Use of the cauliflower *Or* gene for improving crop nutritional quality

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Abstract. Carotenoids are a group of pigments that are essential to human diets. An increasing interest in carotenoids as a nutritional source of vitamin A and health-promoting compounds has prompted the recent progress in metabolic engineering of carotenogenesis in food crops. Current strategies have been mainly focused on manipulating genes encoding carotenogeic enzymes. In many cases, it is difficult to reach the desired levels of carotenoid enhancement. In this chapter, we briefly summarize the recent progress on our understanding of carotenoid biosynthesis. We describe the isolation of a novel gene, the *Or* gene, from a high-β-carotene orange cauliflower mutant. The *Or* gene encodes a plastid-targeted protein containing a cysteine-rich zinc finger domain and appears to be plant-specific. The insertion of a *copia*-like LTR retrotransponson in the *Or* gene confers high levels of carotenoid accumulation in the normally low-pigmented tissues. Rather than directly regulating carotenoid biosynthesis, the *Or* gene controls carotenoid accumulation by inducing the formation of chromoplasts, which provide a metabolic sink to sequester and deposit carotenoids. Examination of the *Or* transgenic potato tubers confirms that the *Or*-induced carotenoid accumulation is associated with the formation of a metabolic sink. Thus, the *Or* gene offers a new molecular tool to complement current approaches for nutritional enhancement in agriculturally important crops.

Keywords: carotenoids, cauliflower, *Or* gene, β-carotene, chromoplast, metabolic sink, crop nutrition enhancement.

Introduction

Carotenoids are a large group of natural pigments produced by all chlorophyll-containing photosynthetic organisms, some bacteria, and many species of fungi. Carotenoids are indispensable to animals and humans in providing precursors for vitamin A synthesis. Vitamin A fulfills many physiological functions in humans such as vision, reproduction, and cell proliferation [1]. Its deficiency, which affects over 140–250 million children under five years of age (World Health Organization), remains one of the most noticeable nutritional problems in many parts of the world. In addition, carotenoids as antioxidant compounds provide additional health benefits. Dietary intake of carotenoid-rich foods has been implicated in reducing the

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incidence of cancer and cardiovascular diseases [2,3]. Some carotenoids also offer protection against age-related eye diseases, such as macular degeneration, the leading cause of age-related blindness. Apparently, developing carotenoid-enriched food plants will be the most effective approach to maximize their nutritional and health potential. Therefore, elucidation of the mechanisms underlying carotenoid biosynthesis and metabolic manipulation of carotenogenesis in food crops has become one of the most interesting research areas in current plant science and biotechnology.

Most of our knowledge on carotenoid biosynthesis in plants has been derived from studies of *Arabidopsis thaliana*, cyanobacteria, tomato, and a few other plant species. In the past decades, a complete set of genes and enzymes of carotenoid biosynthesis has been isolated and characterized by a combination of molecular, genetic, biochemical, and genomic approaches [4–6]. The availability of a large number of carotenogenic genes from bacteria and plants along with the information on carotenoid metabolism has opened the door to genetic engineering of carotenoid content and composition in food plants to benefit humans [5,7–9]. So far, the popular strategy is to overexpress plant or bacterial genes that control the committed steps of carotenoid biosynthesis in food crops. Golden Rice is one of the best-known examples in using this strategy for improving crop nutritional value. In the late generation of Golden Rice 2, overexpression of genes in a mini-carotenoid biosynthetic pathway leads to the accumulation of up to 37 μg/g total carotenoids in rice endosperm, a level adequate to provide most of the recommended dietary allowance of vitamin A for children in an average daily consumption of rice [10]. However, in many other cases, alteration of expression of carotenoid biosynthetic genes is insufficient to drastically enhance carotenoid levels in transgenic plants [5], or even leads to unexpected phenotypic changes [11]. This makes it essential to search for novel genes controlling plant carotenogenesis and to gain a better understanding of the mechanisms underlying carotenoid biosynthesis and accumulation in plants.

Cauliflower *Or* gene represents a novel gene mutation. It causes many low-pigmented tissues of the plant, most noticeably the edible curd and the shoot meristem to accumulate high levels of β-carotene and turns these tissues orange [12–14]. The *Or* gene has been isolated by a map-based cloning strategy [15]. This gene appears to represent a regulatory gene in controlling carotenoid accumulation. It functions in increasing the sink capacity rather than altering the expression of genes involved in carotenoid biosynthesis [14–16].

In this chapter, we briefly summarize the progress on carotenoid biosynthesis and metabolic engineering of carotenoids in food crops for improving their nutritional value. We describe the isolation of the *Or* gene from the orange cauliflower mutant and elucidation of its functional role in controlling carotenoid accumulation. The successful use of the *Or* gene to

enhance carotenoid levels in transgenic potato tubers demonstrates that manipulation of sink formation offers an alternative strategy to increase carotenoid content in food crops.

