhanced plastid sink strength for carotenoid biosynthesis and storage.

The mechanism underlying *AtOR*^{His}-induced chromoplast biogenesis remains to be elucidated. Chromoplast biogenesis is associated with membrane proliferation, and remodeling of the internal membrane represents the most prominent change during chromoplast differentiation (Li and Yuan, 2013, Egea et al., 2010). Stacks of membranes in the chromoplasts of the OR mutants are evidenced by TEM from this and previous studies (Li et al., 2001, Paolillo et al., 2004). In addition, our ongoing study reveals that *AtOR*^{His} physically interacts with a number of proteins involved in the plastid protein import apparatus. Based on these studies, a possible mechanism of *AtOR*^{His} in controlling chromoplast biogenesis for carotenoid accumulation is postulated to modulate plastid membrane proliferation and/or chromoplast preprotein import via regulating proteins in the plastid protein import machinery. Future elucidation of the specific mechanism underlying *OR*-regulated chromoplast biogenesis could open up a new area of plastid biology.

An Effective Arabidopsis Callus System for Functional Study of Genes Involved in Chromoplast Biogenesis and Carotenoid Accumulation

Arabidopsis normally does not contain chromoplasts in its tissues, which hinders the progress in the studies of genes, signaling, and metabolic processes involved in chromoplast biogenesis. Previously we observed that calli derived from cauliflower $BoOR_{MUT}$ plants accumulate β-carotene with intense orange color (Li et al., 2006) and $BoOR_{MUT}$ confers chromoplast formation in different plant species (Lu et al., 2006; Lopez et al., 2008). The present study produced Arabidopsis OR^{His} transgenic lines that contained intense orange color with carotenoid accumulation in the calli. Electron microscopy revealed the presence of membranous chromoplasts similar to chromoplasts found from curd of the orange cauliflower mutant (Li et al., 2001; Paolillo et al., 2004). Thus, we generated Arabidopsis lines that contain a tissue with chromoplast development program.

Callus is very responsive to genes involved in carotenoid biosynthesis with different color phenotypes and has been used as a model plant system to study the gene functions in carotenoid metabolism. Maize callus transformed with *PSY* from different plant species shows marked differences in color intensity and was used to identify the most efficacious *PSY* for Golden Rice 2 production (Paine et al., 2005). Arabidopsis callus expressing *AtPSY* gives intense orange color and is used to investigate the effect of PSY on carotenoid sequestration (Maass et al., 2009). Expression of a bacterial *PSY* in different citrus genotypes yields calli of various color with diverse natural carotenoid patterns and provides information to interpret the common appearance of a favored β,β-pathway following an increased expression of *PSY* in plants (Cao et al., 2012). Recently, rice embryogenic callus is developed for rapid functional characterization of genes involved in carotenoid biosynthesis following validating it with known and unknown function genes (Bai et al., 2014).

The Arabidopsis OR^{His} or OR^{Ala} callus system could be used as a unique model for rapid functional study of genes involved in chromoplast biogenesis in addition to carotenoid metabolism. By overexpressing or suppressing the expression of associated genes in these lines, their effects on chromoplast development and carotenoid accumulation can be examined in the callus system following quick visual screening of callus phenotypes. As Arabidopsis can be easily transformed with a large number of gene constructs, this Arabidopsis callus system likely provides an effective and relatively high throughput approach to functionally characterize candidate genes for their involvement in chromoplast biogenesis and carotenoid metabolism.

OR^{His} and OR^{Ala} Serve as New Genetic Tools for Crop Nutritional Quality Improvement

Carotenoids, especially β-carotene, are indispensable to human nutrition and provide the primary dietary source for vitamin A biosynthesis. Low levels of carotenoids in major food crops contribute to the global prevalence of vitamin A deficiency, which leads to significant efforts to generate carotenoid enriched crops either through biotechnology or traditional breeding. By expression of *PSY* or a mini-pathway of carotenoid biosynthesis, numerous transgenic crops with enhanced carotenoid levels are produced (Fraser et al., 2002; Ducreux et al., 2005; Paine et al., 2005; Diretto et al., 2007; Naqvi et al., 2009, Welsch et al., 2010). Selection of favorable alleles that alter carotenoid metabolic flux toward β-carotene results in the breeding of orange maize with high β-carotene content (Harjes et al., 2008; Yan et al., 2010). In contrast to modification of catalytic activity of the pathway, the discovery of *OR* provides an alternative and complementary approach for carotenoid enhancement in food crops via enhancing the storage sink strength (Li and Van Eck, 2007). Moreover, *OR* promotes continuously increased carotenoid accumulation in the $BoOR_{MUT}$ transgenic potato tubers during postharvest storage (Li et al., 2012).

The demonstration that OR^{His} and OR^{Ala} promoted high levels of carotenoid accumulation suggests their potential as new genetic tools for crop carotenoid enhancement. In contrast to $BoOR_{MUT}$ that contains a large retrotransposon insertion with none of the alternatively spliced transcripts showing comparable effect in enhancing carotenoid level as $BoOR_{MUT}$ allele (Lu et al., 2006), OR^{His} or OR^{Ala} contains one or two nucleotide change required for the His or Ala substitution in a WT *OR* gene. Such a change is sufficient to alter *OR* function for carotenoid accumulation. In addition, while homozygous $BoOR_{MUT}$ causes dwarf phenotype in cauliflower (Li et al., 2001), OR^{His} exhibits no deteriorating effects on plant growth and development in melon as well as in the transgenic Arabidopsis.

