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Symposium on 'Enhancing the nutritional value of plant foods'

Biofortification of essential nutritional compounds and trace elements in rice and cassava

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Plant biotechnology can make important contributions to food security and nutritional improvement. For example, the development of 'Golden Rice' by Professor Ingo Potrykus was a milestone in the application of gene technology to deliver both increased nutritional qualities and health improvement to wide sections of the human population. Mineral nutrient and protein deficiency as well as food security remain the most important challenges for developing countries. Current projects are addressing these issues in two major staple crops, cassava (*Manihot esculenta* Crantz) and rice. The tropical root crop cassava is a major source of food for approximately 600 million of the population worldwide. In sub-Saharan Africa >200 million of the population rely on cassava as their major source of dietary energy. The nutritional quality of the cassava root is not sufficient to meet all dietary needs. Rice is the staple food for half the world population, providing approximately 20% of the *per capita* energy and 13% of the protein for human consumption worldwide. In many developing countries the dietary contributions of rice are substantially greater (29·3% dietary energy and 29·1% dietary protein). The current six most popular 'mega' rice varieties (in terms of popularity and acreage), including Chinese hybrid rice, have an incomplete amino acid profile and contain limited amounts of essential micronutrients. Rice lines with improved Fe contents have been developed using genes that have functions in Fe absorption, translocation and accumulation in the plant, as well as improved Fe bioavailability in the human intestine. Current developments in biotechnology-assisted plant improvement are reviewed and the potential of the technology in addressing human nutrition and health are discussed.

Biofortification: Rice: Cassava

A sufficient and balanced diet is possibly the most important contribution to human health and prophylaxis. Diet should not only supply the energy pathways but also the essential amino acids, vitamins and trace elements, particularly the uncommon S-rich amino acids, lysine, methionine, vitamins A, C, D and E, B vitamins, folic acid and the ionic elements I, Fe, Zn and Se. Chronic deficiency of these nutrients can lead to severe symptoms, including developmental disturbances of the fetus, restrictions of cognitive perception in children, lethargy, blindness and acute immunological malfunctions. Since these symptoms are not obviously associated with an inadequate diet, this malnutrition is also termed 'hidden hunger'. It is estimated (World Health Organization, 2002) that about two billion of the world population, primarily women and children, suffer from Fe malnutrition, while about 2·8 million children suffer from severe symptoms of vitamin A malnutrition. Malnutrition is not restricted to developing countries. In Western industrial nations malnutrition not only affects low-income groups in the population but also individuals with special dietary habits, such as children of vegan or macrobiotic mothers (Black, 2003). About 11% of the female population in Switzerland suffers from hidden anaemia as a consequence of Fe deficiency (Eichholzer, 2003). Malnutrition also has economic consequences. In Bangladesh about 1–2% of the gross national product is lost annually as a consequence of Fe deficiency (van den Briel & Webb, 2003).

Deficiency in essential amino acids and trace elements is most widespread in developing countries. The poor in

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particular cannot afford a balanced diet, and this group represents a large proportion of the population of large cities. According to Food and Agriculture Organization (2004) about 30% of these populations are poor and have no possibility of growing any crop plants. Providing staple food fortified with essential amino acids, vitamins and trace elements at no additional cost to the consumer is a provisional solution to the most pressing problem of vitamin and trace element deficiency for the poor in the population.

A number of methods for fortifying food have been suggested, some of which have potential, while others have been unsuccessful for a variety of reasons, i.e. the chemical properties of the substances, unfavourable conditions for their distribution or economic reasons. I or vitamin C, for example, can easily be added to common salt (García-Casal *et al*. 2003), folic acid can be added only with difficulty because of its high water solubility (Shrestha *et al*. 2003) and Fe cannot be added because it oxidizes (Boccio & Lyengar, 2003). For decades distribution programmes for vitamin A have existed without improving the situation substantially, because the distribution does not reach the target populations (Pirie, 1983; Sommer, 1989). An alternative method would be to fortify crop plants directly. Fortification by agronomic practice, e.g. using fertilizers containing trace elements, is very difficult for developing countries (Cakmak, 2002) and completely impossible for complex compounds such as provitamin A. Molecular breeding and gene technology, on the other hand, offer prospects and should be considered as a means of improving the quality of food and thus the health of the consumers (Zimmermann & Hurrell, 2002).

Breeding *v*. gene technology

Conventional and molecular breeding can recombine genes to a limited extent, and thus generate more favourable properties. For new properties that cannot be developed with the available genes in a given gene pool, breeding depends on a few useful mutations. The possibility of achieving changes in the expression pattern of genes through breeding is therefore minimal. Provitamin A (β-carotene), for example, is produced in green parts of all plants as a pigment to protect chlorophyll against light damage, but is not produced in the starch-storing part of the rice endosperm. Historically, one mutation is known that also allows the production of β-carotene in the root, but this mutation occurred in carrots several centuries ago (World Carrot Museum, 2005). The chance of achieving such a result for the production of β-carotene in the endosperm of rice by breeding is negligible.