Carotenoid biosynthesis in plants

Carotenoids are comprised of carotenes and their oxidized form, xanthophylls. The carotenoid biosynthetic pathway (Fig. 1) has been long established by labeling and inhibition studies and mutant analysis, but isolation of carotenogenic genes has only been achieved during the past two decades [4]. Carotenoids are mainly C_{40} isoprenoids synthesized *de novo* essentially in all kinds of plastids except proplastids in plants [17]. Like other plastidic isoprenoids, carotenoid biosynthesis starts with the synthesis of isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) in plastids. Three IPP molecules are added to DMAPP to produce geranylgeranyl pyrophosphate (GGPP), the immediate precursor for biosynthesis of carotenoids, as well as tocopherols, gibberellins, phytol, taxol, etc.

The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP to produce phytoene catalyzed by phytoene synthase. Subsequently, two related enzymes phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) catalyze phytoene into lycopene. Two *cis-trans* isomerases of Z-ISO and CRTISO are needed to convert poly-*cis*-compounds into all-*trans* forms in plants. A single enzyme carotene desaturase (crtI) in bacteria confers the same reactions. Lycopene is the branching point of this pathway and cyclized to yield α- and β-carotene by lycopene ε-cyclase and/or lycopene β-cyclase. These carotenes can undergo hydroxylation and epoxidation reactions to form xanthophylls, such as lutein, zeaxanthin, violaxanthin, and neoxanthin. Xanthoxin, the product that results from violaxanthin/neoxanthin cleavage by 9-*cis*-epoxycarotenoid dioxygenase, provides the direct substrate for phytohomone ABA synthesis [18,19].

Regulation of carotenoid biosynthesis in plants

The composition and relative abundance of various carotenoids in plants are remarkably conserved in green tissues, but vary in a broad range in non-green tissues or organs, indicating that plants have developed complex regulatory mechanisms controlling carotenogenesis. Several processes affect carotenoid accumulation, including those that determine the supply of isoprenoid precursors, the catalytic activity of the pathway, and metabolic turnover or conversion. In addition, carotenoid sequestration is also known to play an important role.

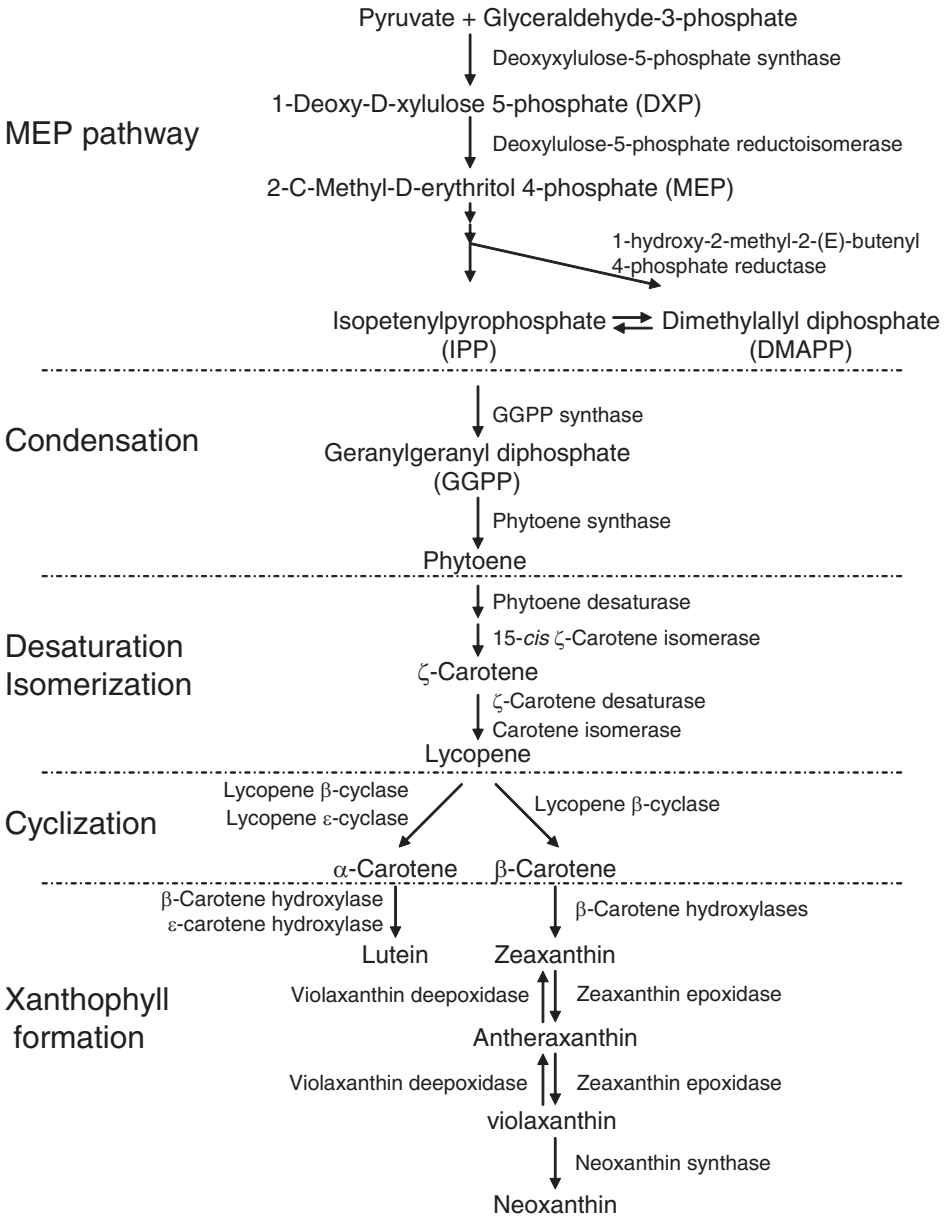

Fig. 1. Carotenoid biosynthetic pathway in plants.