OR is highly conserved among diverse plant species. Genome editing has emerged as potent new tools that enable site specific modifications of a genome (Sander and Joung, 2014). As one or two nucleotide change alters the *OR* function, the rapidly developing genome editing techniques can be utilized to induce the site-specific mutagenesis of a crop *OR* gene. Moreover, genetic diversity among crop

552 species provides an additional resource for discovering the functional variants of *OR*.
553 Indeed, the variable sites of *OR* mutations in $OR^{His/Ala}$ and $BoOR_{MUT}$ suggest the
554 potential to identify new functional alleles from genetic variation of crops. Thus, in
555 addition to biotechnology approach, there is potential to introduce functional alleles of
556 *OR* into staple crops through traditional breeding to improve crop nutritional quality.
557
558
559 **MATERIALS AND METHODS**
560
561 **Plant Materials and Callus Induction**
562 *Arabidopsis thaliana* plants were grown in a computer-controlled growth
563 chamber under 14 h light and 10 h dark at 23 °C. To induce calli, Arabidopsis seeds
564 were surface sterilized in 20% bleach for 15 min and sown on plates of callus
565 induction SDC medium (4.30 g L^{-1} MS basal salt, 0.1% [V/V] Gamborg's vitamin, 1.0
566 g L^{-1} MES, 3% [W/V] sucrose, 0.5 mg L^{-1} 2,4-D, 2 mg L^{-1} indole-3-acetic acid, 0.5
567 mg L^{-1} 2-isopentenyladenine, adjusted pH to 5.8 by 1M KOH, and 0.4% [W/V]
568 Phytagel) as described (Maass et al., 2009). Following stratification for 2 days in dark
569 at 4℃, the seeds were germinated under light (16h light/8h dark) at 23℃ for 5 days
570 and grown in dark for 2 weeks in a growth incubator. Calli were collected, frozen in
571 liquid nitrogen, and stored at -80℃ until use.
572
573 **Site-Directed Mutagenesis (SDM) and Arabidopsis Transformation**
574 To identify the conserved Arg in the OR proteins, the protein sequences of
575 AtOR (At5g61670), AtOR-like (At5g06130), and SbOR (Sb04g033280)
576 (http://www.plantgdb.org/SbGDB/) were aligned with melon CmOR (Tzuri et al.,
577 2015). SDM primers specific to change Arg to other amino acids in these OR proteins
578 were designed using Agilent QuickChange Primer Design program
579 (http://www.genomics.agilent.com/primerDesignProgram.jsp) (Supplemental Table
580 S2). *AtOR*, *AtOR-like* and *SbOR* in pCR™2.1 vector were mutagenized using
581 QuikChange II XL Site-Directed Mutagenesis Kit according to the instruction manual
582 of manufacturer (Agilent Technologies, cat# 200521). The *OR* mutant variants along
583 with their WT versions were cloned into pCAMBIA1300S with CaMV 35S promoter
584 (Zhou et al., 2011) to generate constructs producing AtOR R90A, R90H, R90K, R90F,

R88H, and R102H as well as SbOr R104H.

The plasmid constructs were transferred into *A. tumefaciens* GV3101 by electroporation and introduced into Arabidopsis by floral dip (Clough and Bent 1998). The transformed T1 seeds were screened for positive transformants on plates of MS medium containing 1% [W/V] sucrose, 30 mg L^{-1} hygromycin, 50 mg L^{-1} carbenicillin, and 0.6% [W/V] agar. Several independent homologous T3 lines for each construct were selected and used for phenotype and further analysis.

Carotenoid extraction and analysis

Carotenoids from Arabidopsis calli (200 mg) were extracted and analyzed essentially following the method as described by Lopez et al. (2008). Carotenoids were identified by their characteristic absorption spectra and their retention times in comparison with standards and published spectra, and quantified using a β-carotene calibration curve (Li et al., 2012). Samples were analyzed in triplicate with three biological replicates.

Protein Extraction, Western Blot Analysis, and PSY Activity Assay

Total proteins from Arabidopsis calli were extracted in cold room with a phenol-based protein extraction protocol (Yang et al., 2007). Powdered calli (200 mg) was added with 600 μL extraction buffer (0.5M Tris-HCl pH7.5, 50 mM EDTA, and 0.1M KCl with 10 mM DTT and 1mM PMSF added just before use) and 600 μL Tris-EDTA saturated phenol (pH 6.7). The sample mixtures were vigorously shaken for 30 min and centrifuged at 12,000 rpm for 30 min. Proteins in the upper phenolic phase were collected, precipitated with 5 volumes of 0.1M NH_4AOC dissolved in methanol overnight, and centrifuged at 4,000 rpm for 40 min at 4℃. Pellets were washed with pre-chilled methanol, air dried, and dissolved in 100 μL solution containing 7M urea and 2M thiourea. Protein concentration was measured by adding 1 μL of sample to 499 μL protein assay buffer (Bio-Rad).

For western blot analysis, 50 μg of total proteins were separated by 10% SDS-PAGE gels and blotted onto PVDF membrane (EMD Millipore). Membranes were blocked overnight and included with primary anti-OR antibody (Lu et al., 2006) or anti-PSY antibody followed by with secondary goat anti-rabbit IgG antibody (Bio-rad). Signals were detected using the ECL Prime Western Blotting Detection

Reagent following the manufacturer's manual (GE Healthcare). AtActin was detected in same membrane using anti-Actin antibody (Sigma, A0848) for loading control. PSY western signal was quantified with Actin as internal control using ImageJ (http://www.di.uq.edu.au/sparqimagejblots).

The *in vitro* PSY activity assay was performed with 400 µg plastid membrane proteins isolated from callus samples as detailed (Zhou et al., 2015). Samples were analyzed with two technical trials and two biological replicates.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from 100 mg calli using Trizol reagent (Life technologies). The cDNA was synthesized from 1 µg RNA with oligodT using Superscript III reverse transcriptase (Invitrogen). Real-time qRT-PCR was performed using SYBR Green Supermix (Bio-rad) and gene-specific primers (Supplemental Table S2) in ABI 7500 Real-Time PCR system (Applied Biosystems). *AtUBQ1* gene was used as nuclear gene internal control and relative expression levels were calculated using the $^{\Delta\Delta}$Ct method (Lyi et al., 2007). Values reported represent the averages of three biological replicates with two technical trials.

Y2H and BiFC Assay

Y2H assay was done using the split-ubiquitin system (Zhou et al., 2015)**.** CDS of *AtPSY* without its transit peptide and stop codon was cloned into pMetYCgate vector to produce fusion protein with Cub (C terminal of ubiquitin), while *AtOR* and *AtOR*^{His} CDSs without their transit peptides were cloned into pNXgate to generate fusion protein with Nub (N terminal of ubiquitin). The pMetYCgate constructs were transformed into yeast strain THY.AP5, and pNXgate to stain THY.AP4. Mating of these two strains brought these two vectors to the diploid yeast cells, which were then inoculated to a selective medium (SD-ATHLUM with 0.05 mM Met) for protein interaction tests. β-galactosidase activity in yeast was assayed in triplicate and calculated as nmol oNP min^{-1} OD600^{-1} as described with minor modification to add 20 mg/L Met to the medium (Zhou et al., 2015).

