Metabolic engineering in plants for food security

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Abstract

Over 800 million people around the world do not have enough to eat, and many more endure monotonous diets that fall short of even basic nutritional requirements. Food and nutritional insecurity are perhaps the most pressing and intractable social issues now faced by the world's governments and populations, and they can only be addressed by a combination of measures that aim to improve health, wealth and agricultural productivity in a sustainable fashion.

Genetically modified plants represent one of these measures, and many different strategies can be used to improve the yield, nutritional properties and agronomic performance of our crops. In particular, the metabolic engineering of crop plants is a versatile approach for enhancing the production of beneficial, small metabolites. Such molecules can provide nutritional benefits, protect plants against biotic and abiotic stresses and improve the ripening and storage properties of harvested products. In this review, we focus on two examples – vitamin biosynthesis and polyamine metabolism – to show how advances in plant metabolic engineering can offer hope to the world's poor and hungry.

Introduction

Metabolic engineering is the modification of endogenous metabolic pathways to increase flux towards particular desirable molecules or divert flux towards the synthesis of new molecules (Slater et al. 2003). In the not too distant past, metabolic engineering in plants was restricted to the manipulation of single genes. Although there have been some notable successes using single gene strategies, the impact of this approach is limited by the complex and extensive feedback mechanisms that operate in plants to maintain homeostasis. More recently, the focus of metabolic engineering has shifted from single gene to multiple gene strategies, and novel methods have been developed for the coordinated regulation of larger

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groups of genes (Capell and Christou 2004). While metabolic engineering in plants has been carried out predominantly to enhance the production of industrial or pharmaceutical metabolites (Verpoorte et al. 2000), there have been several recent examples where metabolic engineering has been used to improve agronomic or nutritional characteristics in plants, in the hope of addressing food security issues (Christou and Twyman 2004). We focus on two broad examples in this review, namely the modification of plants to enhance the production of vitamins and the manipulation of the polyamine biosynthesis pathway. These examples have been chosen because they illustrate some of the diverse strategies used in metabolic engineering, i.e. the enhancement of existing metabolic pathways, the extension of endogenous pathways to synthesize new compounds, and the use of a single metabolic engineering approach simultaneously to improve the nutritional properties of plants and to protect them from environmental stresses, thus favoring higher yields. The potential of such enhanced plants to address worldwide food security problems is discussed.

Enhanced vitamin production in transgenic plants

Vitamins are essential components of the human diet, which act predominantly as enzyme cofactors or prosthetic groups or as precursors for important signaling molecules. Both plants and microorganisms synthesize vitamins *de novo*, and plants represent the major dietary source of some vitamins, including vitamins E and K_1. Although the metabolic pathways for vitamin biosynthesis have been more fully elucidated in microbes than in plants, the engineering of plants for enhanced vitamin synthesis is now gaining much attention (Herbers 2003). While there has been some progress in dissecting and regulating the synthesis of vitamins C (ascorbic acid) and H (biotin) in plants (Alban et al. 2000; Conklin 2001), the most significant advances, at least in terms of addressing food insecurity, have been made with the synthesis of vitamins A and E.

Vitamin A

Vitamin A, or 11-*cis*-retinal, is required in all human and animal cells, tissues and organs but is particularly important in the eye, where it functions as the lipid prosthetic group of the visual pigment opsin. Vitamin A deficiency is a significant health threat in the developing world and is the most common (yet preventable) cause of blindness in developing countries (World Health Organization 2001). Humans usually obtain vitamin A directly from animal sources, but can synthesize it if provided with its immediate precursor, provitamin A (β-carotene), which is present at high levels in certain fruits and vegetables. The recommended daily

allowance of vitamin A is expressed as retinol equivalents, and is equal to about 6 mg of β-carotene per day. There is very little β-carotene in cereal grains, which represent the staple diet for many of the world's poorest people.

The synthesis of carotenes in plants begins with the linkage of two geranylgeranyl diphosphate molecules to form the precursor phytoene (Figure 1). The conversion of phytoene into β–carotene requires three further enzymatic steps. All four steps are absent in cereal endosperm tissue, so cereal grains accumulate geranylgeranyl diphosphate but not the downstream metabolic products in the pathway. Therefore, the synthesis of β-carotene in cereals represents an example of metabolic pathway extension, where novel enzymatic activities must be introduced into the plant and expressed in the endosperm to extend the pathway beyond its endogenous endpoint.