Supply of isoprenoid precursors

GGPP serves as a common substrate for biosynthesis of carotenoids, gibberellins, phytol, and other isoprenoid compounds. Alteration of the precursor pool size into carotenogenic pathway is likely to affect carotenoid

levels [20]. For example, the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway synthesizes IPP and DMAPP for the production of plastidic isoprenoids. The null mutation of *CLA1*, which encodes the first enzyme in the pathway, produces less precursors, leading to lack of chlorophylls and carotenoid pigments with a retardation of chloroplast development and an albino phenotype [21,22]. Alteration of the expression of this gene results in different levels of carotenoids as well as other isoprenoids such as chlorophylls, tocopherols, abscisic acid, and gibberellins [23]. Hydroxymethylbutenyl diphosphate reductase (HDR) catalyzes the simultaneous synthesis of IPP and DMAPP in the last step of the MEP pathway. During tomato fruit ripening and *Arabidopsis* seedling de-etiolation, increased carotenoid production is associated with enhanced expression of the *HDR* gene [24]. These studies show the effect of availability of isoprenoid precursors for carotenoid accumulation.

Regulation of carotenoid biosynthetic genes

Among the carotenoid biosynthetic enzymes, *PSY*, which catalyzes the first committed step of carotenoid biosynthesis, is believed to control isoprenoid flux into the pathway [25]. Therefore, *PSY* is a highly regulated point in carotenoid biosynthesis. Light has been shown to have a strong effect on carotenogenesis in photosynthetic tissue while developmental cues affect carotenoid content in flowers and fruits. Studies show that both light and development cues regulate *PSY* expression. Light affects carotenoid biosynthesis by increasing expression of *PSY* in green pepper leaves, mustard, and *Arabidopsis* seedlings [26,27]. Comparison of *Arabidopsis* wild-type and phytochrome mutants reveals that up-regulation of *PSY* expression is mediated by phytochrome [27]. Indeed, the full-length promoter of *Arabidopsis PSY* mediates positive responses towards different light qualities [28]. On the other hand, *PSY* is strongly induced during flower and fruit development in tomato plants, implying unlike that in the leaves, *PSY* is mainly regulated by developmental signals in those tissues [29].

PDS, the second enzyme in carotenoid biosynthetic pathway, is also strongly induced during flower and fruit development. In tomato, high levels of PDS expression are found in organs and tissues where chromoplasts are formed [30]. More importantly, carotenogenesis requires coordinated expression of multiple carotenoid biosynthetic genes as demonstrated during citrus fruit maturation [31].

Metabolic turnover or conversion

Metabolic turnover of carotenoids not only helps to maintain the steady level of carotenoids in plants, but also produces important signaling and accessory apocarotenoid molecules, such as phytohormone ABA and flavor volatiles

beta-ionone, pseudoionone, and geranylacetone for a variety of biological processes [32]. A family of carotenoid cleavage dioxygenases (CCDs) catabolizes oxidative cleavage of carotenoids. The first CCD gene was cloned through analysis of a maize ABA deficient mutant *viviparous 14* [19]. Subsequently, more CCD genes were cloned and identified from other plant species, including *Arabidopsis*, tomato, petunia, cowpea, avocado, and citrus [33].

Analysis of enzymatic activities reveals that CCDs display complicated substrate and cleavage site specificity. In *Arabidopsis*, there are nine members of CCDs, of which AtCCD1 cleaves a variety of carotenoids symmetrically at the 9,10 or 9′,10′ double bond to form a dialdehyde and one or two C_{13} products, whereas AtCCD7 protein catalyzes a specific 9,10 cleavage of beta-carotene to produce apo-beta-carotenal and beta-ionone [34]. Similarly, CitCCD1 protein cleaved β-cryptoxanthin, zeaxanthin, and all-*trans*-violaxanthin at the 9,10 and 9′,10′ positions and 9-*cis*-violaxanthin at the 9′,10′ positions [35]. Another class of CCDs is 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), including VP14, which specifically cleaves 11,12 double bonds [18,35,36]. The third class of CCDs cleaves at the 7,8 double bonds of zeaxanthin [37]. In addition, lycopene cleavage dioxygenase, which is involved in sequential conversion of lycopene into bixin, cleaves lycopene at the 5,6 and 5′,6′ positions [38].

A body of evidence shows that oxidative cleavage of carotenoids is induced under environmental stresses [18,36,39]. Additionally, circadian rhythm has been shown to affect carotenoid catabolism [40]. Moreover, developmental cues also play an important role. The transcript of *LeCCD1* gene, which could confer the formation of the important flavor volatiles in tomato fruit, is accumulated during the ripening process [32].