BiFC (Bimolecular fluorescence complementation) was performed as described (Zhou et al., 2015). Briefly, *AtPSY* CDS without stop codon was cloned to the vector pUC-SPYCE to make a fusion protein with cYFP. *AtOR* and *AtOR*^{HIS} CDSs

without stop codons were cloned to pUC-SPYNE to produce fusion protein with nYFP. Plasmids in *Agrobacteria* GV3101 was used to inject four week old tobacco leaves. Two days after injection, the leaves were observed under confocal microscope (Leica TCS SP5 Laser Scanning Confocal Microscope). The YFP and chlorophyll fluorescence was detected with excitation light wavelength at 488 nm and emission filter for YFP at 520 to 560 nm and for chlorophyll at 620 to 680 nm. Images were taken and processed using Leica LAS AF software.

Transmission electronic Microscopy (TEM)

Arabidopsis calli of Col, *AtOR*, *AtOR*^{His} and *AtPSY* were analyzed by TEM following the protocol described (Cao et al., 2012). The calli were fixed, followed by dehydration and embedding. The embedded samples were sectioned with Leica UC6 ultramicrotome to get ultrathin sections, which were then stained and analyzed by transmission electron microscope (HITACHI H-7650).

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673 **Figure legends**

674

675 **Figure 1.** Characterization of *AtOR* and *AtOR^{His}* overexpressing transgenic lines. A,
676 Alignment of partial OR protein sequences from melon CmOR (G and O with green-
677 and orange-flesh fruit, respectively), Arabidopsis AtOR, cauliflower BoOR, sorghum
678 SbOR, wheat TaOR, rice OsOR, maize ZmOR, and tomato SlOR. Protein accession
679 numbers are given in Supplemental Figure S1 legend. The solid triangle shows the
680 position of Arg to His mutation and the empty triangle indicates the 88th or 102th Arg
681 (R) in AtOR. B, Real-time qRT-PCR analysis of relative expression level of *AtOR* in
682 calli of Col, *AtOR* lines L6 and L25, and *AtOR^{His}* lines L14 and L39. C, Western blot
683 analysis of AtOR protein levels in calli of Col, *AtOR* and *AtOR^{His}* lines. Actin protein
684 levels were analyzed as loading control. D, Phenotype of 22 days old plants of Col,
685 *AtOR* and *AtOR^{His}* lines grown in soil. E, Phenotype of 35 days old plants grown in
686 soil.

687

688 **Figure 2.** *AtOR^{His}* overexpressing transgenic lines accumulate high levels of total
689 carotenoids in calli. A, Calli of Col, *AtOR* and *AtOR^{His}* lines. Bar =1 cm. B, Total
690 carotenoid levels of Col, *AtOR* and *AtOR^{His}* lines measured by HPLC. Data are the
691 average ± SE of three biological replicates. Asterisks indicate significant difference
692 when compared to Col ($p \leq 0.05$, n=3). C, HPLC elute profiles of carotenoids from
693 calli. Black line is for Col, blue line for *AtOR-L6* and red line for *AtOR^{His}-L39*. Peak 1
694 is lutein, and peak 2 is β-carotene.

695

696 **Figure 3**. *AtOR^{Ala}* overexpressing transgenic lines accumulate high levels of total
697 carotenoids in calli. A, Relative expression levels of *AtOR* tested by qRT-PCR in calli
698 of Col and *AtOR^{Ala}* lines. B, AtOR protein levels in calli of Col and *AtOR^{Ala}* lines. C,
699 Callus color of Col and *AtOR^{Ala}* lines. Bar = 1 cm. D, Total carotenoid levels in calli
700 of Col and *AtOR^{Ala}* lines, measured by HPLC. Asterisks indicate significant difference
701 when compared to Col ($p \leqslant 0.05$, n=3).

702

703 **Figure 4**. Expressions of carotenoid biosynthetic genes are not greatly affected in
704 calli of *AtOR* and *AtOR^{His}* transgenic lines. A, Outline of carotenoid biosynthetic
705 pathway. Solid arrows indicate one step, dashed arrows indicate multiple steps. G3P,

706 glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP,
707 2-C-methyl-D-erythritol-4-phosphate; IPP, isopentenyl diphosphate; DMAPP,
708 dimethylallyl diphosphate, GGPP, geranylgeranyl diphosphate; DXS,
709 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate
710 reductoisomerase; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene
711 desaturase; ZDS, ζ-carotene desaturase; Z-ISO, ζ-carotene isomerase; CRTISO,
712 carotenoid isomerase; LCYe, lycopene ε-cyclase; LCYb, lycopene β-cyclase;
713 CYP97C1, Cytchrome P450-type monooxygenase 97C; BCH1/2, β-carotenoid
714 hydroxylase 1/2; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase. B,
715 Relative expression level of carotenogenic genes in Col and *AtOR* and $AtOR^{His}$ lines.
716 Data are the average ± SE of three biological replicates with two technical trials.
717 Asterisks indicate significant difference when compared to Col ($p \leqslant 0.05$, n=3).
718
719 **Figure 5**. $AtOR^{His}$ and AtOR share similar interaction with AtPSY and same effect on
720 regulating AtPSY protein level. A, BiFC of AtOR or $AtOR^{His}$ interaction with AtPSY.
721 Vectors with nYFP (N terminal of YFP) and cYFP (C terminal of YFP), or
722 AtOR-nYFP and AtPSY-cYFP, or $AtOR^{His}$-nYFP and AtPSY-cYFP were
723 co-expressed in tobacco leaves. The fluorescence signals were detected by confocal
724 microscope. Bar = 20 μm. B, Yeast-two-hybrid analysis of AtOR and $AtOR^{His}$
725 interaction with AtPSY. Combinations of vectors with Nub (N terminal of ubiquitin),
726 Cub (C terminal of ubiquitin), Nub-AtOR, Nub-$AtOR^{His}$ or AtPSY-Cub were
727 co-expressed in yeast cells. Yeast were grown on non-selective medium (SD-UTL)
728 and selective medium (SD-ATHLUM, with 0.05 mM Met) with four series of 10
729 times dilution. For the β-galactosidase activity assay, yeast were cultured in liquid
730 medium overnight and tested. C, Western of AtPSY in calli of Col, *AtOR*, $AtOR^{His}$,
731 and $AtOR^{Ala}$ lines. Actin protein level was used as loading control. Relative PSY
732 protein levels were calculated from quantification of western band signals. Asterisks
733 indicate significant difference when compared to Col ($p \leqslant 0.05$, n=3).
734
735 **Figure 6**. TEM images of plastids in Arabidopsis calli of Col, *AtOR*, $AtOR^{His}$ and
736 *AtPSY* transgenic lines as well as in orange curd of $BoOR_{Mut}$ cauliflower mutant.
737 Plastids with plastoglobuli were observed in calli of Col, *AtOR* and *AtPSY* transgenic