Breeding is very difficult in vegetatively-propagated crop plants. In these species the lines and varieties are highly heterozygous, so that the varieties are lost during cross-breeding and the cross-bred products revert to a wild type. This outcome has been recorded for potatoes and native fruit varieties including the grapevine. In tropical and subtropical developing countries it is particularly true for cassava (*Manihot esculenta* Crantz), an important staple crop particularly for the poor.

In these cases, where breeding does not offer a solution, gene technology can help; with gene technology single genes can be isolated and their promoters can be replaced by appropriate promoters that activate the gene in a different organ. Such a chimeric construct of a promoter and a gene can then be brought back by gene transfer into the same or another plant species. Genetic transformation does not change the genetic background of the plant, in contrast to breeding. This process represents the best method of adding a new characteristic to a plant without changing its other properties (Klein & Fitzpatrick-McElligott, 1993; Potrykus *et al*. 1995; Sinclair *et al*. 2004). In the following paper the methodology for genetically modifying a plant will be discussed, with particular reference to the most important approaches for biofortification in rice and cassava.

Gene technology

Before a gene can be used for gene transfer, it has to be identified and isolated. A common tool used in this process is the defective mutation. Molecular methods involving transposons, the so-called 'jumping genes', can be used to create defective mutations. Their phenotype indicates their function (Fig. 1(a)). Since the nucleotide sequence of the transposon is known, the neighbouring gene fragments of the defective gene can be sequenced (Fig. 1(b)). With this information about part of the nucleotide sequence of the gene the complete functional gene can be isolated from the

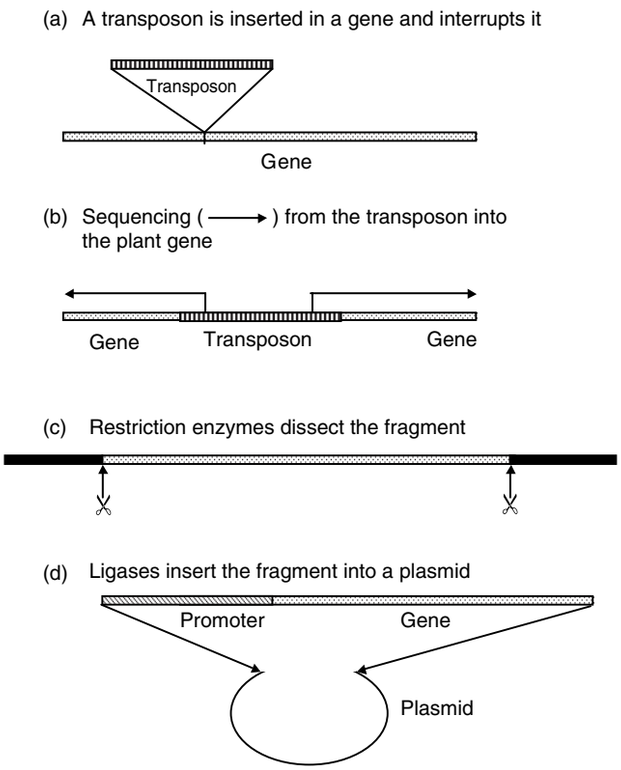

Fig. 1. Molecular methods and gene transfer. ✂, Restriction enzymes.

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wild-type plant. For this purpose natural 'gene scissors' (restriction enzymes) are used that act at a specific position in the nucleotide sequence (Fig. 1(c)), together with natural ligases that link the gene fragments again at their ends (Fig. 1(d)). This method allows the isolation of genes with distinct functions from their natural organism and also the exchange of promoters, i.e. 'switches' that determine when and where the gene is active during development.

Embedded in a plasmid, a ring-shaped genetic structure of bacteria, the construct can be multiplied in bacteria, from where it can then be isolated in large quantities for further studies or for gene transfer.

For gene transfer in plants a natural transfer system is normally used, such as *Agrobacterium*, a ubiquitous soil bacterium, which during wounding of plant tissue transfers part of its own genome into plant cells and integrates it into the plant genome. This section of the bacterial genome naturally contains genes that stimulate the plant to increase its production of plant-growing hormones. This action leads to tumours, so-called 'crown galls' or intensive branching from a single point (termed 'witches' brooms'). If the genes for additional hormone production are replaced with genes that have other properties, then the plant can be supplemented with these new characteristics and the hormone-dependent abnormalities do not occur. Although *Agrobacterium* affects only a limited number of host plants naturally, this method of gene transfer can be used for nearly all important crop plants.

