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Review

Genetic engineering of carotenoid formation in tomato fruit and the potential application of systems and synthetic biology approaches

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ABSTRACT

The health benefits conferred by numerous carotenoids have led to attempts to elevate their levels in foodstuffs. Tomato fruit and its products contain the potent antioxidant lycopene and are the predominant source of lycopene in the human diet. In addition, tomato products are an important source of provitamin A (β-carotene). The presence of other health promoting phytochemicals such as tocopherols and flavonoids in tomato has led to tomato and its products being termed a functional food. Over the past decade genetic/metabolic engineering of carotenoid biosynthesis and accumulation has resulted in the generation of transgenic varieties containing high lycopene and β-carotene contents. In achieving this important goal many fundamental lessons have been learnt. Most notably is the observation that the endogenous carotenoid pathways in higher plants appear to resist engineered changes. Typically, this resistance manifests itself through intrinsic regulatory mechanisms that are "silent" until manipulation of the pathway is initiated. These mechanisms may include feedback inhibition, forward feed, metabolite channelling, and counteractive metabolic and cellular perturbations. In the present article we will review progress made in the genetic engineering of carotenoids in tomato fruit, highlighting the limiting regulatory mechanisms that have been observed experimentally. The predictability and efficiency of the present engineering strategies will be questioned and the potential of more Systems and Synthetic Biology approaches to the enhancement of carotenoids will be assessed.

Introduction

The analyses of carotenoids and their intermediates accumulating in mutants, under different environmental conditions and after inhibitor treatments established the fundamental sequence of biochemical reactions involved in carotenoid biosynthesis by the mid 1960's [1]. Subsequently during the 1970's and 80's cell-free systems were developed which shed light on the rudimentary properties of many of the enzymatic reactions contributing to carotenoid formation [2]. Although solubilisation and partial purifications were reported [2,3], the labile nature and practical logistics of specific enzyme assays made it virtually impossible to attain and utilise protein sequence information as a means to isolate DNA sequences encoding the biosynthetic enzymes. Despite these difficulties, advances in molecular genetic strategies such as tagged mutants, complementation and improved sequence resources facilitated the isolation of genes encoding carotenoid enzymes from bacteria [4,5], fungi [6], algae [7,8] and higher plants [9] by the mid 90's. The ever growing suite of genes and concurrent development of transformation systems provided the toolkits to embark on genetic manipulation of the carotenoid pathway, subsequently termed metabolic engineering. Bacteria [10], yeasts [11], algae [12], higher plants [3] and animals [13] have all now been engineered to either synthesise non-endogenous carotenoids or enhance existing contents.

The genetic engineering (GE)[1] of higher plant has received the most attention because many crops represent staple foodstuffs from which essential dietary components such as carotenoids are acquired. In addition, they are a cheap renewable resource that in comparison to microbial production are environmentally and economically favourable. Golden Rice (GR) [14,15] is the most publicised example of a staple crop plant engineered to produce carotenoids. This is because of its potential humanitarian benefits in combating vitamin A deficiency in the developing world. It is estimated that GR could prevent 1–2 million childhood deaths annually in South East Asia that would result from Vitamin A deficiency [16]. Besides rice, numerous other crop plants such as potato, canola, carrot and tomato have been engineered to enhance carotenoid con-

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[1] *Abbreviations used*: ABA, abscisic acid; ARE, antioxidant response elements; GE, genetic engineering; GR, golden rice, IL, introgression line; MAS, marker assisted selection; ODEs, ordinary differential equations; PPARs, peroxisome proliferator activated receptors; PAGE, polyacrylamide gel electrophoresis; RIL, recombinant inbred line; TAP, tandem affinity purification; TSNI, time series network interference.

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tents. In fact, transformation of a crop plant to alter carotenoid content is no longer a frontier in carotenoid science but an established application of technology. Characterisation of these transgenic resources has and will continue to be invaluable in advancing our fundamental understanding not only of carotenoid formation but plant metabolism in general. In this review attention will be focussed on tomato fruit because of the wealth of information describing the health benefits associated with diets rich in tomato products, the body of work performed on carotenoid biosynthesis and its genetic manipulation in tomato as well as the emerging "omic" platforms customised for tomato. Notable successes in enhancing fruit carotenoid content will be described, whilst highlighting the many gaps in our knowledge that remain. Finally, the potential application of Systems and Synthetic Biology to the enhancement of carotenoid content in crop plants will be discussed as a means improving experimental predictability and operating at optimal biosynthetic capacity.

Health benefits and potential industrial use of tomato carotenoids

Ripe tomato fruit and its products provide 85% of the lycopene found in the diet [17]. A wealth of epidemiological studies has found that higher dietary intake of tomato products are associated with a reduced risk of prostate cancer [18]. In addition, higher serum levels of lycopene have also been shown to inversely correlate with the occurrence of prostate cancer [19]. Such levels of lycopene can be achieved through increased consumption of tomato fruit or products with elevated lycopene content [20]. Besides prostate cancer, the benefits of high tomato diets have been linked to the prevention of cardiovascular disease, skin and eye health as well as other cancers [3]. Research during the 1990's associated the health benefits of lycopene to its potent antioxidant properties. However, the precise mechanism by which lycopene exerts its health benefits remains unclear. More recent studies now suggest that lycopene itself is not the bioactive agent but a metabolite(s) or oxidation product(s) of lycopene are responsible for its ability to prevent the onset of disease states [21]. Potential bioactive products derived from lycopene include "lycopenoids" which are formed from the action of the carotenoid cleavage enzyme CMO-II. This dioxygenase shows homology to CMO-I which forms all-*trans* retinal from β-carotene. The known products of CMO-II include apo-8′-lycopenal and apo-12′-lycopenal [21]. It is also possible that CMO-II could act on other acyclic tomato carotenoids such as phytoene and phytofluene to generate other bioactive apo-carotenes. Lycopene oxidation products such as lycopene 5, 6-epoxide have also been identified in human serum [22,23]. In contrast to its antioxidant properties, lycopene metabolites are believed to exert their beneficial effects as agonist or antagonists of nuclear receptors such as Peroxisome Proliferator Acivated Receptors (PPARs) or active response elements such as transacting antioxidant response elements (ARE) [24].