738 lines. Characteristic membranous chromoplasts found in curd of *BoOR*$_{Mut}$ cauliflower
739 mutant were observed in the *AtOR*^{His} transgenic lines. Bar = 0.5 µm.
740

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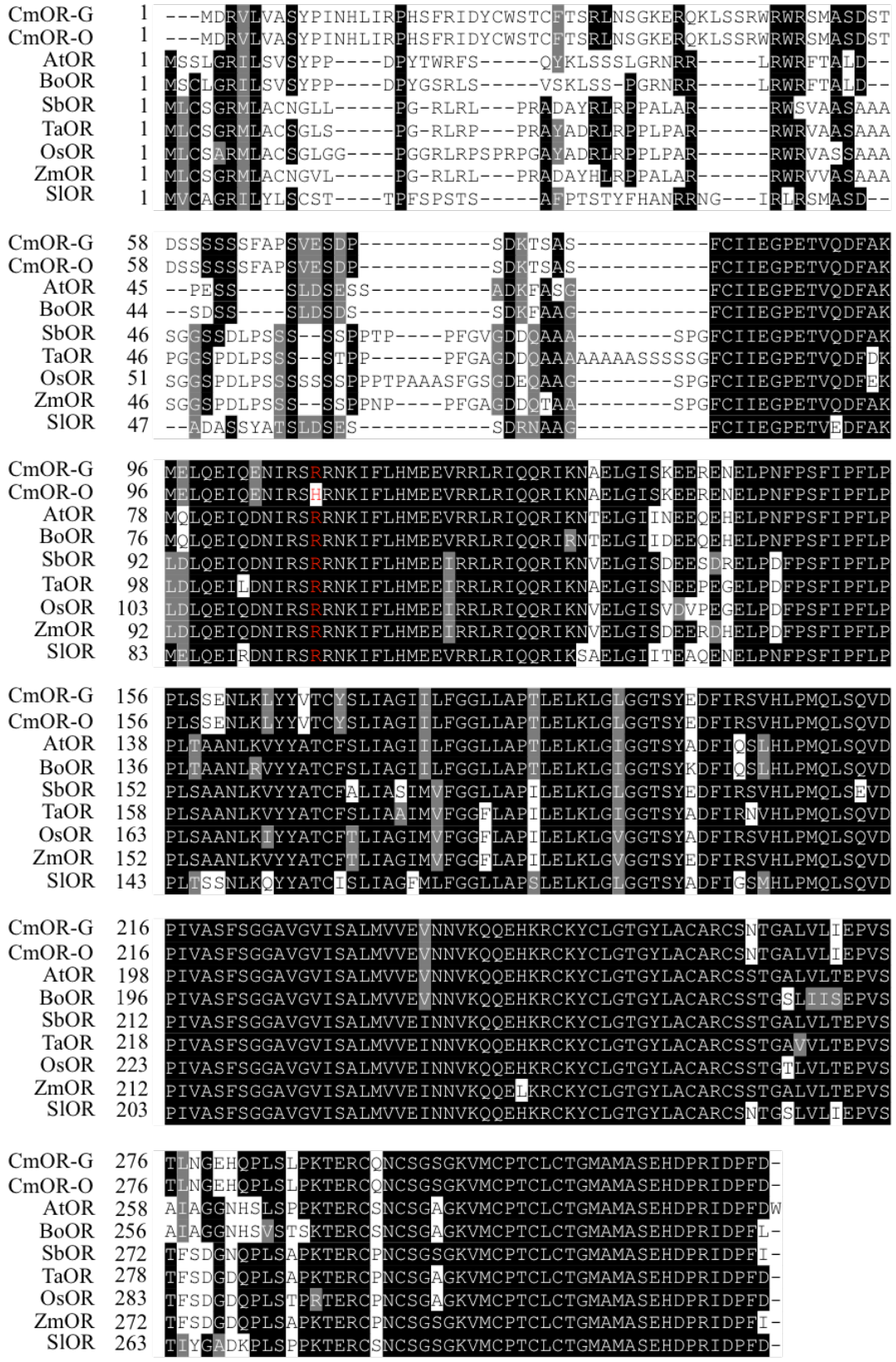
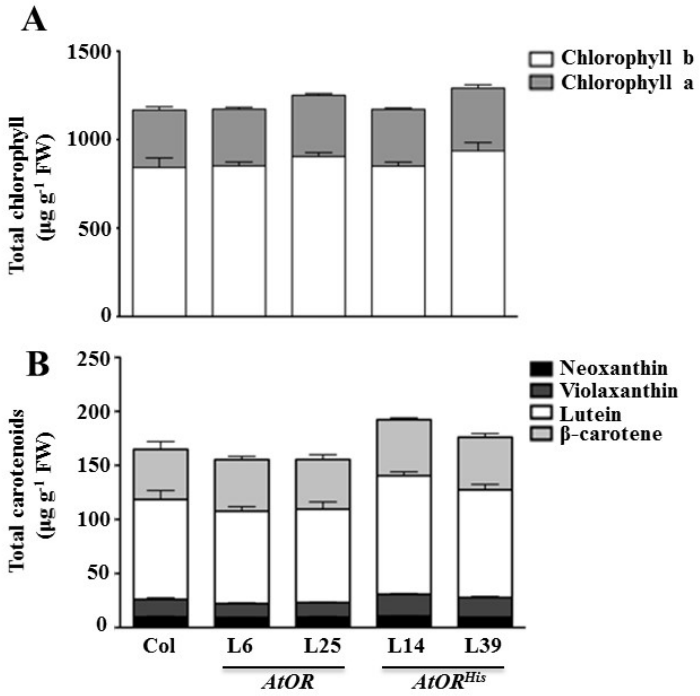
Supplemental Figures and Tables

