RICE GENETICS: ENGINEERING VITAMIN A

Eleanore T. Wurtzel
Lehman College and The Graduate Center of The City University of New York, USA

1. Introduction

Carotenoids, derived from plant food sources, are converted in humans to vitamin A and other important compounds needed for growth and development. Certain carotenoids have been distinguished in protection against cancer, in promoting immune responses, as antioxidants and as photoprotectants. Carotenoids are also useful as natural colouring agents in foods and cosmetics. Endosperms of food crops, such as maize and wheat, are low in provitamin A (1–10%) as compared with non-provitamin A carotenoids (Graham, 1997). Rice, an important food staple worldwide, accumulates no carotenoids in its endosperm and is, therefore, associated with vitamin A deficiency affecting 250 million children in developing countries (Underwood & Arthur, 1996). It has been estimated that improved vitamin A nutrition would eliminate approximately 1.3–2.5 million annual deaths (Humphrey *et al.*, 1992). The effects of vitamin A deficiency are manifested as xerophthalmia (visual impairment), blindness, increased mortality due to heightened severity of childhood diseases and greater risk of maternal transmission of viruses such as HIV (Semba *et al.*, 1994). Vitamin A intervention programmes have proven effective over the short term, but difficulties have been found in reaching children at the highest risk and at maintaining serum levels required to eliminate vitamin deficiency for the long term (Underwood & Arthur, 1996).

In the 1990s, molecular biology research led to the discovery of genes encoding enzymes required for carotenoid biosynthesis. Such an achievement provided a successful alternative to alleviating worldwide vitamin A deficiency through the use of genetic engineering to improve levels of provitamin A carotenoids in rice. This biotechnological achievement is a success both for genetics and for public health. This accomplishment can be similarly applied to improving the carotenoid content and composition of other important food staples such as wheat and maize.

2. What are carotenoids?

Carotenoids are a large class of yellow, red and orange pigments derived from isoprenoids. Carotenoids are synthesized by all photosynthetic organisms, as well as some bacteria and fungi. Animals do not have the ability to synthesize carotenoids, but must obtain these nutritionally important compounds through dietary sources, typically plants. In plants, the biosynthesis of carotenoids is essential for plant growth and development; carotenoids function as accessory pigments in photosynthesis, as photoprotectors preventing photooxidative damage and as precursors to the plant hormone, abscisic acid. The presence of carotenoids in endosperm tissue also adds nutritional value; in humans and animals, dietary carotenoids are essential precursors to vitamin A and to retinoid compounds needed in development (Lee *et al.*, 1981; Bendich & Olson, 1989). Other non-provitamin A carotenoids, such as lycopene, lutein, zeaxanthin and others, also play beneficial roles in human health (Giovannucci *et al.*, 1995; Kohlmeier *et al.*, 1997; Sommerburg *et al.*, 1998). Carotenogenic bacteria and other non-photosynthetic organisms synthesize carotenoids to provide protection in high-light, oxygen-containing environments.

3. The carotenoid biosynthetic pathway in bacteria and plants

(i) Location of the biosynthetic pathway

In plant cells, the biosynthetic pathway takes place in the plastid compartment of the cell and the enzymes of the pathway are encoded by genes located in the nucleus (Cunningham & Gantt, 1998).

(ii) Carotenoids are terpenoids derived from isopentenyl pyrophosphate

Carotenoids are derived from a five-carbon isoprenoid building block, isopentenyl pyrophosphate (IPP), which is common to all terpenoid compounds such as rubber, menthol and taxol. All plastids have the ability to manufacture these IPP precursors through a plastid-specific biosynthetic route that is also found in bacteria (Lichtenthaler, 1999). Four molecules of IPP are combined to produce the 20-carbon isoprenoid GGPP (geranylgeranyl pyrophosphate), the first precursor to carotenoids and to a variety of other isoprenoid-derived pathways, including gibberellins, the phytol chain of chlorophyll, prenylquinones, tocopherols and other natural products (Chappell, 1995).

(iii) From GGPP to the first carotenoid intermediate, phytoene

The biosynthesis of all carotenoids (**Figure 1**) begins with the combination of two molecules of GGPP to produce phytoene, the first compound specific to the carotenoid biosynthetic pathway (Cunningham & Gantt, 1998). This step is catalysed by the enzyme PSY (phytoene synthase).

(iv) From phytoene to lycopene

Phytoene, a colourless compound, undergoes the addition of double bonds resulting in the red-coloured carotenoid intermediate, lycopene, most notably seen in red tomato fruits. In higher plants and cyanobacteria, these steps are catalysed by two enzymes, PDS (phytoene desaturase) and ZDS (zeta-carotene desaturase), while in bacteria, such as *Erwinia uredovora*, only one enzyme, CRTI, is required to convert phytoene to lycopene.