Tomato products are also a good dietary source of β-carotene (provitamin A). Deficiency in provitamin A can cause xerophthalmia, blindness and premature death [25]. In addition to carotenoids, it is important to note that tomato is an excellent source of other health promoting phytochemicals such as flavonoids, phenylpropanoids, vitamin C, vitamin E and FruHis [26]. This is probably why the intake of tomato and its products have greater preventative effects than administering supplements solely [27].

The massive accumulation of carotenoids in tomato fruit is associated with a very active endogenous isoprenoid biosynthetic pathway in the plastid. This offers the potential to use tomato fruit as a cell factory for the production of non-endogenous high-value isoprenoid related commodities. For example, feed supplements such as ketocarotenoids [28] or anticancer agents, the taxanes [29]. Genetic engineering (GE) approaches can utilise fruit-specific promoters to use fruit for product sequestration without perturbing essential plant processes associated with vegetative tissues.

Present and potential strategies used to enhance the carotenoid content of tomato fruit

It is clear that ripe tomato fruit are an important dietary source of carotenoids beneficial to human health. Consumer demands along with governmental recommendations [30] for more nutritious food products have generated considerable industrial interest in the development of fruits and vegetables with improved consumer health quality traits. Conventional plant breeding of tomato has introduced many important traits into elite varieties. More recently the tomato introgression (IL) and recombinant inbred line (RIL) populations are proving very useful in exploiting modern marker-assisted selection (MAS) as a means of introducing traits into elite varieties [31]. However, these approaches are labour intensive and time-consuming. They also rely upon sexually compatible species, which limits the traits that can be introduced, as often the biodiversity of the species is not sufficiently broad enough to provide the required trait. Presently, the size of the region on which the QTL of interest is localised means that inevitable gene drift arises, which potentially could result in the simultaneous transfer of detrimental traits along with the QTL of interest. In contrast, GE approaches are more focussed, enabling more targeted manipulations. They are comparatively less labour intensive and time-consuming and no limitations on the origin of the cDNA that can be introduced occur. In 2006, the worldwide acreage of GE crops was 252 million acres grown by 10.3 million farmers in 22 countries [32]. Virtually no GE crops, however, are grown in Europe, as the general public's reluctance to accept the technology prevents commercialisation. The GE crops that are cultivated are predominantly *Brassica napus*, corn, cotton and soybean all engineered to contain traits conferring herbicide or insect tolerance. Herbicide tolerant (HT) soybeans represent 87% of the total US soybean production [33]. It is estimated that approximately 80% of US processed food could contain a GE-derived ingredient [34]. However, there are very few commercially available whole foods such as fruits and vegetables typically termed LMOs (Living Modified Organisms) in the market place. This situation is likely to change in the coming years with numerous disease resistance GE potato, tomato and grape varieties in the pipeline. Other GE crops with greater yield and productivity, sources of renewable fuels/energy and reduced environment impact are being developed. Fruits and vegetables containing edible vaccines and reduced allergens have been reported [35] and staple crops with improved nutritional properties such as Golden Rice are in the process of deregulation [36]. Therefore, despite the European consumer's suspicions of GE foods there is great potential for the technologies in the future especially with the development and cultivation of crops for renewable energy sources, generating economic pressure on land usage for the production of food.

Carotenoid biosynthesis and its regulation during tomato fruit ripening

The sequence of reactions involved in carotenoid biosynthesis in tomato fruit are fundamentally the same as any other higher plant and are summarised in Fig. 1 [3]. However, regulation of the biosynthetic pathway at specific stages in fruit development and ripening means that qualitative and quantitative differences in carotenoid content are found in vegetative, flower and fruit tissues. Carotenoids are synthesised in the plastid; chloroplasts in

