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Review

The carotenoid biosynthetic pathway: Thinking in all dimensions

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ABSTRACT

The carotenoid biosynthetic pathway serves manifold roles in plants related to photosynthesis, photoprotection, development, stress hormones, and various volatiles and signaling apocarotenoids. The pathway also produces compounds that impact human nutrition and metabolic products that contribute to fragrance and flavor of food and non-food crops. It is no surprise that the pathway has been a target of metabolic engineering, most prominently in the case of Golden Rice. The future success and predictability of metabolic engineering of carotenoids rests in the ability to target carotenoids for specific physiological purposes as well as to simultaneously modify carotenoids along with other desired traits. Here, we ask whether predictive metabolic engineering of the carotenoid pathway is indeed possible. Despite a long history of research on the pathway, at this point in time we can only describe the pathway as a parts list and have almost no knowledge of the location of the complete pathway, how it is assembled, and whether there exists any trafficking of the enzymes or the carotenoids themselves. We discuss the current state of knowledge regarding the "complete" pathway and make the argument that predictive metabolic engineering of the carotenoid pathway (and other pathways) will require investigation of the three dimensional state of the pathway as it may exist in plastids of different ultrastructures. Along with this message we point out the need to develop new types of visualization tools and resources that better reflect the dynamic nature of biosynthetic pathways.

Contents

1. Introduction

What level of understanding is required to predictably manipulate a plant biosynthetic pathway, or controllably breed plant varieties to achieve a desired chemical profile? Recent research in metabolic engineering demonstrates feasibility for altering plant secondary metabolism, but reveals the inadequacies of having an incomplete picture of metabolic regulation. The functional alteration of biosynthetic pathways has the potential to improve the nutritional or survival characteristics of plants, which is of significant importance in addressing food security in the face of climate change. Yet, the more that is explored in control of plant metabolism, the more questions that arise. Metabolon formation may be influenced by unique combinations of enzymes for which interaction may alter activity and/or localization [1,2]. The ability to conduct predictable metabolic engineering is limited by understanding of the dynamic landscape of metabolons, for which research would benefit from improved three-dimensional modeling and visualization of plant metabolism.

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An example of how fundamental research on a pathway improved traditional breeding is that of plant carotenoid biosynthesis. Carotenoids are essential hydrophobic plant compounds usually of yellow, orange or red color, and play important roles in photosynthesis and photoprotection, as well as in production of phytohormones [3]. Certain carotenoids, such as α- and β-carotene, are precursors for vitamin A, good antioxidants and necessary for human health. Carotenoid derivatives participate in signaling in plant developmental programs and responses to abiotic and biotic stress, and mediate response to the presence of beneficial and non-beneficial organisms. Carotenoids and their derivatives impart colors and fragrance to flowers and other plant parts. Volatile apocarotenoids mediate plant–animal interactions (e.g. attracting insect pollinators) and enhance the flavor characteristics of food crops. Nutritional value of certain food crops and stress tolerance of any plant are directly related to carotenoid content.

2. Carotenoid biosynthetic pathway enzymes and model systems

In plants, enzymatic formation of carotenoids occurs on plastid membranes and is mediated by nuclear-encoded enzymes [3]. The pathway per se starts with condensation of geranylgeranylpyrophosphate (GGPP), a precursor from the upstream methylerythritol (MEP) pathway, to produce the C_{40} 15-cis-phytoene. This step is catalyzed by phytoene synthase (PSY), a rate-limiting enzyme for the pathway. Phytoene undergoes consecutive modifications such as desaturation and isomerization to form lycopene, which is later cyclized to carotenes. Carotenes are oxygenated to xanthophylls. A generalized pathway of plant carotenoid biosynthesis can be found through online web portals such as PlantCyc (http://pmn.plantcyc.org/PLANT/NEW-IMAGE?type=PATHWAY&object=CAROTENOID-PWY).

Extensive study of carotenoid biosynthesis in *Zea mays* (maize), a major food crop, has benefitted from the diverse germplasm collection, phenotypic mutants, genetic and physical map, and quantitative trait loci (QTL) affecting carotenoid biosynthesis (as reviewed in [4]). Other important models for understanding carotenogenesis include Arabidopsis (as reviewed in [5]), tomato, pepper, and daffodil (as examples: [6–8]). However, there is much that we do not understand about the regulation of carotenoid biosynthesis in the context of the manifold roles of carotenoids in plants. Most importantly, the location of the "fully assembled" biosynthetic pathway is unknown. Therefore, how can we really achieve predictable metabolic engineering of this important pathway?