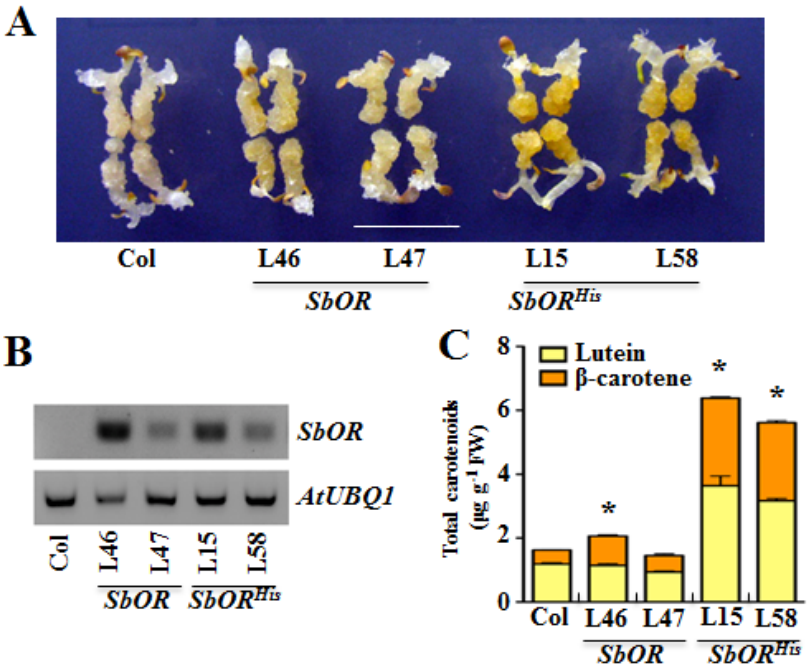


Supplemental Figure S1. Alignment of full length of OR proteins from CmOR-G

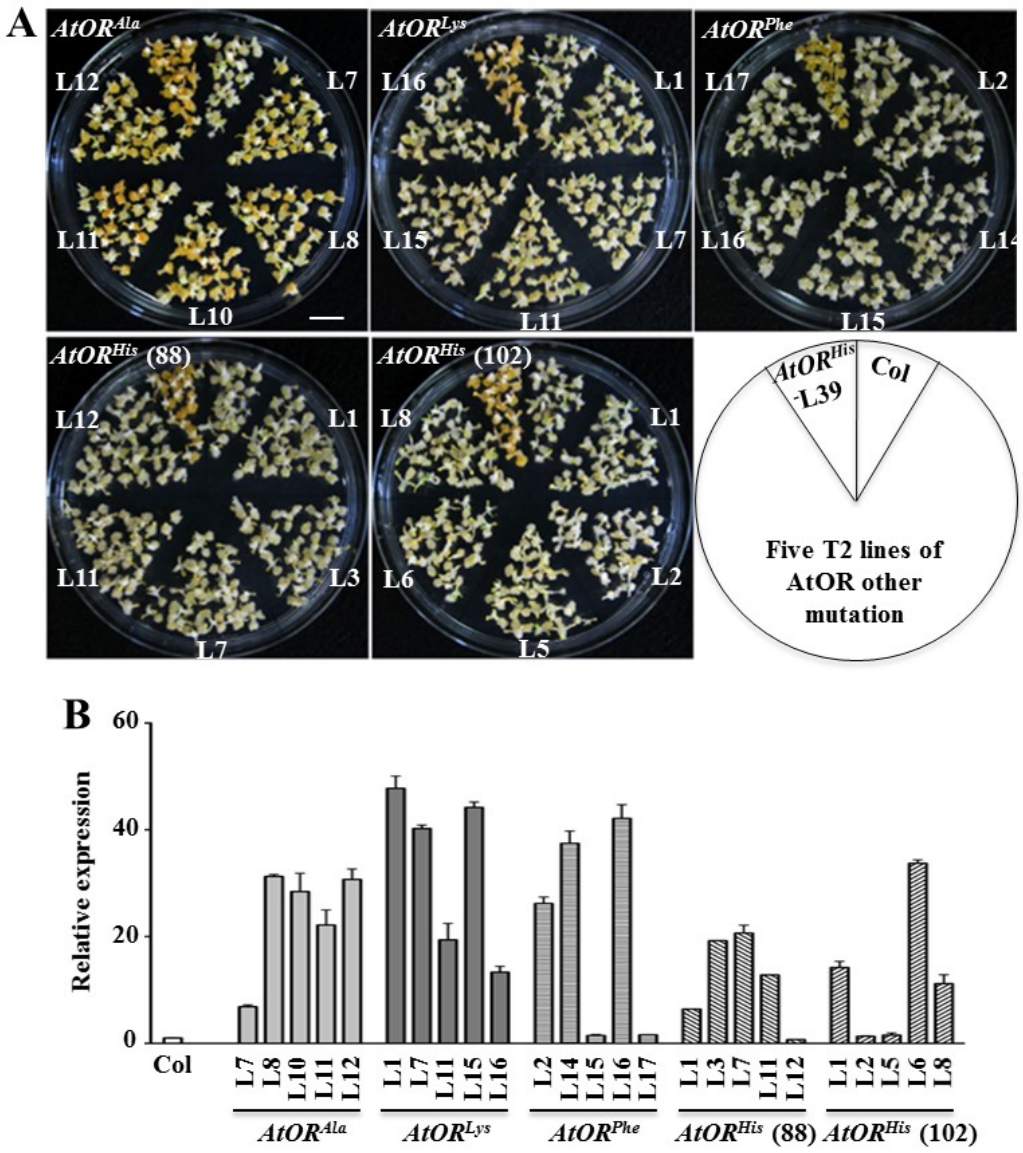
(*Cucumis melo*, XP_008467325, with green-flesh fruit), CmOR-O (with orange-flesh fruit), AtOR (*Arabidopsis thaliana*, NP_200975), BoOR (*Brassica oleracea*, ABH07405), SbOR (*Sorghum bicolor*, XP_002452827) and TaOR (*Triticum aestivum*, Traes_6AL_9CEDE1FC0.1, http://plants.ensembl.org/Triticum_aestivum/Info/Index). OsOR (*Oryza sativa*, NP_001047593), ZmOR (*Zea mays*, ACN31016), SlOR (*Solanum lycopersicum*, XP_004235195). The red letter in the protein sequence is the site of Arg to His mutation.