Carotenoid sequestration

One of the major carotenoid storage organelles within plant cells is chromoplasts. High levels of carotenoid accumulation in chromoplasts contribute the red, orange, and yellow color found in many flowers, fruits, and vegetables. While chromoplasts frequently derive from fully developed chloroplasts, as seen during fruit ripening of tomato and pepper, they also arise from other non-photosynthetic plastids, such as in carrot and squash [41]. Chromoplasts develop lipoprotein-sequestering substructures to sequester and retain large quantity of carotenoids [42]. Based on their ultrastructures, the carotenoid-sequestering structures within chromoplasts fall into globular, crystalline, membranous, fibrillar, and tubular types [43]. The various compositions of carotenoids, polar lipids, and proteins may determine the type of these structures.

Most of what we have learned about the carotenoid sequestration comes from studies of globular, fibrilar, or tubular-type chromoplasts. Several genes encoding carotenoid-associated proteins in chromoplasts have been cloned

and identified [42]. The first *fibrillin* (*fib*) gene was isolated from pepper fruits [44]. Shortly after, another cDNA encoded the chromoplast-specific carotenoid-associated protein (CHRC) was cloned from *Cucumis sativus* corollas [45]. Both proteins carry a hydrophobic hairpin domain, which might be involved in interactions with carotenoids. More homologs have continually been characterized from different types of plastids and species [43,46], suggesting that they are involved in sequestration of not only carotenoids but also other hydrophobic compounds.

The expression of genes encoding carotenoid-associated proteins is well associated with chromoplast development and carotenoid accumulation. The *fib* gene was shown to be strongly induced during fruit ripening [44]. The *ChrC* message was detected only in cucumber corollas, where its level increased in parallel to flower development [45]. Overexpression of the pepper *fib* gene in tomato fruits leads to a twofold increase in carotenoid content [47], while down-regulation of *LeCHRC* causes transgenic tomato flowers to accumulate 30% less carotenoids [48].

Metabolic engineering of carotenoids in crops

Significant progress has been achieved in quantitative and qualitative manipulation of carotenoids in food crops for enhancing their nutritional quality and health benefits [5,7–9]. The strategy commonly used is to alter expression of the key enzymes or several enzymes of a mini-biosynthetic pathway in specific tissues such as in seeds, fruits, or other storage organs. Phytoene synthase is one of the key metabolic enzymes involved in carotenogenesis, and thus a prominent target for genetic manipulation of carotenoid content in food plants. Overexpression of the phytoene synthase gene from either bacteria or plants results in significant increase in total carotenoid levels in tomato fruit [49], potato tuber [50], and canola seed [51]. Expression of multiple biosynthetic genes in the carotenoid biosynthetic pathway leads to a profound increase of β-carotene in Golden Rice [10,52] and ''golden'' potato [53], as well as the accumulation of zeaxanthin in tomato fruit [54]. Qualitative modification of carotenoid composition in food crops by tissue-specific regulation of endogenous genes in the pathway is achieved for β-carotene or zeaxanthin enhancement in potato [55–57] and in tomato [58]. Expression of β-carotene ketolase gene in ketocarotenoid biosynthesis confers the accumulation of astaxanthin, a new and high-economic value carotenoid, in potato tubers [59] and in model plants [60–62].

In a few cases, alteration of the expression of genes involved in light signal pathway also leads to enhanced levels of carotenoid accumulation. Cryptochromes are blue light photoreceptors found in plants, bacteria, and animals. Overexpression of the tomato *CRY2* gene results in overproduction of anthocyanins and chlorophyll in leaves and of flavonoids and lycopene in fruits along with other phenotypic changes [63]. In tomato, fruit-specific

suppression of *DET1* which encodes a nuclear regulatory protein in photomorphogenesis significantly increases carotenoid and flavonoid contents [64]. These results undoubtedly show that carotenoid biosynthesis is affected by other signal modules, indicating an alternative approach in carotenoid enhancement.

The basic concept of metabolic engineering of carotenogenesis is to increase the metabolic flux towards carotenoid biosynthesis. However, in some cases, the increased flux into carotenogenesis can alter or reduce flux in other competing pathways, leading to unexpected phenotypic changes. For example, overexpression of *PSY* in canola and *Arabidopsis* seeds causes not only a high level of accumulation of carotenoids, but also leads to a delayed germination phenotype due to an increased content of the carotenoid-derived ABA [51,65]. Constitutive expression of tomato *PSY-1* results in a stunted growth due to an insufficient synthesis of gibberellins and producing pleiotrophic effects such as premature pigmentation of seed coats and cotyledons [11].

Although it is possible to produce transgenic crops with significant increased levels of carotenoids via alteration of the expression of genes involved with carotenogenesis, the extent is limited due to a lack of fully understanding of the mechanisms underlying carotenogenesis and its interaction with other metabolic processes [7]. Novel carotenoid gene mutations will provide not only the new insights into the control mechanisms, but also possible genetic tools to manipulate carotenogenesis in food crops. Recent studies on the *Or* gene from an orange cauliflower mutant has offered such an endeavor for carotenoid enhancement.