The four enzyme activities in the β-carotene synthesis pathway missing in cereal grains are phytoene synthase, phytoene desaturase, ζ-carotene desaturase and lycopene β-cyclase (Figure 1). The first major breakthrough was the development of rice grains accumulating phytoene. Burkhardt et al. (1997) described rice plants transformed with the daffodil (*Narcissus pseudonarcissus*) phytoene synthase gene which accumulated high levels of this metabolic intermediate. Further work by the same group (Ye et al. 2000) produced transgenic rice plants expressing the daffodil genes encoding phytoene synthase and lycopene β-cyclase, and the *crt*I gene from the bacterium *Erwinia uredovora* which encodes an enzyme with both phytoene

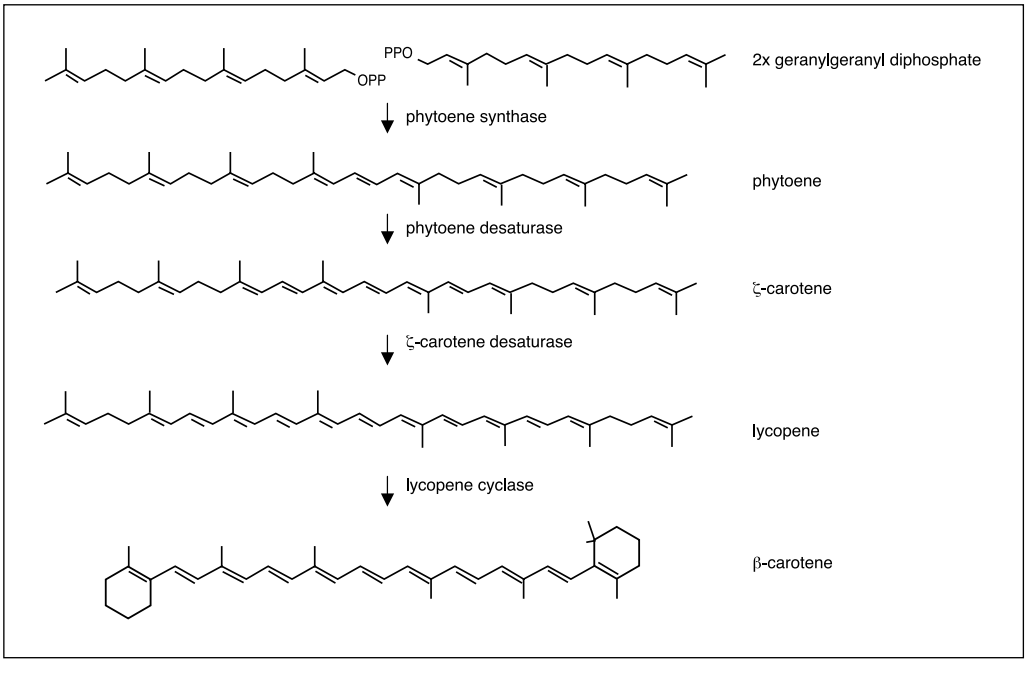

Figure 1. Enzymatic steps and metabolic products in the β-carotene biosynthesis pathway which are missing in cereal grains.

desaturase and ζ-carotene desaturase activities. The daffodil genes were expressed under the control of the rice glutelin-1 promoter, which is endosperm specific, while the bacterial gene was controlled by the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter. This ground-breaking, multi-gene engineering approach resulted in golden-colored rice grains containing up to 2 μg g^{-1} of β-carotene, in which case a moderate rice meal of 100 g would represent about 10% of the RDA for vitamin A. Interestingly, similar results were achieved in rice plants containing phytoene synthase and the bacterial *crt*I gene but lacking lycopene β-cyclase (Beyer et al. 2002). This suggested that rice grains either contain a residual endogenous lycopene β-cyclase activity or that the endogenous enzyme is dormant in wild-type grains but induced in the transgenic grains by the high levels of metabolic intermediates.