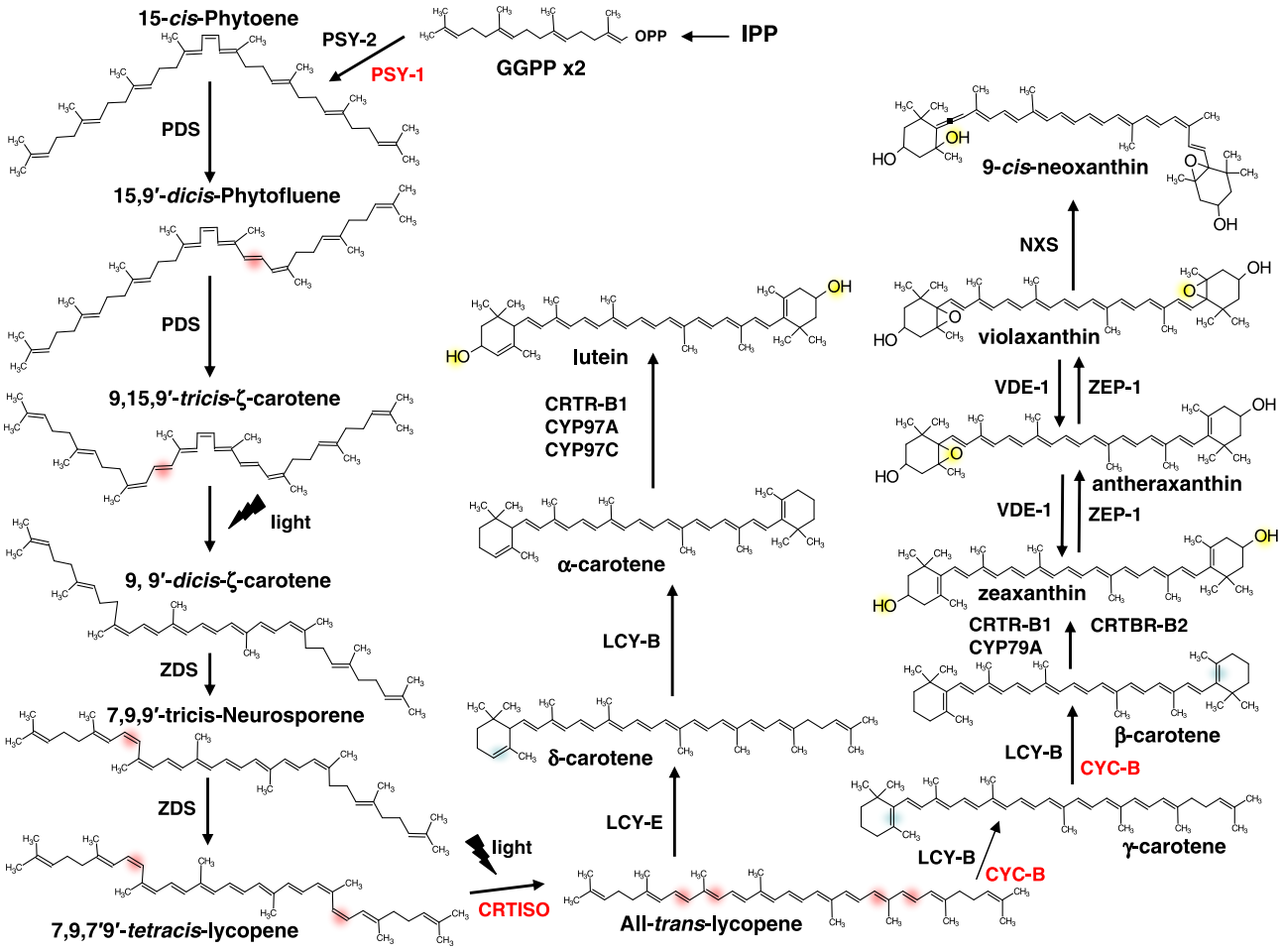

Fig. 1. Representative scheme of carotenoid biosynthesis in higher plants. Enzymes assigned to reactions are provided, those in red indicate that they are tomato fruit ripening specific or enhanced. The introduction of double bonds into the carotene molecules are indicated by red shading. The characteristic double bond within the carotenoid end group is shaded in blue, while oxygen/hydroxyl moieties introduced into the carotenoid skeleton are shaded in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

photosynthetic tissues and chromoplasts in ripe fruit and flowers. The IPP (C_5) from which carotenoids are derived is synthesised by the plastidial mevalonate independent pathway in a virtually exclusive manner in tomato fruit [37]. Geranylgeranyl pyrophosphate (GGPP), (C_{20}) is the immediate ubiquitous isoprenoid precursor utilised in the formation of carotenoids. Two molecules of GGPP are condensed in a head to tail manner to form phytoene (C_{40}). This molecule is the first dedicated step in carotenoid formation. The enzyme responsible for this conversion is phytoene synthase (PSY). Phytoene exists as the 15-*cis* geometric isomer and possesses three conjugated double bonds. A series of desaturation reactions is responsible for extending this series of conjugated double bonds to create the characteristic carotenoid chromophore. Phytoene desaturase (PDS) is the first desaturase enzyme, introducing a double bond at the 9′ of the phytoene molecule to create 15, 9′-di *cis*-phytofluene. Another double bond is then introduced at the 9 position forming 9, 15, 9′-*tricis*-ζ-carotene. This molecule with seven conjugated double bonds has a yellow/green colouration. Further desaturation requires isomerisation to yield 9, 9′-*dicis*-ζ-carotene, whether this reaction is enzymatic or non-enzymatic induced by light is still an area that must be unambiguously resolved. Following the formation of 9, 9′-*dicis*-ζ-carotene desaturation to 7, 9, 7′, 9′-*tetracis*-lycopene (prolycopene) via 7, 9, 9′-*tricis*-neurosporene proceeds catalysed by ζ-carotene desaturase (*ZDS*) [38,39]. Cyclisation of lycopene to form either β- or α-carotene via γ- or δ-carotene, respectively, can not proceed until an *all-trans* configuration of lycopene is generated. This reaction is catalysed by a carotene isomerase (CRTISO) in non-green tissue, while in photosynthetic tissues light and chlorophyll (acting as a sensitiser) carry out the conversion [38,39]. Lycopene has eleven conjugated double bonds and the chromophore formed conveys the red coloration to ripe tomato fruit. The enzymes responsible for the cyclisation of lycopene are the β-cyclase (LCY-B, CYC-B) and ε-cyclase (LCY-E) forming β-carotene and α-carotene, respectively. The introduction of oxygen moieties into the cyclic carotenoids results in the formation of xanthophylls. Zeaxanthin is generated from β-carotene through the introduction of hydroxyl groups at position 3 and 3′ in the β-ionone rings. Two enzymes zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), acting in tandem, then regulate the formation of violaxanthin. Conversion of violaxanthin to 9-*cis*-neoxanthin occurs via the action of neoxanthin synthase, neoxanthin being the precursor of the phytohormone abscisic acid (ABA). Lutein, the major carotenoid in photosynthetic tissue, is formed from α-carotene by the action of β- and ε-hydroxylases {CYP97A, CYP97C, CRT-B1 and B2, [reviewed in 40]}.