Functional analysis of the *Or* gene from cauliflower

Characteristics of the orange cauliflower (Brassica oleracea var. botrytis) mutant

The eye-appealing orange cauliflower (Fig. 2A) was first discovered in Bradford Marsh in Canada in 1970. Through many years of work by Professor Emeritus Michael Dickson at Cornell University and by other breeders, the orange cauliflower is now commercially available.

The orange cauliflower results from a spontaneous mutation of a single gene, designated as *Or* for *Orange* gene [12]. The *Or* gene mutation causes not only the normally white curd to turn orange, but also imparts visible orange coloration in other parts of the plant, such as in the shoot meristems, the pith of the stem, and the vasculature at the base of the petioles [14]. The *Or* homozygous plants have an intense orange coloration in these tissues and exhibit stunted growth with a small curd and a delayed curd formation. The *Or* heterozygous plants are less pigmented and exhibit normal growth as wild-type plants. HPLC analysis reveals that while the wild-type curd tissue

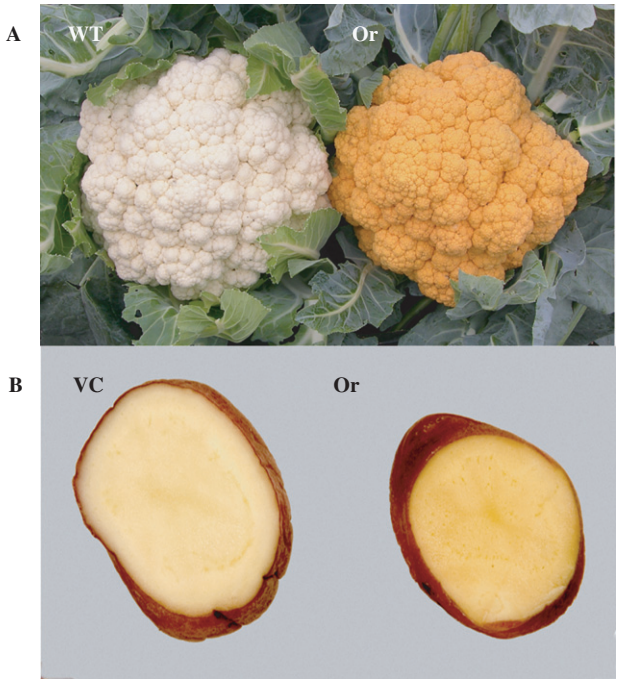

Fig. 2. Effect of the *Or* gene on carotenoid accumulation in cauliflower (A) and transgenic potato tubers (B). (The color version of this figure is hosted on Science Direct.)

contains negligible amounts of carotenoids, the *Or* plants accumulate high levels of mainly β-carotene. None of the precursors of β-carotene was detected in either genotype. The outer curd tissues of *Or* homozygous plants accumulate approximately 8 μg/g fresh weight β-carotene, a level several hundred fold higher than that detected in comparable wild-type tissues [14]. No differences in leaf carotenoid content and composition were observed between wild-type and *Or* homozygous plants.

Microscopic analysis shows that the β-carotene accumulation occurs within chromoplasts and is of several forms, predominantly as sheet structures [14]. These sheets occur as helical ribbons reminiscent of carotenoid-sequestering sheets in carrot root [66] and appear to be highly ordered structures. Based on the substructures, the *Or* chromoplasts are classified as membranous chromoplasts [67].

While each shoot apical meristem or curd inflorescence meristem cell of wild-type plants contains numerous colorless proplastids or leucoplasts, only one or two large chromoplasts are found in each affected cell in these *Or* tissues, suggesting a limited plastid division in the *Or* mutant [14]. The one or two chromoplasts constitute the entire plastidome in these cells [67].

The expression of carotenoid biosynthetic genes including *PSY*, *PDS*, *ZDS*, *LCYB*, *LCYE*, and *CHYB* is not dramatically altered by the mutation; neither is the expression of isoprenoid biosynthetic genes, including *DXS*, *DXR*, *IPI*, and *GGPS* [14]. The calli derived from the *Or* mutant seedlings accumulate significantly higher levels of carotenoids than those from wild-type seedlings, but when treated with norflurazon, a specific inhibitor of PDS, both calli synthesized comparable levels of phytoene. No major differences in carotenogenic gene expression were observed between the wild-type and *Or* calli. These results imply that the *Or* gene may exert its effect on carotenoid accumulation through a novel mechanism rather than increasing the capacity of biosynthesis [68].

Map-based cloning of the Or gene

To gain more insights into the mechanism underlying *Or*-induced β-carotene accumulation, a map-based cloning strategy was employed to isolate the *Or* gene. Initially, 10 amplified fragment length polymorphism (AFLP) markers closely linked to the *Or* gene were identified in a mapping population consisting of 195 F_2 individuals. Three AFLP markers that flank the *Or* gene were successfully converted into PCR-based sequence-characterized amplified region (SCAR) markers [69]. Two of which were used later for the analysis of a large segregating population consisting of 1,632 F_2 individuals and a high-resolution genetic map was developed. By screening a cauliflower bacterial artificial chromosome (BAC) library and through chromosome walking, the *Or* locus was defined in a single BAC clone [70]. This BAC was sequenced and nine putative genes were predicted. Fine genetic mapping identified a single candidate gene that cosegregates with the *Or* locus. Sequence analysis revealed that a 4.7 kb *copia*-like LTR retrotransposon is inserted in the mutant allele. Introduction of the genomic fragment containing the candidate gene with the retrotransposon insertion into wild-type cauliflower plants turns the white color of curd tissue into orange with the accumulation of β-carotene, which confirms the candidate gene to be *Or* [15].