The "Golden Rice" project represented not just a technological breakthrough but also a model of humanitarian science that serves as an example for the deployment of other crops addressing food insecurity and malnutrition. From the beginning, the clear aim of the project organizers was to maintain freedom to operate and to provide the technology free of charge to subsistence farmers in developing countries, a feat that required careful negotiation over more than 100 intellectual and technical property rights (Potrykus 2001). Golden Rice fulfils an urgent need: it complements traditional interventions for vitamin A-deficiency and provides a real opportunity to address a significant world health problem. It was developed to benefit the poor and disadvantaged and will be provided to subsistence farmers with no attached conditions. It requires no additional inputs compared with other rice varieties. To avoid potential biosafety concerns (real or perceived), Golden Rice lines have been generated with the innocuous metabolic selection marker *mpi*, which allows regenerating plants to grow when mannose is the only carbon source (Lucca et al. 2001).

While β-carotene synthesis in rice has the greatest potential to address real food security and health problems, experiments in other plants have revealed further useful information about this important metabolic pathway. Transgenic tomatoes have been described expressing *Erwinia uredovora* phytoene synthase (*crt*B) (Fraser et al. 20020) and *crt*I (Romer et al. 2000) as well as a β-cyclase gene from *Arabidopsis thaliana* (Rosati et al. 2000). In the first case, fruit-specific expression of *crt*B was achieved using the tomato polygalacturonase promoter, and the recombinant protein was directed to the chromoplasts using the tomato phytoene synthase-1 transit sequence. Total fruit carotenoids were found to be two- to four-fold higher than in wild-type plants. Romer et al. (2000) expressed *crt*I constitutively, under the control of the CaMV 35S promoter. This, unexpectedly, reduced the total carotene content by about 50% but the levels of β-carotene increased three-fold to 520 μg g^{-1} dry weight. This probably reflects the existence of complex feedback mechanisms acting at several different levels, a possibility discussed in detail by Giuliano

et al. (2000). Rosati et al. (2000) used the fruit-specific tomato phytoene desaturase promoter to express the *A. thaliana* β-lycopene cyclase gene and increased β-carotene levels in transgenic fruits to 60 μg g^{-1} fresh weight. Work is ongoing to determine how the β-carotene pathway is regulated and what steps need to be taken to overcome feedback control.

Vitamin E

Vitamin E is actually a group of eight hydrophobic compounds: α-, β-, γ- and δ-tocopherol, and the unsaturated equivalents α-, β-, γ- and δ-tocotrienol. Dietary vitamin E is obtained mainly from seeds, and its function in the body is to prevent the oxidation and polymerization of unsaturated fatty acids. Deficiency leads to general wasting, kidney degeneration and infertility. The α-, β-, γ- and δ-derivatives differ in the number and position of methyl groups around the chroman ring as shown in Figure 2. The most potent vitamer is RRR-α-tocopherol, but common dietary sources of natural vitamin E such as soy oil are much richer in γ-tocopherol which has only 10% of the activity of α-tocopherol, while α-tocopherol itself is only a minor component. Natural vitamin E-supplements, which account for 10-15% of the total vitamin E market, are produced mainly from soy oil by chemically converting γ-tocopherol to α-tocopherol (Subramanian et al. 2000).

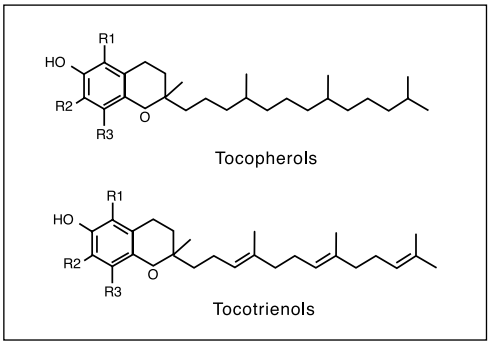

Figure 2. Structure of vitamin E. For α-derivatives, R1, R2 and R3 are methylated. For β-derivatives, R1 and R3 are methylated. For γ-deriviatives, R2 and R3 are methylated. For δ-derivatives only R3 is methylated.