The accumulation of carotenoids in ripe tomato fruit is co-ordinated with fruit development and ripening. During this process many cellular changes arise including a transition from a chloroplast present in immature and mature green fruit to a chromoplast present in ripe fruit. This occurrence is accompanied by a quantitative and qualitative change in the carotenoid profile. In chloro-

plast-containing tissues xanthophylls predominate, reflecting their association with photosynthetic function. In chromoplast-containing tissues, however, acyclic carotenoids (phytoene, phytofluene, ζ-carotene, neurosporene and lycopene) accumulate [41]. At the gene level it has been shown that with the exception of *Pds* and *Zds* an alternative set of carotenoid biosynthetic genes exists exerting their effects during the onset of fruit ripening. For example, *Ggpps-2*, *Psy-1*, *CRTISO*, *CYC-B* and *CrtR-b1* all show ripening-enhanced expression patterns [42,43]. This suggests that the coarse regulation of carotenoid formation in ripening tomato fruit occurs at the level of transcription and is then relayed to increased protein and enzyme activities. At a pathway level flux coefficients demonstrate that phytoene synthase is responsible for increasing products into the pathway, while a concurrent restriction of cyclisation results in lycopene accumulation.

Ethylene governs multiple aspects of fruit development and ripening. The transcription of the carotenoid biosynthetic genes *Psy-1* and *Pds* has been shown to be dependant on ethylene-related events, while transcription of genes involved in cyclisation (*CYC-B*) is ethylene independent [44]. Light intensity and type has also been shown to influence carotenoid accumulation in tomato fruit [45].The effects are mediated via phytochromes [45], cryptochromes [46] and light signal transduction components [47,48]. Although light can affect the transcription of certain biosynthetic genes it would appear that this regulatory mode of action is not solely responsible for the phenotypes. For example, plastid biogenesis is likely to be a key factor in the accumulation of carotenoids [47,49]. Many regulatory aspects involved in the fine control of the pathway have been elucidated as a consequence of genetic engineering and will be described in the following section.

Metabolic engineering of carotenoid formation in tomato fruit

Tomato lines and varieties have been generated with enhanced levels of lycopene, β-carotene and xanthophylls (zeaxanthin and lutein) [3]. In addition, low levels of non-endogenous carotenoids such as ketocarotenoids have been reported [28]. Table 1 summarises the plants, the target transgenes, promoters, phenotypes and stability. These are notable achievements, demonstrating that a highly regulated, interconnected pathway, that is compartmentalised, membrane bound and involved in essential plant processes can be engineered to enhance carotenoids beneficial to human health in crop plants. Over the last decade the route to our present understanding has not been straightforward, but through careful characterisation many fundamental aspects of the carotenoid pathway have been revealed. Interestingly, many of these regulatory mechanisms are not evident until a perturbation is introduced into the pathway, in effect "silent" until homoeostasis is affected. Such findings have not just had a bearing on the engineering of carotenoid contents in plastids but also our general understanding of plant metabolism. In order to illustrate how these regulatory mechanisms can have an important bearing on the outcomes of GE and how they can be overcome several examples will be highlighted.

The construction of transgenic lines over-expressing the endogenous *Psy-1* demonstrated the unpredictability associated with the engineering of plant metabolic pathways. These plants displayed numerous unintended effects such as co-suppression or sense-suppression yielding loss of plant vigour, yellow coloured fruit devoid of carotenoids or blotchy fruit colorations and premature pigmentation in seeds, young shoots, roots and young fruit. Severe dwarfism also occurred due to reduced gibberellin (GA) levels, which were a result of plastidial GGPP being diverted from GA into unscheduled carotenoid formation [50].

Carotenoids, GA's and GGPP are all biosynthetically related through IPP. The pathway is comprised of a common core responsible for the formation of prenyl phosphate intermediates acting as precursors which are diverted into specific branches of the pathway [50]. The pathway can be regulated in a developmental or process manner and is often specific to a certain cellular species. For optimal plant performance the pathway must deliver these compounds in significant quantities at specific stages of plant development or in response to environmental cues. This requires complex multiple levels of regulation and a fine balance between precursor supply and utilisation. Genetic engineering of the carotenoid pathway can alter this balance in some tissues and subsequent effects can be far reaching, as illustrated with constitutive *Psy-1* over-expression. Besides GA formation other isoprenoid derived phytohormones are affected in vegetative tissues as well as chlorophyll and tocopherol contents in immature and mature fruit. Other examples of perturbed isoprenoid synthesis due to GE include the high β-carotene varieties of tomato generated through the constitutive over-expression of a bacterial phytoene desaturase (*CrtI*) [51]. In this instance tocopherol levels were elevated via an unknown mechanism. In order to minimise or avoid these detrimental effects it is essential to use tissue-specific promoters. In the case of tomato ripening enhanced promoters are used as controlled expression at this stage facilitates co-ordination with endogenous carotenoid formation and reduced competition with competing branches of the isoprenoid pathway.