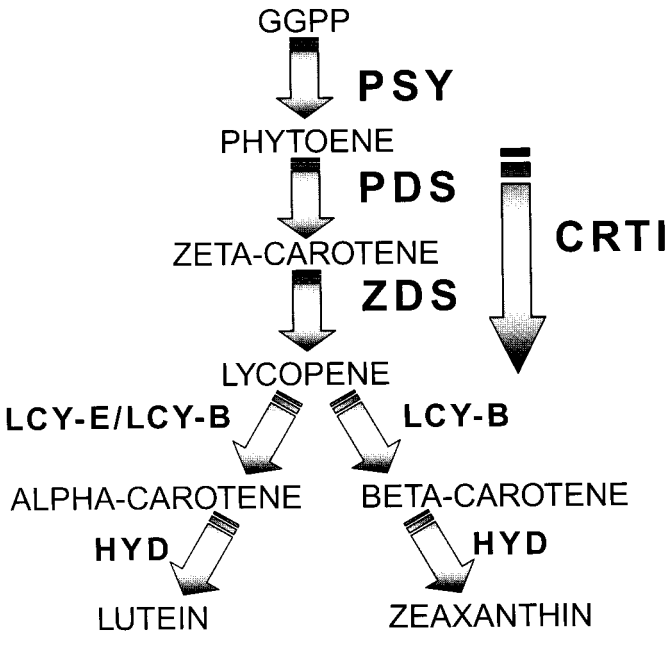

Figure 1 Carotenoid biosynthetic pathway. Enzymes are in bold and arrows point from enzyme substrate to product. Compounds: GGPP, geranylgeranyl pyrophosphate. Enzymes: PSY, phytoene synthase; PDS, phytoene desaturase (plant type); ZDS, zeta-carotene desaturase; CRTI, phytoene desaturase (bacterial type); LCY-B, lycopene beta cyclase; LCY-E, lycopene epsilon cyclase; and HYD, hydroxylase enzymes.

(v) From lycopene to provitamin A carotenes

Rings added by the enzyme LCY-B (lycopene beta cyclase) to both ends of the lycopene molecule result in the most active provitamin A carotenoid, beta-carotene, having two "beta" rings. Alternatively, LCY-E (lycopene epsilon cyclase), in combination with LCY-B, catalyses the biosynthesis of alpha-carotene, with one "epsilon" ring and one "beta" ring. In humans and animals, the central cleavage of beta-carotene results in two molecules of vitamin A (see **Figure 2**); cleavage of alpha-carotene results in only one molecule of vitamin A, which is derived from that half of alpha-carotene having the "beta" ring. As a result of the "epsilon" ring, alpha-carotene has only *half* the provitamin A activity compared with that of beta-carotene. Therefore, it is after lycopene formation that the pathway diverges, producing either more or less provitamin A active carotenoid, depending on the relative levels of the two cyclase enzymes LCY-E and LCY-B.

(vi) Conversion of provitamin A carotenes to non-provitamin A xanthophylls

After ring addition, both beta-carotene and alpha-carotene undergo addition of oxygen by HYD (hydroxylase) enzymes, giving rise to xanthophylls (oxygenated carotenoids) such as lutein (derived from alpha-carotene) or zeaxanthin (derived from beta-carotene). However, addition of oxygen further diminishes provitamin A activity. Other types of structural modifications give rise to the diversity of carotenoid compounds found in nature.

(vii) Summary of genes and enzymes needed for provitamin A carotenoid accumulation

Biosynthesis of the provitamin A carotenoid beta-carotene requires enzyme activity of PSY, PDS, ZDS and LCY-B (**Figure 1**). Alternatively, the bacterial enzyme CRTI takes the place of the two plant enzymes, PDS plus ZDS. HYD and LCY-E enzyme activities diminish the provitamin A value of carotenoids. In order to genetically engineer the pathway for provitamin A accumulation in any organism, the manipulation of the genes encoding the above enzymes is critical, as are genes encoding enzymes needed for accumulation of the carotenoid building blocks, IPP and GGPP.