Tocopherol synthesis in plants requires input from two metabolic pathways. The shikimate pathway generates homogentisic acid, which forms the aromatic ring of the compound, whereas the side chain is derived from phytyldiphosphate, a product of the methylerythritol phosphate (MEP) pathway (Figure 3). These precursors are joined together by the enzyme homogentisic acid prenyltransferase (HPT) to form the intermediate 2-methyl-6-phytylbenzoquinol (MPBQ). MPBQ is the substrate for two enzymes, tocopherol cyclase and MPBQ methyltransferase. The former enzyme produces δ-tocopherol while the latter introduces a second methyl group to form 2,3-dimethyl-5-phytylbenzoquinol. The action of tocopherol cyclase on this intermediate produces γ-tocopherol. Both δ-tocopherol and γ-tocopherol act as substrates for the enzyme γ-tocopherol methyltransferase (γ-TMT), producing α- and β-tocopherol, respectively. The relative abundance of

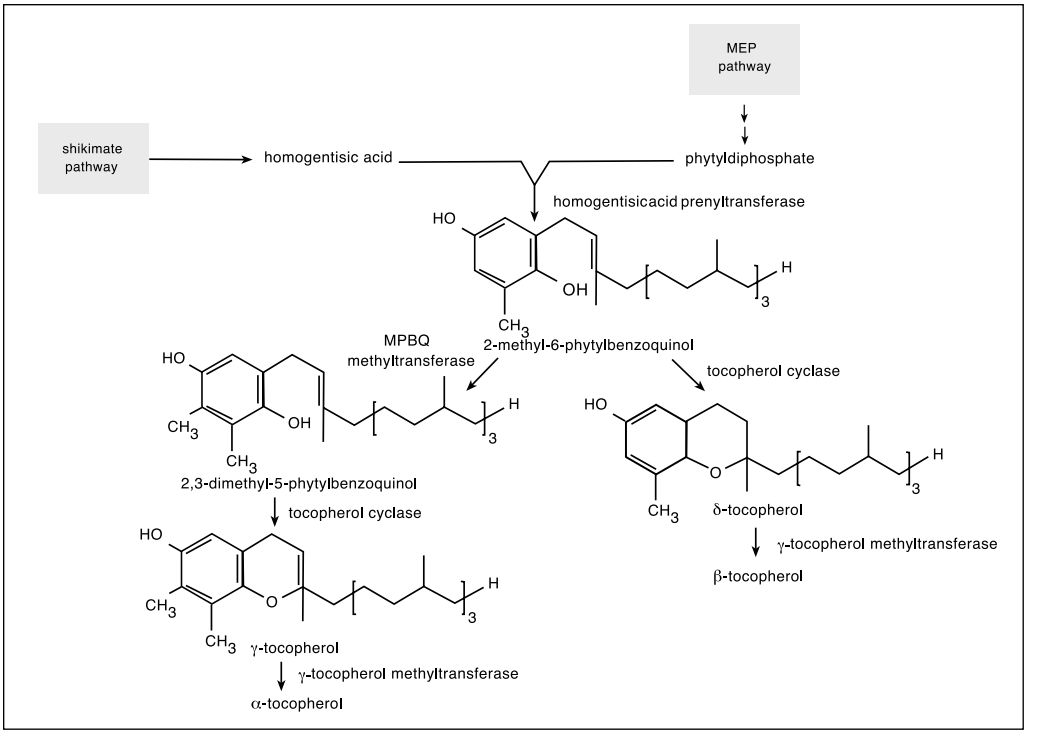

Figure 3. Late steps in tocopherol biosynthesis. MPBQ = 2-methyl-6-phytylbenzoquinol.

the four tocopherols in different plants is dependent on the activities of the enzymes discussed above.

Recent work carried out using the model plant *A. thaliana* has shown how the levels of vitamin E activity can be increased in the seeds of this plant, either by increasing the total amount of vitamin E or by shifting the vitamer profile towards the most potent form, α-tocopherol. Shintani and Della Penna (1998) expressed the *Synechocystis* PCC6803 and *A. thaliana* genes encoding γ-TMT in *A. thaliana* seeds using the carrot seed-specific DC3 promoter. This resulted in a radical shift from γ- to α-tocopherol (and from δ- to β-tocopherol) showing that nutritional enhancement in plants was possible without altering total vitamin A levels. In contrast, Savidge et al. (2002) overexpressed *A. thaliana* HPT, producing twice the level of vitamin E found in normal seeds, while Geiger et al. (2001) expressed the *E. coli tyrA* gene, which encodes a dual function enzyme (chorismate mutase and prephenate dehydrogenase), resulting in up to three times the normal level of vitamin E.