A sub-population of *Psy-1* over-expressers, with no loss in plant vigour and small elevations in fruit carotenoid content, has been generated and inheritable stability of the phenotype demonstrated. Detailed characterisation of the carotenoid pathway at the transcript, enzyme activity and metabolite level during the mature green fruit stage has revealed numerous regulatory aspect of the pathway [52]. Firstly transcriptional up-regulation of a gene does not always correlate to increased protein or enzyme activity and thus post-translational mechanisms must operate within the pathway. Biosynthetic steps in the pathway prior to or after the point of engineering appear to sense and react to alterations in precursor and/or product levels in an attempt to maintain homeostasis within the pathway. Examples of these phenomena include varieties containing a bacterial derived phytoene synthase (*CrtB*) expressed in a fruit-specific manner; here the subsequent desaturation step in the pathway is reduced in response to high phytoene synthase activity [53]. In contrast, transgenic lines expressing a bacterial desaturase have reduced phytoene synthase transcription and enzyme activity. Deoxy-D-xylulose 5-phosphate Synthase (*Dxs*) over-expression elevates phytoene formation in ripe fruit but desaturation limits progression through the pathway [37]. These observations are linked to forward-feed regulation whereby the original end-products of the pathway may change due to induction or enhancement of biochemical steps in the pathway beyond the point of engineering. In the case of *Psy-1* over-expression, a lycopene cyclase (*CYC-B*) is induced resulting in increased enzyme activity generating β-carotene as an unintended end-product [52]. A similar occurrence is witnessed in the *CrtI* (bacterial desaturase) expressing lines where cyclase induction yields high β-carotene levels. In addition to forward-feed, feedback inhibition has also been observed in plants expressing the *CrtI* enzyme. Here the elevated β-carotene levels that resulted appear to reduce phytoene synthase activity by transcriptional down-regulation of *Psy-1* [51].

Determination of enzyme activities within the pathway often reveals that the steps subjected to manipulation typically display a high elevation in enzyme activity [53,54]. This activity does not correlate with increased product formation. Besides the ability of steps in the pathway to react and alter activity to control overall pathway flux, it is also likely that substrate channelling operates. Evidence for protein interactions creating isoprenoid biosynthetic complexes comes from observations of radiolabeled incorporation

Table 1
Examples of genetic engineering for enhanced carotenoid content in tomato.

Inserted target gene/cDNA and source	Gene function	Promoter	Fruit phenotype	Reference
Dxs from *E. coli* overexpression	Plastidial IPP formation	CaMV* 35S and Fibrillin from pepper	Increased phytoene and other carotenoids approaching 2-fold elevation	[37]
CrtB from *Erwinia uredovora* overexpression	Carotenoid biosynthesis phytoene synthase	Polygalacturonase (PG) from tomato	Increased (up to 4-fold) levels of carotenoids (phytoene, lycopene and β-carotene)	[53]
PSY-1 cDNA from tomato overexpression	Carotenoid biosynthesis phytoene synthase	CaMV 35S	Increased (1.5-fold) β-carotene content	[50,52]
CrtI from *E.uredovora* overexpression	Carotenoid Biosynthesis phytoene desaturase	CaMV 35S	Increased (up to 3-fold) β-carotene content. Reduced Lycopene and phytoene content	[51]
LCY-B from *Arabidopsis* overexpression	Carotenoid biosynthesis β-lycopene cyclase	*Pds* from tomato	Increased (7-fold) β-carotene content without affects on lycopene levels.	[77]
LCY-B from tomato overexpression	Carotenoid biosynthesis β-lycopene cyclase	CaMV 35S	Increased (31.7-fold) β-carotene content reduced lycopene content	[78]
CYC-B from tomato overexpression	Carotenoid biosynthesis β-lycopene cyclase	CaMV 35S	Increased β-carotene	[42]
CrtY from *E.herbicola* overexpression	Carotenoid biosynthesis β-lycopene cyclase	Plastid transformation, plastid *atpI* promoter	Increased (4-fold) β-carotene without a reduction in lycopene levels	[73]
Antisense LCY-B from tomato	Carotenoid biosynthesis β-lycopene cyclase	*Pds* from tomato	Increased (1.3-fold) lycopene content	[77]
Antisense *CYC-B* from tomato	Carotenoid biosynthesis β-lycopene cyclase	CaMV 35S	Increased lycopene	[42]
LCY-B and *CHY-B* from *Arabidopsis* and Pepper respectively	Carotenoid biosynthesis β-lycopene cyclase and β-carotene hydroxylase	*Pds* from tomato	β-Cryptoxanthin and zeaxanthin produced	[54]
*Crt*W and *CrtZ* from marine bacteria (*Paracoccus* species)	xanthophyll biosynthesis 4,4′ β-oxygenase and 3,3′ β-hydroxylase	CaMV 35S	Ketocarotenoid formation in leaf, low levels detected in the fruit	[28]
CRY-2	Blue light photoreceptors tomato cryptochrome	CaMV 35S	Increased (2-fold) fruit carotenoid content	[46]
RNAi *DET-1*	Light signal transduction Tomato *DE-ETIOLATED-1*	Ripening enhanced promoters, (P119, TFM7 and 2A11)	Increased β-carotene content up to 10-fold, elevated lycopene up to 4-fold and increased flavonoid content	[48]
COP1LIKE	Light signal transduction Tomato *COP1LIKE*	Constitutive promoter	Increased carotenoid content approaching 2-fold.	[47]
CUL4	Light signal transduction, tomato DDB1 interacting protein (Cullin).	Ripening enhanced (TFM7)	Increased (2-fold) carotenoid content	[79]
ySAMdc	Polyamine biosynthesis	E8 ripening enhanced	Increased lycopene content 3-fold	[59]
FIBRILLIN tomato	Lipid associated protein	Tomato Fibrillin	Increased (2-fold) carotenoids and derived volatiles	[80]