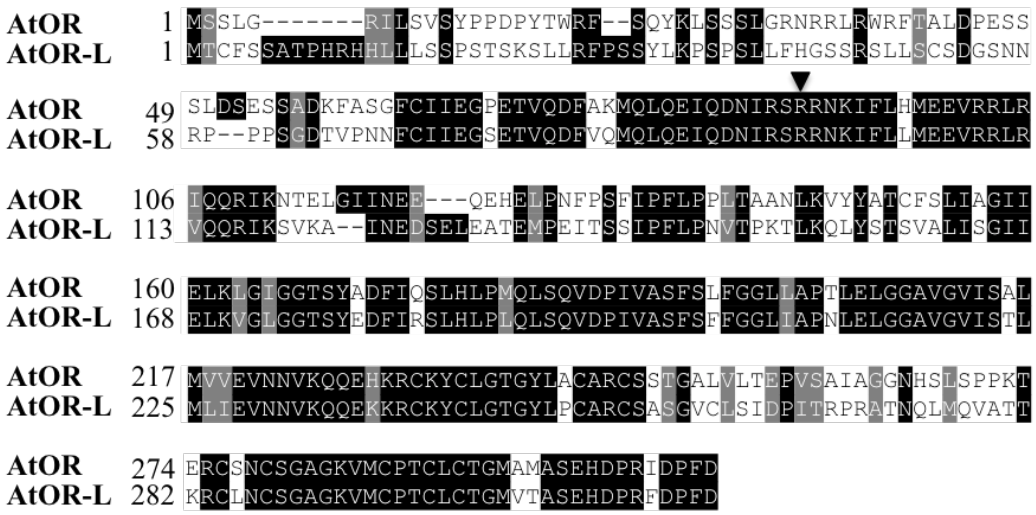
Supplemental Figure S2. Leaf chlorophyll and total carotenoid contents in the *AtOR* and *AtOR*^{His} lines were similar to those in Col. A. Leaf chlorophyll content in 20 days old plants. Leaf powders (20 mg) ground in liquid nitrogen were extracted with 1 ml of 80% acetone, and the supernatants were analyzed spectrophotometrically at 545 and 663 nm. B. Leaf carotenoid content in 20 days old plants. Values are the average ± SE of three biological replicates.



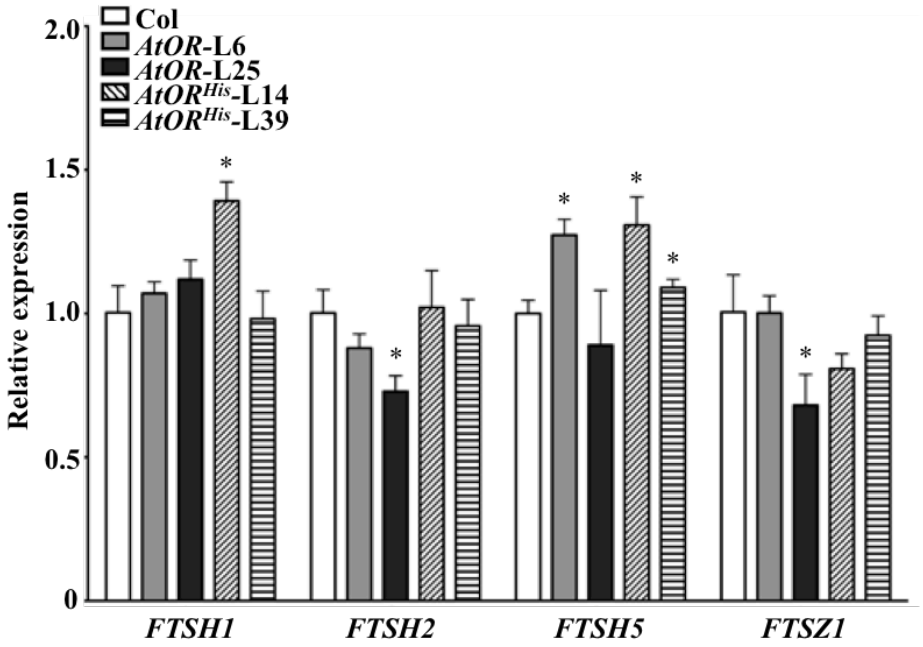
Supplemental Figure S3. *SbOR*^{His} overexpressing transgenic lines accumulate high level of total carotenoids in Arabidopsis calli. A, Callus color of Col, *SbOR* and *SbOR*^{His} lines. Bar = 1 cm. B, *SbOR* transcript level in calli shown in A. *AtUBQ1* was used as control. C, Total carotenoid content in calli of Col, *SbOR* and *SbOR*^{His} lines, measured by HPLC. Asterisks indicate significant difference when compared to Col ($p \leq 0.05$, n=3).