Analysis of the Or gene and the encoding protein

The *Or* gene is a single-copy sequence in the cauliflower genome. Comparison of the genomic sequence and full-length cDNA of *Or* reveals that the *Or* wild-type gene contains eight exons and seven introns. The insertion of a *copia*-like LTR retrotransponson in exon 3 results in the production of three alternatively spliced *Or* mutant transcripts following excision of the retrotransponson. All of the spliced transcripts can be read through and share the same start codon and stop codon with the wild-type gene. RT-PCR analysis revealed that these three transcripts are expressed at different levels in the mutant cauliflower, while only wild-type transcript is expressed in the

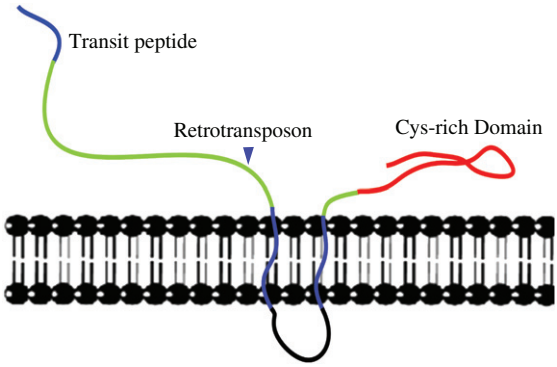

Fig. 3. Topology of the OR protein. Transit peptide: M1-S22; Unknown domain: V23-Y145; Transmembrane domain 1 (TM1): Y146-E168; Transmembrance domain 2 (TM2): D195-V217; Cysteine-rich domain: H225-L305. The arrow indicates the retrotransposon insertion site.

wild-type plants. In both mutant and wild-type plants, *Or* transcripts are expressed highly in young leaves, curds, and flower buds, and low in mature leaves and roots, indicating the insertion of retrotransponson does not change its pattern of tissue-specific expression [15].

The wild-type gene encodes a protein of 305 amino acid with an estimated molecular mass of 33.5 kD. The predicted protein has two putative transmembrane domains and is predicted to be plastid targeted (Fig. 3). Indeed, the Orwt:GPF fusion protein was found to be associated with non-colored plastids, such as leucoplasts in the epidermal cells of young leaves and amyloplasts in the young developing seeds of transgenic *Arabidopsis*. The characteristic of the OR protein is that it carries a cysteine-rich zinc finger domain in its C-terminal. This domain exists in DnaJ-like molecular chaperones, which participate in protein folding, assembly and disassembly, and translocation into organelles [71,72]. As OR lacks the typical N-terminal J domain of the molecular chaperones, OR is more likely a novel protein with a unique cellular function. OR is hypothesized to exert its function through protein–protein interaction mediated by its cysteine-rich domain [15]. The OR protein appears to be plant-specific and its homologs are present in all plant species examined. OR and its homologs share an exceptional sequence conservation (Fig 4), which suggests an important role of this protein in plant growth and development.

Possible functional role of Or in inducing carotenoid accumulation

The *Or* gene is not one of the carotenoid biosynthetic genes and appears to exert no direct effect on carotenoid biosynthesis. By contrast, the spatial and temporal expression patterns of the *Or* gene and subcellular localization of