* CaMV, Cauliflower mosaic virus.

of precursors and products analysis as well as the behaviour of the enzymes during purification [55] and native gel-electrophoresis [56]. However, Tandem Affinity Purification (TAP) tagging experiments or yeast two hybrid experiments have to date failed to show specific interactions (unpublished data). In transgenic plants it also appears that the transgenic gene products may in many cases not even be in the same cellular micro-environment as the endogenous pathway [53,54].

The engineering of multiple steps in the carotenoid and isoprenoid precursor pathways simultaneously creating a heterologous "mini-pathway" has been successful in potato [57] but in tomato many of the same regulatory mechanisms found with the manipulation of single biochemical steps are still experienced [28]. As an alternative to pathway engineering, phenocopies of the tomato high-pigment mutants (*hp1* and *2*) have been created [46–48]. In these examples cryptochromes and components of the light signal transduction pathway have been altered. These approaches have the advantages of not only elevating the carotenoid content of the fruit but other important health related phytochemicals such as phenylpropanoids and flavonoids [48]. The mechanisms leading to the accumulation of these compounds are not fully understood, but there is strong evidence that a contributory factor is the increased plastid area per cell, suggesting plastid biogenesis is a target. Recently altered ABA contents have also been shown to affect carotenoid levels via altered plastid characteristics [58].

Traditionally, carotenoid formation has been treated as a defined unit, but the demonstration that the manipulation of components sensing environmental cues and other processes such as fruit longevity [59] can elevate tomato fruit carotenoids has indicated that carotenoids are integral components of metabolism. Therefore in order to evaluate and exploit these changes in a more "global" manner characterisation must start to include "omic" technologies developed in recent years. For example, metabolomic approaches on *Psy*-1 over-expressers demonstrated that alterations to the carotenoid pathway can affect isoprenoids, sectors of intermediary metabolism and cellular components [52]. The effect of altering carotenoid content on ripening related characteristics of fruit has also been demonstrated [G. Giuliano, personnel communication].

In summary, carotenoids beneficial to human health can be elevated in tomato fruit through pathway engineering or improved sequestration machinery. Despite these achievements, characterisation has shown that the plant can adapt and resist to the changes enforced upon its endogenous metabolic pathways. Typically "silent" intrinsic regulatory mechanisms operate to maintain metabolic homeostasis. This raises several questions. How predictable are the current GE approaches? How efficient is engineering in achieving the targeted end-products? Are the engineered changes optimal? With the emerging disciplines of Systems and Synthetic Biology we have the opportunity to address these questions and potentially take the engineering of desired traits to a new, improved level.

The potential of systems and synthetic biology to the production of useful carotenoids in plants

Characterisation is an important aspect of genetic engineering experiments enabling assessment and the design of further strate-

gies as well as fulfilling important safety criteria. Initial approaches focused on the transgenes and end-products of interest, which subsequently evolved into a more pathway level evaluation. Today, the advances in "omic" technologies facilitates characterisation of genetic engineering outputs at several levels (phenomics, transcriptomics, proteomics, metabolomics and fluxomics). In tomato, microarray platforms are publicly available in an cDNA (www.ted.bti.cornell.edu) and Affymetrix GeneChip™ format (www.affymetrix.com/). Metabolomic platforms using NMR [60], GC–MS [61] and LC–MS [62] have been established in tomato, as well as more targeted metabolite profiling approaches [63]. The utility of both NMR and GC-MS techniques to the development of fluxomic procedures has also been demonstrated [64]. In comparison, quantitative proteomics has proven more problematic to achieve routinely. Despite these difficulties, quantitative analysis of the tomato proteome has been achieved either through a two-dimensional PAGE based format or multi-dimensional chromatography MS/MS format [65].

These "omic" technologies generate large datasets. A key objective to be addressed now is how to integrate this data; so that exploitable outputs that impact on the design of further genetic engineering strategies and their predictability can be achieved. In