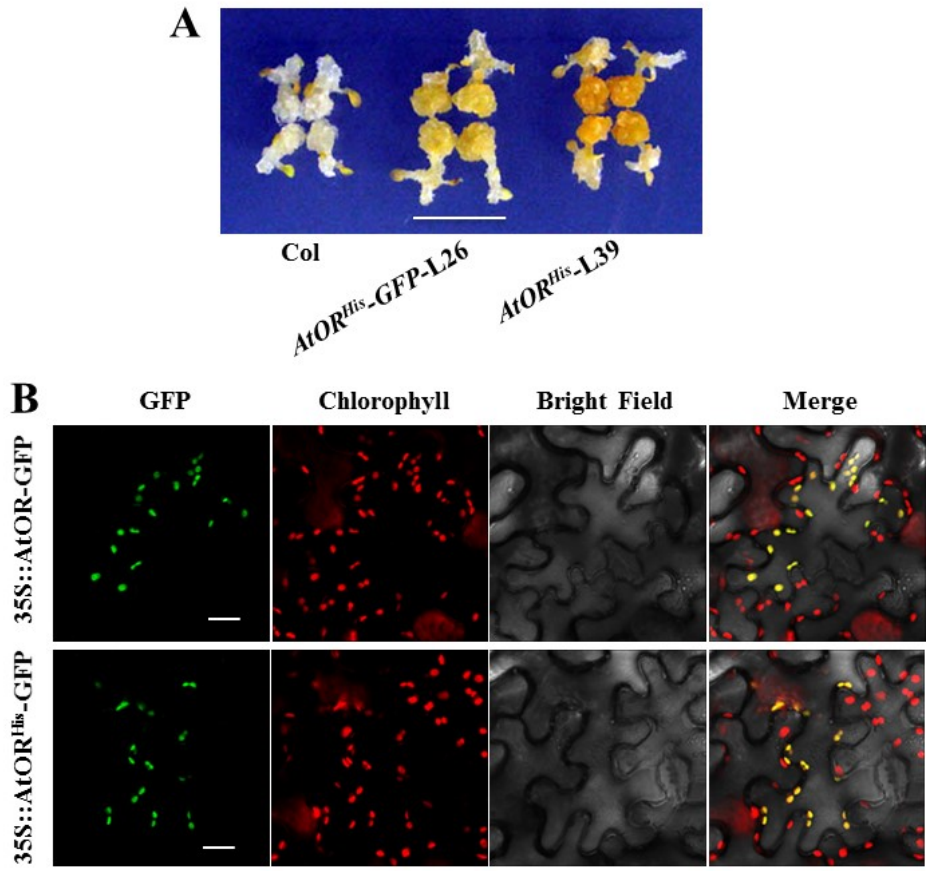
Supplemental Figure S4. Phenotype of T2 calli of *AtOR* mutation lines and their *AtOR* expression level. A, Callus color of five T2 lines of $AtOR^{Ala}$, $AtOR^{Lys}$, $AtOR^{Phe}$, $AtOR^{His}$*(*R88H*)* and $AtOR^{His}$*(*R102H*)*, with Col and $AtOR^{His}$-L39 as control. Only $AtOR^{Ala}$ T2 lines had intense orange calli. Bar = 1 cm. B, Relative expression level of *AtOR* in calli shown in A. For each T2 line, all calli were pooled to extract RNA, and *AtOR* expression was detected by real-time qRT-PCR. Values are the average ± SE of three biological replicates with two technical trials.



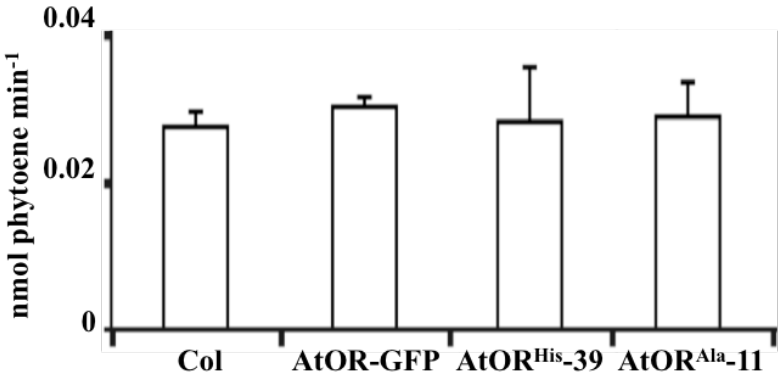
Supplemental Figure S5. Alignment of the protein sequences of AtOR and AtOR-L (AtOR-Like). Amino acids sequence of AtOR (At5g61670) and AtOR-Like (At5g06130) were aligned by ClustalW and then colored by Boxshade. The triangle shows the amino acid position of Arg to His mutation, which is the 90th in AtOR, and 97th in AtOR-Like.



Supplemental Figure S6. Relative expression levels of Arabidopsis *Pftf* homologous genes *FtsH1*, *2*, *5* and *FtsZ1* in AtOR and AtOR^{His} calli were similar to those of Col. Callus cDNA used for carotenogenic genes expression were used here.



Supplemental Figure S7. AtOR^{His} has the same subcellular localization in chloroplasts as AtOR. A, Calli of Col, *AtOR^{His}-GFP*-L26 and *AtOR^{His}*-L39. B, Subcellular localization of AtOR-GFP and AtOR^{His}-GFP. Vectors of AtOR-GFP and AtOR^{His}-GFP were injected into tobacco leaves, and the fluorescence signals were observed using confocal microscope. Bar = 20 μm.



Supplemental Figure S8. PSY activity in Col, AtOR, AtOR^{His} and AtOR^{Ala} calli. Plastids were isolated from calli as described in Zhou et al. (2015). After lysis and subfractionation, 400 µg membrane protein was used per reaction.