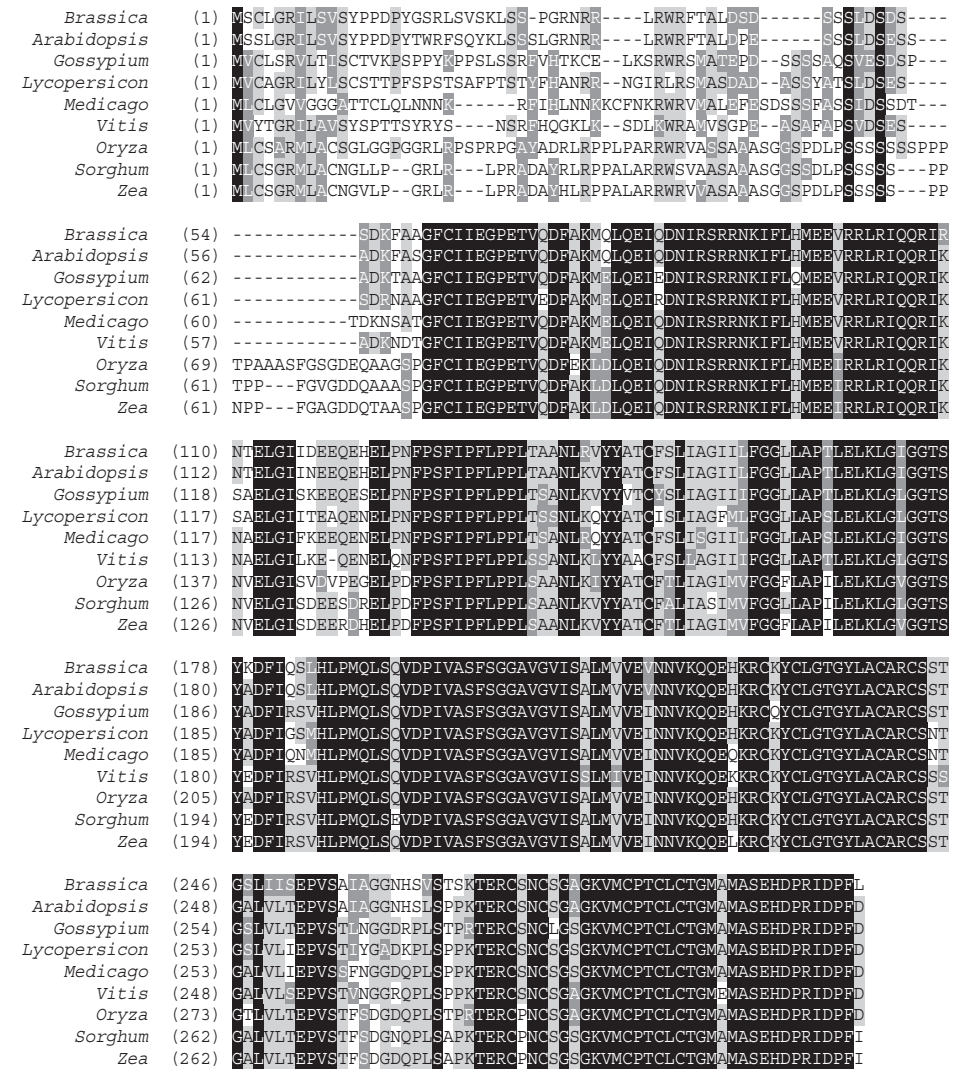

Fig. 4. Alignment of the predicted amino acid sequences of OR homologs from higher plants. The amino acid sequences were obtained from GenBank and the Institute for Genomic Research (TIGR) Plant Transcript Assemblies Database. The accession numbers are: *Arabidopsis thaliana* NP_200975, *Brassica oleracea* var. *botrytis* ABH07405, *Vitis vinifera* CAO62305, *Oryza sativa* NP_001047593, *Lycopersicon esculentum* TA6503_4081, *Gossypium hirsutum* TA2322_3635, *Medicago truncatula* TA4160_3880, *Glycine max* TA6293_3847, *Hordeum vulgare* TA5326_4513, *Triticum aestivum* TA19897_4565, *Sorghum bicolor* TA4814_4558, and *Zea mays* TA16877_4577.

the protein are associated well with differentiation of plastids and carotenoid accumulation. The cauliflower homolog of plastid fusion/translocation factor (*Pftf*) known to be involved in chromoplast differentiation [73] is expressed highly in the *Or* mutant curd, indicating enhanced differentiation of chromoplasts in the mutant [15]. Moreover, introduction of the *Or* gene into white cauliflower results in the formation of large membranous chromoplasts in the curd cells with increased levels of β-carotene. Taken together, genetic and cellular analysis suggests that the functional role of *Or* is associated with the differentiation of proplastids and/or other non-colored plastids into chromoplasts, which provide a metabolic sink for carotenoid accumulation [15].

Use of the Or gene to enrich carotenoids in transgenic potato tubers

Enriching carotenoid levels in major staple crops is expected to have a broad and significant impact on human nutrition and health. Initially to see whether *Or* functions in another plant species, the *Or* gene was transformed into the *Arabidopsis ap1-1/cal-1* "cauliflower" mutant. This mutant was chosen because it forms inflorescence meristems sharing the same structure as curd tissue of cauliflower. Expression of the *Or* transgene in the *Arabidopsis* mutant resulted in production of "orange-yellow" color instead of the normal pale-green hue in the inflorescence meristems (Li *et al.*, unpublished data). HPLC analysis confirmed that the color shift is indeed associated with enhanced carotenoid accumulation. These results provide evidence that *Or* works across species to enhance carotenoid accumulation.

The *Or* gene under the control of a tuber-specific promoter was transformed into potato plant. Visual examination of the flesh color of some transgenic tubers reveals that they exhibit a deep orange yellow-flesh hue (Fig. 2B). The subsequent HPLC analysis of these tubers showed that these transgenic tubers contain not only increased levels of violaxanthin and lutein normally present in the non-transformed controls, but also accumulate significant levels of β-carotene and three other metabolic intermediates of phytoene, phytofluene, and ζ-carotene, which are not detected in the controls. The total levels of increase were over sixfold. Interestingly, while carotenoids in potato tubers were reported to be stable or decrease during long-term cold storage [74,75], cold storage greatly increases the total levels of carotenoids as well as β-carotene in the *Or* transgenic tubers. Further, the high-carotenoid trait in the *Or* transgenic tubers is stable in a subsequent generation [16]. These results prove that the *Or* gene functions in the storage tissue of food crops to enhance carotenoid accumulation.