A very recent study has shown how the results obtained from experiments in *A. thaliana* can be used to produce soybean with enhanced nutritional properties, which have the real potential to address food and nutritional insecurity. As for Golden Rice, the success of this study relied on the simultaneous expression of mul-

tiple genes, in this case the *A. thaliana VTE3* and *VTE4* genes, encoding MPBQ methyltransferase and γ-tocopherol methyltransferase (Van Eenennaam et al. 2003). The transgenic soybeans showed a significant elevation in the total amount of vitamin E activity (five-fold greater than wild-type plants) which was attributable mainly to an eight-fold increase in the levels of α–tocopherol, from its normal 10% of total vitamin A to over 95%. In this case, multiple gene transfer was used not to extend a metabolic pathway, but to regulate the existing steps to increase flux towards desired metabolites.

Enhanced polyamine synthesis in transgenic plants

Polyamines are small, polycationic compounds that are found in all living organisms and are thought to be involved in a wide range of physiological functions, including the control of growth and cell division. Although humans can synthesize polyamines from the amino acid ornithine, this is an insufficient source and further polyamines must be obtained in the diet. The nutritional benefits of polyamines have been widely studied and are particularly noted for their impact on cell regeneration and growth (Bardocz 1993, 1995). In animals, polyamine-supplemented diets have been shown to provide instant energy for use in the small intestine (Bardocz et al. 1998), to promote weight gain and feeding efficiency by countering the effect of anti-nutritional factors (Grant et al. 1990; Smith 1990; Sousadias and Smith 1995; Mogridge et al. 1996) and to promote the maturation of glycan chains on proteins synthesized by intestinal cells (Greco et al. 2001).

In plants, the simplest polyamine (putrescine) can be synthesized from either ornithine or arginine through the activities of the enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). The other major polyamines, spermidine and spermine, are synthesized from putrescine (Figure 4). Spermidine is formed by the addition of an aminopropyl group donated by decarboxylated S-adenosylmethionine in a reaction catalyzed by spermidine synthase (SDE). Spermine is formed by the addition of a second aminopropyl moiety to spermidine, a reaction catalyzed by spermine synthase (SME) (Tiburcio et al. 1990). As in animals, polyamines in plants are thought to be involved in many different physiological processes (Walden et al. 1997; Malmberg et al. 1998) and are thought to be particularly important in stress responses (Bouchereau et al. 1999). Therefore, the manipulation of polyamine metabolism in plants promises dual benefits, i.e. enhanced nutritional properties and improved stress responses, allowing survival in more extreme environments and promoting higher yields. Rarely are scientists presented such an opportunity to "kill two birds with one stone".

Studies on the polyamine biosynthesis pathway have been carried out predominantly in tobacco and rice, with many enzymatic steps being perturbed either by overexpression or antisense suppression (Kumar et al. 1997; Malmberg et al. 1998; Kakkar and Sawhney 2002). The main aim of these studies has been to investigate