The additional gene is inherited like any other gene according to Mendelian laws. Providing the GM plants are homozygous with reference to the transgene and they are crossed with a plant that is also homozygous for the same gene, then the transgene no longer segregates, i.e. all progeny will also possess the transgene. In terms of its molecular properties, the additional gene does not differ basically from the other, previously present, internal genes. Thus, GM plants should be assessed according to their phenotype like traditionally-bred plants. Indeed, before market release GM plants are assessed much more strictly for their characteristics as compared with any traditionally-bred plant. Thus, the putative health hazards alleged by pressure groups are excluded.

Rice

Rice feeds more than half the human population worldwide, most of whom live in developing countries and many have (at least during certain seasons) no other or virtually no other diet. Rice contains small amounts of Fe and provitamin A in the aleurone layer of the seeds, which is polished away in order to prevent the kernels becoming rancid. Consequently, vitamin A and Fe deficiencies are widespread. Although certain rice varieties have been reported to contain more provitamin A or Fe in the central part of the seeds (Welch & Graham, 2004), traditional breeding has not yet solved the problem. Although for provitamin A the problem is close to being solved, for Fe a solution is currently actively being sought. These studies will be reviewed.

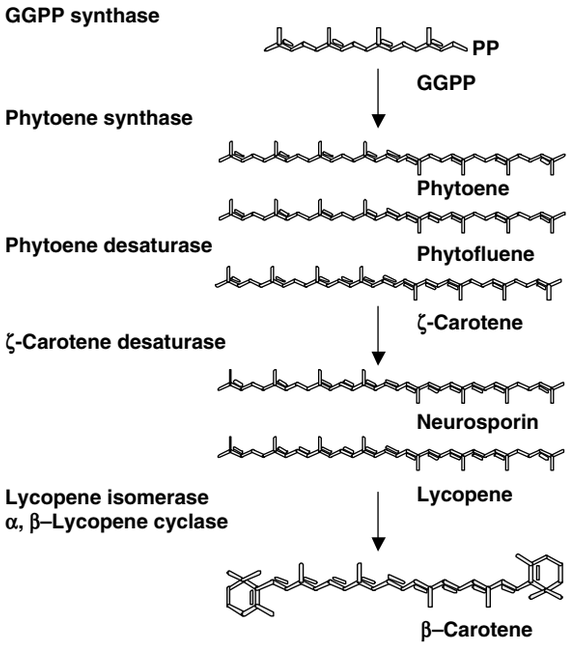

Fig. 2. Biosynthesis of carotene. GGPP, Geranylgeranyl diphosphate.

Provitamin A

Geranylgeranyl diphosphate is present in rice kernels as a substrate for provitamin A production (Fig. 2), being modified by phytoene synthase to phytoene (Burkhardt *et al*. 1997). Subsequently, two desaturases transform phytoene first to phytofluene and then via ζ-carotene to neurosporin, which spontaneously changes into lycopene. Lycopene isomerase and α,β-lycopene cyclase produce the final product β-carotene (provitamin A; Ye *et al*. 2000), which is dissolved in membrane lipids and after ingestion is split into two molecules of retinol (vitamin A) in man.

The breakthrough in the project to develop provitamin A-containing Japonica rice was the transfer of several genes for enzymes of the carotene biosynthesis pathway (Ye *et al*. 2000). This study has shown that not only single genes and their characteristics, such as insect resistance or herbicide tolerance, but also complete metabolic pathways can be supplemented with several enzymes. These genes have now been introduced into Indica rice varieties, which are the main rice genotypes grown in the twenty-six countries with widespread vitamin A deficiency, including India, Indonesia, Thailand and China. Moreover, these new lines provide sufficient levels of provitamin A at normal dietary intakes (Paine *et al*. 2005), which invalidates an earlier criticism that GM rice does not contain enough provitamin A for adequate intake in a normal daily diet (Dawe *et al*. 2002).

Field tests in the USA during summer 2004 have shown that GM rice varieties produce sufficient provitamin A in their kernels even when grown outdoors under natural conditions. This field test has yielded for the first time a substantial amount of material that can now be used for feeding trials (Ingo Potrykus, personal communication).

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Provitamin A is lipophilic and can be resorbed in the intestine only if dissolved in lipids. Comparative studies with carrots are misleading, as in the storage root of carrots most of the provitamin A is not dissolved in membranes but is present in a crystalline form (Herrmann *et al*. 1992) and has to be dissolved in lipids for resorption in the intestine. Other concerns, including the yellow colour of provitamin A-enriched rice, are issues of consumer behaviour. This aspect can only be tested by offering it on the market, when in contrast to their theoretical response in polls consumers often decide differently when they make the practical choice in a supermarket. This issue of choice is probably similar for small-scale farmers in the developing world.