4. Genetic engineering of carotenoid accumulation in rice and other plants

Survival of all photosynthetic organisms, including rice, depends on the production of carotenoids in green tissue. Therefore, one can assume that rice and other cereal crops all possess the genes needed for carotenoid biosynthesis, although these genes may not necessarily be expressed in seed tissue. From research on the pathway, it is understood that if pathway precursors are present, the accumulation of carotenoids such as beta-carotene is dependent on expression of the messenger RNAs encoding pathway enzymes. When efforts were first made to engineer the rice endosperm, it was determined that the plastid-containing endosperm lacked carotenoids, but did contain the carotenoid building block GGPP, making this tissue biochemically competent to biosynthesize carotenoids. To test this hypothesis, a gene encoding PSY from daffodil flowers was introduced into rice so that it was only expressed in endosperm tissue. As a result, phytoene accumulated in the rice endosperm (Burkhardt *et al.*, 1997). This indicated a potential for carotenoid accumulation in endosperm, but also that later pathway enzymes were still missing, since no compounds downstream of phytoene accumulated. Subsequently, the genes encoding daffodil PSY, LCY-B and a bacterial CRTI were introduced into rice and resulted in "golden rice" which accumulated beta-carotene (Ye *et al.*, 2000). However, it was surprising that some of the engineered rice also accumulated significant levels of xanthophylls, oxygenated carotenes that could only have been produced if the rice endosperm contained HYD enzyme activity. Since the HYD genes were not introduced as part of the genetic engineering effort, this result suggested that

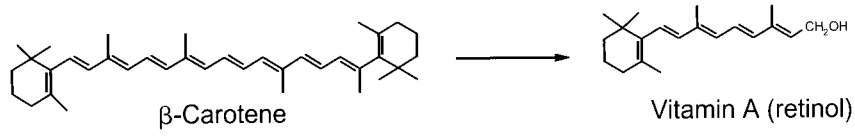

Figure 2 Conversion of betacarotene to vitamin A.

the rice endosperm already contained HYD enzyme activity.

In another study, where canola seeds were similarly engineered with the gene encoding PSY, a 50-fold increase in carotenoids was achieved (Shewmaker *et al.*, 1999). These transgenic (genetically engineered) canola seeds contained primarily beta-carotene and alpha-carotene. Compared with non-transgenic rice endosperm, which lacked carotenoid, non-transgenic canola seeds contained some carotenoids – mainly the xanthophyll lutein. The presence of xanthophylls indicated that non-transgenic canola seeds must have had HYD enzyme activity. However, this activity was insufficient to keep pace with a pathway engineered with an increased level of PSY enzyme, since the transgenic canola seeds accumulated mostly carotenes and little xanthophyll.

In contrast to the engineered rice endosperm where several pathway enzymes beyond PSY were required to confer carotenoid accumulation, increased carotenoid in canola seed was accomplished by introducing only one enzyme, PSY, suggesting that in non-transgenic canola seed PSY levels limit pathway throughput. This is not always the case, as was seen in the genetic engineering of a model bacterium where enhanced carotenoid accumulation was accomplished simply by increasing production of the carotenoid building block IPP (Matthews & Wurtzel, 2000).

5. Implications and future prospects

These first experiments to manipulate the carotenoid biosynthetic pathway are exciting and encouraging as they point to the enormous potential of introducing biochemical traits that will have a great impact on worldwide nutrition. From the rice and canola studies, it is clear that other important food crops, such as maize and wheat, will be good candidates for manipulation. The success in rice is the first example of a genetic engineering accomplishment in plants whereby a trait of nutritional value has been introduced and has potential for global impact. The pathway must be further introduced into native varieties, and other gene combinations incorporated to maximize pathway output for specific provitamin A carotenoids.

However, while the scientific problem appears to have been solved, there are still potential commercial problems. The technology used to create this rice involved as many as 32 companies holding 70 patents, each of which represents a barrier to further commercial development. Furthermore, the goal of the inventors of golden rice was to provide it at no cost to farmers in developing countries. In an effort to set a good example, Monsanto announced that they would grant patent licenses at no charge to developers of the "golden rice".

Another biotechnological success of engineering tobacco flowers to accumulate astaxanthin, a commercially valuable carotenoid (Mann *et al.*, 2000), points to a future of manipulating plants as biochemical factories instead of using traditional methods of organic synthesis, which sometimes provides impure mixtures. Plants genetically engineered for improved carotenoid composition will provide access to improved nutrition that is otherwise unavailable to much of the world's population.

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GLOSSARY

endosperm nutritional tissue surrounding the embryo in most seeds

biosynthetic pathway metabolic pathway in which small molecules are synthesized into larger organic molecules

isoprenoids compounds composed of one or more "isoprene units", a five-carbon saturated hydrocarbon

pathway engineering the application of recombinant DNA technology to alter or construct a sequence of enzymatically catalysed biochemical reactions that result from the activity of several gene products

See also **Super rice for increasing the genetic yield potential** (p.663)

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