Potato tubers normally accumulate carotenoids in amyloplasts [76]. Further examination of the cellular contents of these transgenic tubers by

light microscopy showed that in addition to various sizes of amyloplasts found in the controls, the *Or* transgenic tubers also contain intact chromoplasts with orange structures of helical sheets and fragments [16]. Those orange structures share common features including color, overall range of forms, and dichroism with the carotenoid-sequestering structures released from chromoplasts of orange carrot roots. The similarity suggests that like carrot, the *Or* transgene induces the formation of carotenoid-sequestering structures within chromoplasts. The fact that the *Or* transgene induces the formation of chromoplasts in the transgenic potato tubes further confirms that the *Or* gene acts as a *bona fide* switch to trigger chromoplast differentiation for carotenoid accumulation.

Carotenoids accumulate in high levels in chromoplasts in plants. Chromoplasts develop a unique mechanism to accumulate mass amounts of carotenoids by generating carotenoid-lipoprotein structures that function as a deposition sink to sequester and deposit carotenoids [42,77]. Indeed, the availability of such a sink for carotenoid deposition has been shown to be directly associated with carotenoid accumulation [42,78,79]. The *Or* case, along with previous studies on red pepper, cucumber, daffodil, and maize, demonstrates that the formation of a suitable sink plays an important role in conferring carotenoid accumulation [15,44,45,80,81].

Creating a metabolic sink is a new strategy to enrich carotenoids in staple food crops

Accumulation of carotenoids or many other metabolites is a net result of several processes, such as biosynthesis and degradation. Enhanced levels of accumulation can be achieved by increasing the rate of biosynthesis or reducing the rate of turnover. Characterization of the *Or* gene and successful demonstration of use of *Or* to increase carotenoid content in transgenic potato tubers, the most important non-cereal staple crop, reveal that manipulation of chromoplast formation to create a metabolic sink exerts a profound effect on carotenoid accumulation. Thus, the *Or* gene offers an alternative new approach to complement effects relying on expression of carotenogenic genes for enhancing carotenoid levels in food crops, particularly in the storage tissues of staple crops.

Many seeds or roots of important crops such as wheat, rice, barley, maize, canola, and cassava synthesize and accumulate low levels of carotenoids in amyloplasts or elaioplasts [17]. The low levels of accumulation could be due to a number of possible reasons, including low metabolic flux into the pathway, limited catalytic activity of particular enzymes in the pathway, and high turnover rate. In some cases, lack of a suitable metabolic sink for sequestering carotenoid end products could be the major cause, as in the case of the white cauliflower curds. Although the genes involved in carotenoid biosynthesis are expressed in the white curd tissue, the absence of a suitable

sink structures restrains the accumulation of carotenoids [14]. Thus, creating a suitable metabolic sink to effectively sequester endproducts provides another strategy to enrich carotenoid levels in food crops.

The *Or* gene has been demonstrated to act as a *bona fide* switch to trigger the differentiation of non-colored plastids into chromoplasts in the normally white or low-pigmented tissues [15,16]. It provides a new molecular tool to enrich carotenoid content in storage tissues of staple crops by inducing the formation of chromoplasts, which provide a potential potent metabolic sink for carotenoid sequestration and deposition. Although the *Or* gene has been shown to confer high levels of carotenoid accumulation in cauliflower and transgenic potato tubers, it should be noted that the extent of carotenoid enrichment via enhancing sink capacity in food crops may be limited by a number of factors such as the maximal potential catalytic activity of carotenoid biosynthetic pathway in particular tissues or organs of crops [82]. Thus, concomitant manipulation of sink capacity to effectively sequester and deposit carotenoids along with the catalytic activity of this pathway to increase the metabolic flux would be a more promising strategy to quantitatively and qualitatively modify carotenoids in food crops to meet the requirement for optimal human nutrition and health.

Conclusions

Significant progress has been made in genetic manipulation of carotenoid biosynthesis. Current advances to enhance carotenoid accumulation in food crops have been mainly focused on manipulation of the genes encoding the metabolic enzymes in the pathway. Such an approach is effective, but in many cases it has been proved to be difficult to reach to desired levels of carotenoid enhancement in plants. To develop better breeding and engineering strategies for carotenoid enrichment, it is essential to gain more insight on the overall regulation of carotenoid biosynthesis and interactions between carotenoid biosynthetic pathway and other signaling pathways in the plant cells. The successful cloning of a cauliflower *Or* gene reveals that manipulation of chromoplast formation to provide an effective metabolic sink for carotenoid sequestration and deposition exerts a profound effect on carotenoid accumulation. The demonstration of use of the *Or* gene to increase carotenoid content in transgenic potato illustrates an alternative new approach to complement effects relying on expression of carotenogenic genes for enhancing carotenoid levels in food crops. Creating an effective metabolic sink for carotenoid accumulation along with manipulation of the catalytic activity of this pathway can be especially useful for metabolic engineering of carotenoids in low-pigmented tissues of staple crops.

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