3. Phytoene synthase: at the head of an enzyme metabolon

PSY is considered a key enzyme in carotenoid biosynthesis. It has been extensively studied and engineered in numerous plants where modifying carotenoid content is desired [9,10]. In maize, nutritionally important carotenoids are accumulated in edible seed endosperm. Maize PSY is encoded by three paralogous genes. In certain genotypes containing an insertion in the *PSY1* gene promoter [11,12], *PSY1* expression is anomalously induced in endosperm and thus conditions the characteristic yellow endosperm color. *PSY1* is also expressed in leaves along with *PSY2*. The expression of *PSY3* is limited to roots and induced by stress conditions [13–15]. Discovery of multiple *PSY* genes has been extended to many plant families. Based on studies in maize, it appears that *PSY* gene paralogs show tissue-specificity of expression that might impart the ability to control carotenogenesis independently of photosynthesis or in response to certain stresses.

Seeds of another grass staple, rice (*Oryza sativa*), lack expression of endosperm-specific *PSY*. In an attempt to add bioavailable carotenoids into the rice seed endosperm, a functional carotenoid biosynthetic pathway was introduced into rice by transforming plants with endosperm-expressed maize *PSY1* and *CrtI* (a multifunctional bacterial enzyme able to convert phytoene to lycopene) [16]. The result of such modification is Golden Rice 2 accumulating high amounts of β-carotene [17]. It took many years to successfully create Golden Rice 2. The first attempt utilized PSY from daffodil, which did not give a sufficient amount of carotenoids in rice seeds [16]. Through extensive trials with phytoene synthases from different plant species, it was discovered that in plant calli, PSY1 from maize was most effective to increase carotenoid production [17], and maize and rice PSY1 enzymes were most successful in promoting endosperm carotenoid accumulation in rice plant transformants. The reason of such a preference for PSYs is not clear.

What was so special about the maize PSY1 enzyme that made it optimal for carotenoid production in certain tissues? As recently discovered [18], the maize PSY1 isozyme differs from others in localization to a distinct plastid compartment. While the majority of phytoene synthases, such as maize PSY2 and PSY3, rice and *Arabidopsis* PSYs, are found to localize to plastoglobuli, maize PSY1 localization depends on its allelic variant. Three maize PSY1 variants differ from each other by only 1–2 amino acids at positions 168 and 257. Amino acid #257 may be any of three different residues: proline, serine or threonine. $Threonine_{257}$ in combination with $asparagine_{168}$ is the variant encoded by *PSY1* in all maize varieties with yellow endosperm. This was likely the allele that gained the promoter insertion mutation that activated endosperm expression and therefore became a selected trait for maize breeding. The $threonine_{257}$ version of PSY appears as a soluble protein in plastids [18], and interestingly, this was the particular variant effectively expressed in Golden Rice 2 endosperm (while other PSY1 allelic variants were not tested). The localization of the $proline_{257}$ variant of PSY1 in maize plastids is different from the $threonine_{257}$ version. When expressed in protoplasts, the $proline_{257}$ variant of PSY is accompanied with formation of fibrils, a sign of high concentration of carotenoids [18]. Similar fibrils are observed inside chromoplasts of fruits such as tomato, loquat and papaya [19,20], or carrot roots [21,22], where carotenoids are naturally accumulated and stored. Would a $proline_{257}$ variant increase carotenoid production in rice, if used instead of maize PSY1 with $threonine_{257}$?

PSY1 variants, forming different types of plastoglobuli – globular or fibrillar – might direct carotenoid biosynthesis to different suborganellar locations. As a consequence, perhaps PSY isozyme choice might affect carotenoid storage and bioavailability which is related to storage location [21,22].

4. Localization of the carotenoid biosynthetic pathway – where is it?

Early biochemical studies made clear that carotenoid enzyme location is critical for activity [23,24]. Unexpected results in metabolic engineering indicate the need for better understanding of carotenoid pathway regulation at many levels [25]. Carotenoids are localized on membranes of plastids, and plastids are known to be the site of carotenoid biosynthesis. However, depending on the tissue and plastid type, plastids are architecturally unique, being dynamic organelles that may go through a developmental program altering the ultrastructure, chemistry, and structural aspects of the carotenoid biosynthesis machinery. Carotenoids on the envelope may have a different destiny (e.g. conversion to apocarotenoids involved in mediating signaling) as compared to carotenoids that function as structural components for photosynthesis or