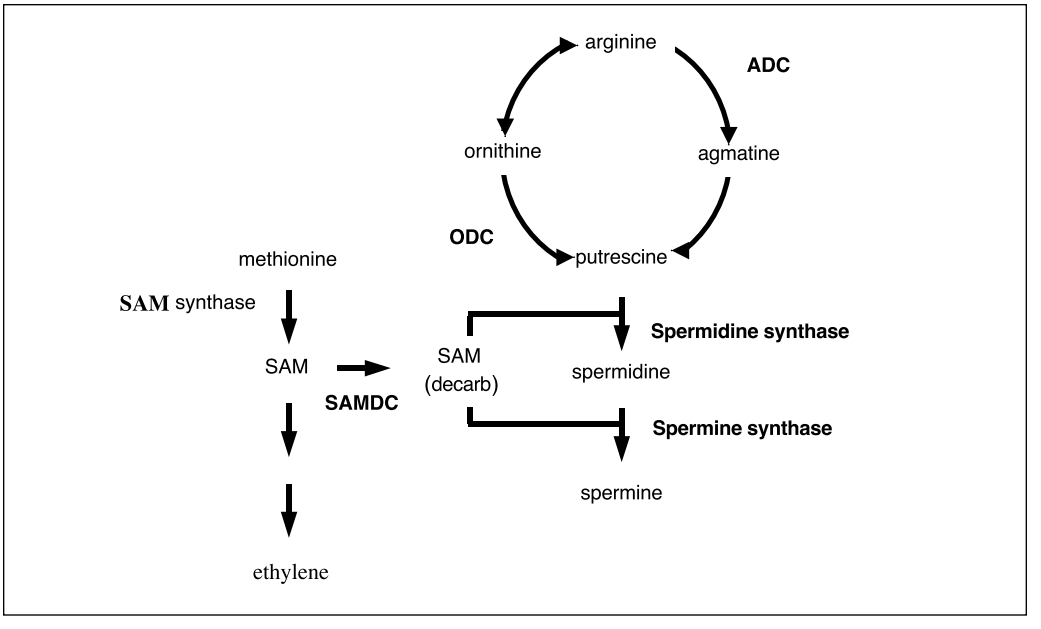

Figure 4. Polyamine biosynthesis pathway in plants. ADC = arginine decarboxylase, ODC = ornithine decarboxylase, SAM = S-adenosylmethionine, SAMDC = SAM decarboxylase.

the effect of increased (heterologous) enzyme activity or suppressed endogenous enzyme activity on the activities of other enzymes in the pathway, the levels of the three major polyamines and the overall impact on phenotype.

The primary targets have been the ADC and ODC enzymes which are required for the synthesis of putrescine. Burtin and Michael (1997) expressed the oat gene encoding ADC in tobacco plants under the control of the constitutive CaMV 35S promoter. Although this increased the total ADC activity in the plants and promoted the accumulation of agmatine (the immediate product of ADC and the direct precursor of putrescine), there was no change in overall polyamine levels nor in the activities of the enzymes ODC or SAMDC (S-adenosylmethionine decarboxylase). In contrast, inducible expression of the same gene in tobacco significantly increased the levels of putrescine and at high levels this produced an abnormal vegetative growth phenotype that resembled the effects of osmotic stress in oat leaves (Masgrau et al. 1997).

In rice, we showed that the constitutive expression of oat ADC using the CaMV 35S promoter resulted in a four-fold increase in ADC activity and a concomitant four-fold increase in putrescine levels, but there was no increase in the levels of spermidine or spermine in these callus lines (Capell et al. 1998). In contrast, when the CaMV 35S promoter was replaced with the stronger maize ubiquitin-1 promoter, a general increase in the levels of all three polyamines was observed in regenerating shoots, supporting a "threshold model" in which a certain trigger level of putrescine

must be achieved to drive metabolic flux towards the downstream polyamines (Bassie et al. 2000). There was no significant change in polyamine levels in the mature vegetative tissues or seeds of these plants, with the exception of one particular transgenic line described in more detail by Noury et al. (2000). In this line, there was a dramatic increase in putrescine levels in seeds (up to ten times normal levels) and also a 1.5-fold increase in spermine.

Chattopadhyay et al. (1997) showed that endogenous ADC activity in a salt-resistant rice cultivar is induced by salt stress whereas ADC activity declines in a salt-sensitive cultivar under the same conditions. Furthermore, Roy and Wu (2001) showed that rice plants expressing the oat gene encoding ADC under the control of a stress-induced promoter were able to maintain biomass accumulation under salt stress while wild-type plants fared poorly. Therefore, we generated transgenic rice plants expressing the *Datura stramonium adc* gene and investigated their response to drought stress compared to wild-type. Wild-type plants responded to the onset of drought stress by increasing endogenous putrescine levels, but this was insufficient to trigger the conversion of putrescine into spermidine and spermine (the agents that actually protect plants under stress). In contrast, transgenic plants expressing *D. stramonium adc* produced much higher levels of putrescine under stress conditions, promoting spermine and spermidine synthesis and ultimately protecting the plants from drought (Capell et al. 2004). Again, this supports a threshold model in which a sufficient level of putrescine, perhaps acting as a stress warning signal, must accumulate before higher polyamines with protective effects are synthesized. Such plants have a great potential to address food insecurity by providing drought tolerance and allowing the cultivation of marginal soils to grow food crops.