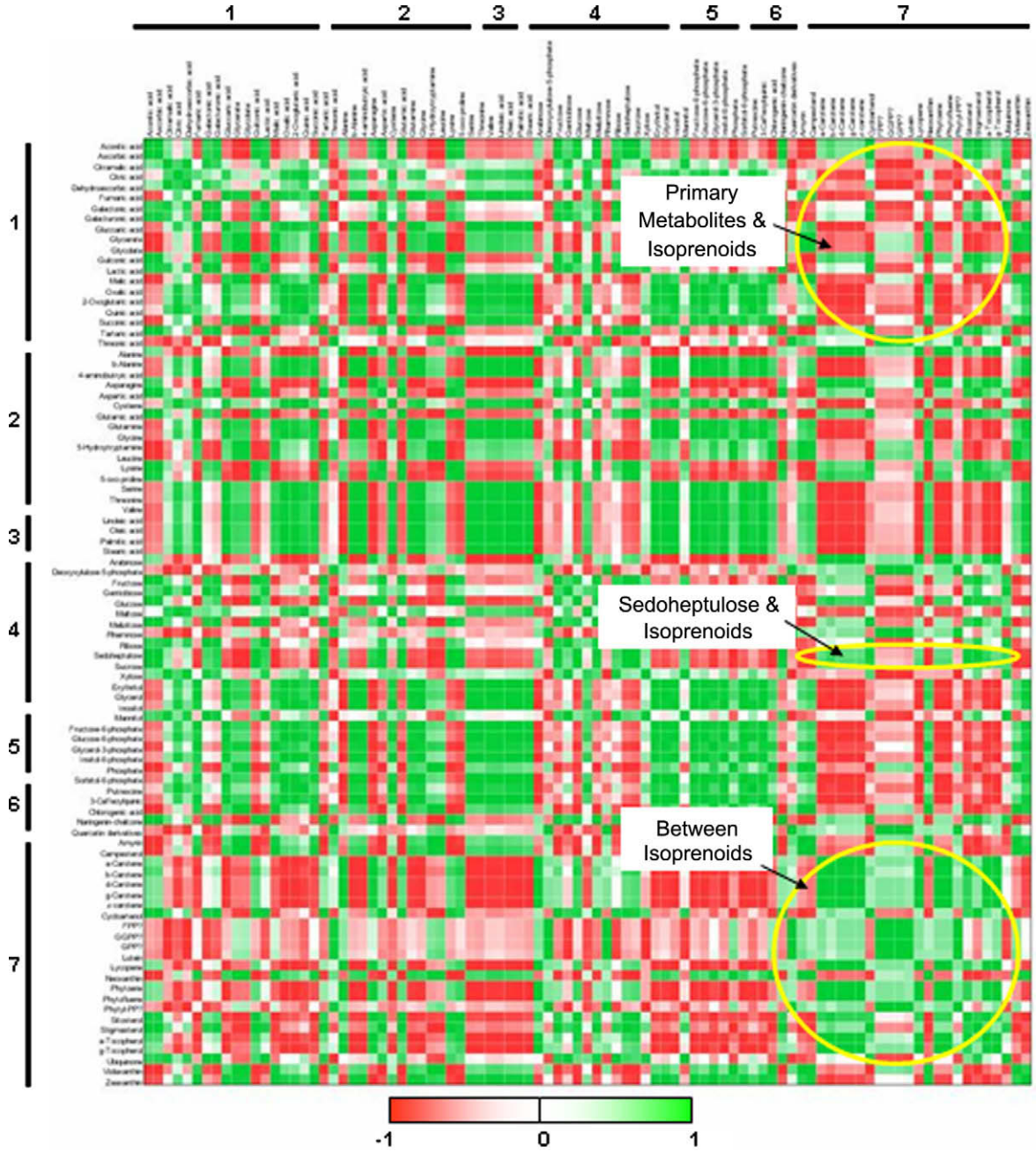

Fig. 2. Heatmap of metabolite x metabolite correlations associated with relative changes following *Psy*-1 overexpression compared to the wild type. Data was generated from three biological experiments and three technical replications at the mature green to ripe stages. Metabolites have been grouped by compound class, 1-organic acids, 2-amino acids, 3-fatty acids, 4-sugars and carbohydrates, 5-phosphates, 6-phenylpropanoids and flavonoids, 7-isoprenoids. Known unknowns have not been included. Pearson correlation coefficients (r) were calculated using BioSynlab software (www.biosynlab.com). The degree of correlation is provided in a false colour scale. Each cell has an r-value as indicated in the scale insert provided. A significance of $P < 0.05$ was used for r. Positive correlations are represented by green and negative red coloration. Examples of key metabolite correlations with isoprenoids are circled and labelled on the heatmap. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

addition, such a level of characterisation will greatly improve the safety evaluation process of both GMO's and LMO's. In order to fully integrate and decipher these datasets mathematical biology will be a key component of the workflow. Mathematical approaches that are being used include co expression analysis, Time Series Network Interference (TSNI), Bayesian analysis and Ordinary Differential Equations (ODEs). Co-expression analysis typically uses clustering approaches, the rationale being that biological entities (e.g. genes or metabolites) will have a good probability of behaving in a similar manner if functionally related. The degree of similarity can be assessed by pairwise similarity functions such as Pearson or Spearman correlation coefficients [66]. Fig. 2 illustrates how correlation analysis between metabolites can be used to identify sectors of metabolism altered by a perturbation engineered into the carotenoid pathway. In this instance data were generated from *Psy*-1 overexpressing fruit at the mature green developmental stages to ripe fruit stages [52]. One hundred and twenty metabolites were determined in a quantitative manner using GC–MS and targeted profiling techniques. The metabolites have been processed relative to the control (wild type) levels. The data have been statistically analysed using student *t*-tests both at the individual metabolite stage and during the determination of correlation coefficients in order to judge significance. A heat map format shows both positive (green) and negative (red) correlations between metabolites (see scale insert). The most significant positive correlations occur between carotenoids and other isoprenoids, with the exception of non-plastid derived isoprenoids such as amyrins and the phytosterol cycloartenol. Negative correlations between carotenoid biosynthesis and the majority of primary metabolites were observed with the exception of the Calvin cycle intermediate sedoheptulose phosphate. In addition to metabolite correlations, the same approach can be used with transcripts utilising transcriptomic data as well as metabolite to transcript interaction combining both transcriptomic and metabolomic datasets. Performing such analyses on genetically engineered varieties will further our understanding of plant metabolic pathways and how they react to perturbations in carotenoid/isoprenoid pathway. In this way sectors of metabolism can be identified that are not directly associated with the target pathway but have the potential to affect levels of the useful products targeted.