Supplemental Table S1. Site-directed mutagenesis in *AtOR* and *SbOR*

Mutation	Mutation site in CDS	Condon change	Amino acid change
AtOR R90H	269-270th, GA to AT	C**GA** to C**AT**	Arg to His
AtOR R90A	268-269th, CG to GC	**CG**A to **GC**A	Arg to Ala
AtOR R90K	268-269th, CG to AA	**CG**A to **AA**A	Arg to Lys
AtOR R90F	268-270th, CGA to TTC	**CGA** to **TTC**	Arg to Phe
AtOR R88H	263-264th, GA to AT	C**GA** to C**AT**	Arg to His
AtOR R102H	305-306th, GG to AT	C**GG** to C**AT**	Arg to His
SbOR R104H	311-312th, GC to AT	C**GC** to C**AT**	Arg to His

Supplemental Table S2. Primer sequences used in this study

	Name	Sequence
For CDS cloning	AtOR-F	AAAAGGTACCATGTCATCTTTGGG
	AtOR-R	AAAACTGCAGTCAATCGAAAGGGTCG
	AtOR-GFP-F	CTCGAGATGTCATCTTTGGGTAGG
	AtOR-GFP-R	CCATGGAATCGAAAGGGTCGATACG
	SbOR-F	GGTACCAATGCTCTGCTCCGGCCGCAT
	SbOR-R	TCTAGATCAAATAAACGGATCGATCCGG
	AtOR2-F	GGTACCATGACGTGCTTCTCTTCTGC
	AtOR2-R	TCTAGACTAATCGAAAGGGTCAAAGCG
For SDM	AtOR-R90H-SDM-F	ATGTGCAAGAAGATCTTGTTTCGATGGCTTCGAATGTTATCTTGAATC
	AtOR-R90H-SDM-R	GATTCAAGATAACATTCGAAGCCATCGAAACAAGATCTTCTTGCACAT
	AtOR-R90A-SDM-F	GATTCAAGATAACATTCGAAGCGCACGAAACAAGATCTTCTTGCAC
	AtOR-R90A-SDM-R	GTGCAAGAAGATCTTGTTTCGTGCGCTTCGAATGTTATCTTGAATC
	AtOR-R90K-SDM-F	GAGATTCAAGATAACATTCGAAGCAAACGAAACAAGATCTTCTTGCACAT
	AtOR-R90K-SDM-R	ATGTGCAAGAAGATCTTGTTTCGTTTGCTTCGAATGTTATCTTGAATCTC
	AtOR-R90F-SDM-F	AGAGATTCAAGATAACATTCGAAGCTTCCGAAACAAGATCTTCTTGCACATGG
	AtOR-R90F-SDM-R	CCATGTGCAAGAAGATCTTGTTTCGGAAGCTTCGAATGTTATCTTGAATCTCT
	AtOR-R88H-SDM-F	ATGCAATTACAAGAGATTCAAGATAACATTCATAGCCGACGAAACAAGATC
	AtOR-R88H-SDM-R	GATCTTGTTTCGTCGGCTATGAATGTTATCTTGAATCTCTTGTAATTGCAT
	AtOR-R102H-SDM-F	CTTGCACATGGAAGAGGTGCATAGGCTAAGAATACAACAACG
	AtOR-R102H-SDM-R	CGTTGTTGTATTCTTAGCCTATGCACCTCTTCCATGTGCAAG
	AtOR2-R97H-SDM-F	CAGCTTCAGGAAATCCAAGATAACATAAGGAGTCATCGCAATAAGATATTTCTCC
	AtOR2-R97H-SDM-R	GGAGAAATATCTTATTGCGATGACTCCTTATGTTATCTTGGATTTCCTGAAGCTG
	SbOR-R104H-SDM-F	GAGATTCAAGATAACATTAGGAGCCATCGAAACAAGATTTTCTTGCATATGG
	SbOR-R104H-SDM-R	CCATATGCAAGAAAATCTTGTTTCGATGGCTCCTAATGTTATCTTGAATCTC

For qRT-PCR	AtOR-F663	CGTAAAGCAGCAAGAGCACA
	AtOR-R812	GTTTTGGGCGGTGATAGAGA
	AtUBQ1-F221	TCTACACTTCATCTTGTGTTGAGGC
	AtUBQ1-R500	CACTGAAACAAGAAAAACAAACCCT
	SbOR-F790	GCACCAATCCTGGAACTTAAAC
	SbOR-R894	CACAATGGGATCTACCTCACTC
	DXS-F	ATGACCAGGTGGTCCATGAT
	DXS-R	TGTGAAAAGCTCCACACTGC
	DXR-F	ACTGGCCTAGCACCAGAAGA
	DXR-R	TGCGATAAACATCGAAACGA
	GGPPS11-F	GCCAATCTGAACCATCCTCT
	GGPPS11-R	ACGGAGAGGAACAGCTGAAT
	PSY-F	ATGATCGATGCGGTGAAGTTTGCG
	PSY-R	TGAAGCATTTGGCCCATCCACAAG
	PDS-F	CGAGATGCTGACATGGCCAGA
	PDS-R	GTCGGTCACGCGCTCAGGTA
	ZDS-F	CCATCGTCACGAGGCCTAGAA
	ZDS-R	TGTGTATGAACCGGCGAGGA
	CCR2-F	ACTCATTGAACTCCCTGGAAC
	CCR2-R	GGATCCTTTATGTACTTCCGAGC
	LCYb-F	AGGATGAACCATTCCTGCTG
	LCYb-R	ATTGAGGAAGACGAGCGTTG
	LCYe-F	ACCTTAGCTCGAAAGTTGACAG
	LCYe-R	CACCAACTTCGTATTGCAAGAG
	LUT1-F	CGAAATCCCAATCATGGGTCA
	LUT1-R	GCACCTCCGAGGAGATCAGC
	BCH1-F	GGCACGCTTCTCTATGGAATATGCATGA
	BCH1-R	GAATCCATAAGAGAGGAGACCAATCGCT

BCH2-F	TGGAAGAACGGAAACAAAGC
BCH2-R	AGCTTGTCGTGCTTTCTGGT
VDE-F	ACCGCTCCGCTGTTGCTAAA
VDE-R	TGGCAATGCACTTTGCGAGT
FTSH1-F	TCACGGGTGCTGACTTACAAAACC
FTSH1-R	TCATCCCCAAAGATCACCTCCTCTG
FTSH2-F	AGGAGAACGCTCCTTGCATTGTC
FTSH2-R	TGCCGGCATGAACCTTGAGAATATC
FTSH5-F	CCATGAGGCTGGGCATGCTTTAG
FTSH5-R	CGCTGTTGCCATCGAGTAATCCTTC
FTSZ1-F	GTCGATGCGATTGAGGTGTTCCTTC
FTSZ1-R	TGCCAAGCCCACGAGTTAAAAGTTC