Experiments with transgenic plants expressing ornithine decarboxylase have also provided insight into the regulation of polyamine biosynthesis. Tobacco root cultures expressing yeast ODC showed a two-fold increase in putrescine and the alkaloid nicotine, which is derived from putrescine (Hamill et al. 1990). The authors commented that despite the use of a very strong promoter, the levels of metabolites were only doubled, suggesting tight regulation of the pathway. The mouse ODC gene, expressed in tobacco and carrot, significantly increased the levels of putrescine in callus (DeScenzo and Minocha 1993; Bastola and Minocha 1995; Andersen et al. 1998). In carrot, the increased putrescine promoted somatic embryogenesis, confirming the role of polyamines in the control of plant growth and morphogenesis (Bastola and Minocha 1995). Studies in carrot suggested that increases in ODC activity has no effect on the ADC branch of the pathway, but our experiments with the antisense suppression of ADC activity nevertheless suggest that the two branches are metabolically linked. In rice cell lines transformed with the oat gene encoding ADC in the antisense direction, reductions were seen in the activities of the endogenous ADC and ODC genes, resulting in a significant reduction in the accumulation of putrescine in the seeds of transgenic rice plants, but very little impact on vegetative tissues (Capell et al. 2000). In rice plants constitutively expressing

human ODC, the levels of all three polyamines were increased in seeds and in vegetative tissues, contrasting with our earlier results for plants expressing ADC and suggesting that ODC rather than ADC is the major regulatory step in putrescine synthesis (Lepri et al. 2001). Polyamine accumulation specifically in the rice grains was possible by expressing ODC under the control of the wheat seed-specific, low molecular weight glutenin promoter, and such lines could be very useful to improve the nutritional properties of rice grains without affecting the growth or development of vegetative parts of the plant.

Another key enzyme in polyamine biosynthesis, SAMDC, has also been targeted in transgenic strategies. We set out to establish whether the overexpression of a heterologous SAMDC gene in rice would directly affect the levels of spermidine and spermine by influencing the later stages of the biosynthetic pathway. The *D. stramonium* SAMDC gene was expressed in rice under the control of the strong constitutive ubiquitin-1 promoter, and the expected increase in SAMDC activity increased the levels of spermidine, but not of spermine, in leaves, whereas both of these polyamines accumulated in seeds (Thu-Hang et al. 2002). As in other studies (Bassie et al. 2000; Lepri et al. 2001), these results show distinct, tissue-specific effects on polyamine accumulation that reflect regulatory processes adapted for different tissues. Roy and Wu (2002) expressed SAMDC in transgenic rice plants and found that, like their ADC transgenics, these plants were protected from salinity stress. When the yeast *SAMDC* gene was expressed in tomato under the control of a ripening-fruit-specific promoter, elevated spermine and spermidine levels were detected in fruits resulting from increased conversion of putrescine into these higher polyamines (Mehta et al. 2002). This led to an increase in the levels of lycopene, prolonged the vine life and increased the nutrient value of the tomato juice.

Conclusions

In this review, we have discussed three major metabolic pathways – β-carotene synthesis, tocopherol synthesis and polyamine synthesis – whose genetic manipulation exemplifies the way in which metabolic engineering could contribute to worldwide food and nutritional security in the future. The β-carotene and tocopherol cases show how the growing sophistication of multi-gene engineering allows existing metabolic pathways to be modulated or extended to generate foods with nutritional properties tailored to address specific nutrition deficiencies. In particular, the Golden Rice project provides a shining and virtuous example of how metabolic engineering can be carried out for the good of hungry and malnourished people without getting bogged down in technical and intellectual property issues. The polyamine case shows how different problems can be addressed by tackling the same pathway in different ways. By modifying single steps in the pathway and con-

trolling when and where the genes are expressed, we have succeeded in producing nutritionally enhanced, transgenic rice grains with increased levels of all three major polyamines. We have also produced rice plants constitutively expressing one of the genes in the polyamine pathway that provides protection against osmotic stress, allowing growth in soils that are too dry to support the growth of wild-type plants. These plants are currently being studied in the field in two diverse agro-climatic environments.

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