Bayesian networks are used to interrogate large "omic" derived datasets. The procedures use a graphical model for probabilistic determination of relationships [67]. They can be performed using steady state or time series dynamic datasets. Because the approach is based on probabilities the outputs must be tested experimentally. Such an approach has been used to determine gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cell cultures [68], identifying several new targets for future pathway engineering. ODEs represent a deterministic approach not based on estimation of probabilities, whereby the relationships of the entities in the network to each other are ascertained [67]. An advantage of ODE over other approaches is that once the parameters are known the equation devised can be used to predict the behaviour of the network under different conditions. Thus, the opportunity for more predictive modelling is feasible. Typically this approach is utilised in a focussed manner such as a pathway where extensive knowledge of the components is known. TSNI is similar to ODE, using differential equations to represent the rate of synthesis of network entities as a function of the concentrations of every other network component, typically following perturbation [67]. Recently a kinetic mathematical model that accurately simulates the developmental patterns of monoterpenoid accumulation in peppermint has been created using ODEs [69]. This work demonstrates the potential of the approach to predictive engineering of the terpenoid pathway and need for fundamental pathway information such as enzyme kinetics as well as advanced "omic" datasets.

The integration of "omic" datasets using mathematical tools truly represents a Systems Biology approach. In order to readily evaluate, display and deposit these datasets and their outputs software packages will be necessary. To date, several programmes exist that enable pictorial display of "omic" datasets over biochemical pathways (i.e. AraCyc [70], MAPMAN [71], KaPPA-View [72] and BioSynLab (www.biosynlab.com) and mathematical analysis {i.e. BL-SOM (www.kanaya.naist.jp/SOM/), BANJO (www.cs.duke.edu/~amink/software/banjo), ARACNE (amdec-bioinfo.cu-genome.org/html/caWork-Bench/upload/arcane.zip), BioSynLab (www.biosynlab.com) and NIR/MNI (tgardner@bu.edu)}. Further developments can be envisaged as the discipline of Systems Biology matures.

The application of Systems Biology to genetic engineering has now been termed "Synthetic Biology", where nature is re-designed for human benefit. Although many of the defined aspects of Synthetic Biology involving component standardisation and reconfiguration, modelling, and experimental testing and validation are still emerging with respect to carotenoid biosynthesis, notable aspects of synthetic biology have already been applied to carotenoid/isoprenoid formation. For example, the construction of mini-pathways both by nuclear [57] and plastid transformation [73], optimised synthetic genes [57], molecular evolution [74] and network engineering [75]. Most cases of synthetic biology are likely to be classified as transgenic (transformed with DNA from a different plant family) or xenogenic (transformed with synthetic DNA or from a different kingdom). Therefore, in the case of LMOs intended for dietary consumption, commercialisation is presently unlikely as most intragenic and cisgenic (transformed only with genetic elements from within a sexually compatible group) approaches are still awaiting clearance through the regulatory process in a timely and cost-effective manner. Therefore, in the case of tomato and other edible plant hosts it is likely that synthetic biology applications will initially be limited to industrially important carotenoids such as astaxanthin and canthaxanthin used as feed supplements or the potential use of carotenoids as biofuels [76].

Conclusions

Over the last decade rapid advances have been made in the genetic engineering of carotenoid biosynthesis within food crops, as exemplified by tomato. This has created a valuable genetic resource that can be utilised to ascertain the benefits and properties associated with enhanced levels, as well as used in the application

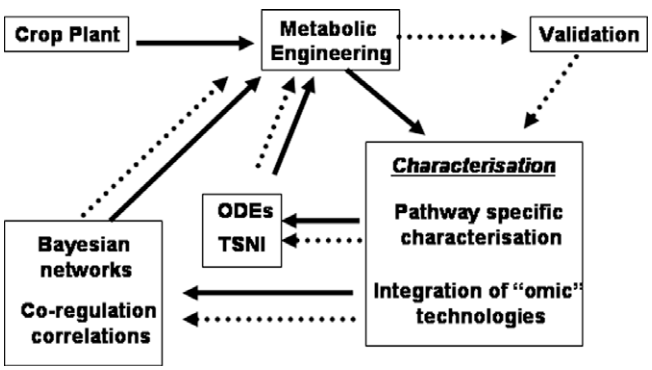

Fig. 3. Potential Systems/Synthetic biology workflows applied to the production of target carotenoids/isoprenoids in suitable hosts such as crop plants. Solid arrows indicate the first phase of characterisation and evaluation; dotted arrows illustrate cyclic routes of iterative data generation and evaluation.

of Systems Biology approaches. In most cases "omic" technology platforms have been developed and validated facilitating the application of Systems and Synthetic Biology approaches to carotenoid formation in plants. In the future these approaches will hopefully enable us to make the engineering process more predictable and standardised. Fig. 3 illustrates a potential workflow that could be employed in an iterative systems approach. In addition, the intrinsic regulatory mechanisms that operate within the pathway and have hindered optimal biosynthetic capacity will hopefully be addressed. Despite these projected advances, however, public perception of GMOs and LMOs are likely to remain limiting factors to commercialisation. Ironically a systems approach would greatly improve the regulatory safety assessment of novel foods that presently exists.

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