It is often claimed that intellectual property rights make the commercial seed too expensive for poor subsistence farmers to afford. Indeed, the development of a GM plant can involve up to seventy patents from thirty different companies (Kryder *et al*. 2000). This issue of the protection of intellectual property has been resolved in the case of 'Golden Rice' in a way that could become an example for the release of further transgenic lines in developing countries; the companies have agreed to forgo their royalties, providing the farmer or seed producer do not make a profit of >US$10 000/year with those lines. Thus, small-scale farmers and small seed producers are exempt, whereas royalties are required from large-scale profits, e.g. if 'Golden Rice' is sold as a 'designer' food in industrial countries.

Even according to conservative estimates 'Golden Rice' promises an increase in gross national product as compared with the fortification of wheat flour with provitamin A (Dawe *et al*. 2002). Zimmermann & Qaim (2002) suggest that this increase should be in the range of US$137 $\times 10^6$ for the Philippines. Thus, it seems surprising that governments are not more progressive in permitting the release of these rice plants, especially since biosafety is not a major issue in this case.

Recently, studies have been undertaken to produce vitamin A directly in the GM plants, although the results have not yet been published. In this case it has to be recognized that the presence of vitamin A can lead to hypervitaminoses, while that of provitamin A does not.

Iron

The Fe content of the rice endosperm is low (between 7 and 24 mg/kg; Graham *et al*. 1999). This factor could explain the low success rate in breeding for improved Fe content in the endosperm. Fe is present in sufficient amounts in nearly all soils, but it is not accessible for plants because it is either chemically bound or it is present as Fe^{3+} (plants can only take up Fe^{2+}). Furthermore, unlike provitamin A, Fe cannot be produced by the plant. Thus, the approach to increasing the Fe content of the plant is more complex. First, the intake into the plant must be facilitated, then the transport of the Fe into the kernels and, finally, the storage in the kernels has to be improved. In addition, absorption of Fe in the intestine can be reduced by anti-nutrients, one of which is phytate, a derivative of a sugar that serves as storage for phosphate in the kernels of cereals (Fig. 3). In rice Fe as Fe^{2+} is bound by phytate, which inhibits its resorption in the intestine. On the other hand, some amino acids, e.g. cysteine, improve intestinal resorption (Poletti *et al*. 2004). Currently, the possibility cannot be excluded that in plants the Fe content is regulated by homeostasis, which can lead to even more complexity. The approach that is being followed therefore includes genes that are involved in all these physiological processes, as summarized in Fig. 4.

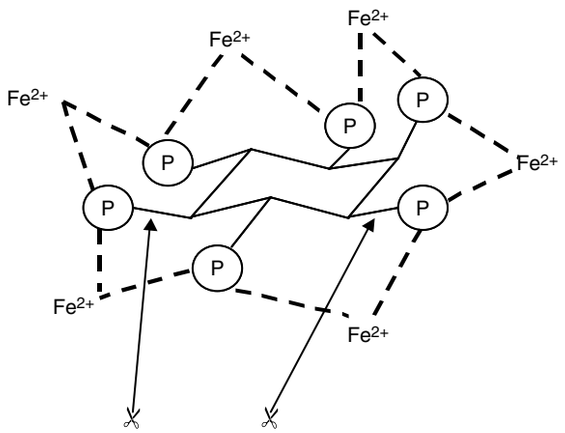

Fig. 3. Structure of phytic acid. Inositol hexaphosphate binds divalent metal ions (Fe^{2+}) via phosphate groups (P). Phytase dephosphorylates (✂) phytate and thereby releases iron for its resorption in the intestine.

When genes for the Fe-storage protein ferritin from leguminous plants (common field bean (*Phaseolus vulgaris*) and soyabean) are expressed under the control of an endosperm specific promoter in rice, the Fe content in the endosperm increases by ≥3-fold (Goto *et al*. 1999; Lucca *et al*. 2002; Vasconcelos *et al*. 2003). On the other hand, Takahashi (2003) has used the gene for nicotianamine synthase, which can stimulate the production in the roots of siderophores, which when exuded from the root complex the Fe as a chelate and improve its uptake into the plant. Uptake and transport are also increased by a Zn transport protein from *Arabidopsis thaliana*, as has been demonstrated in barley (Ramesh *et al*. 2004). Phytase degrades phytate, which inhibits Fe absorption in the intestine, and phytase from a fungus (*Aspergillus fumigatus*) has been expressed in rice, although the heat-stable enzyme loses most of its activity during cooking (Lucca *et al*. 2002). However, an alternative is the highly-heat-resistant phytase that has long been used by the feed industry as an additive to animal feed. Finally, when the gene for a cysteine-rich metallothionine is over-expressed in rice, the cysteine content in the soluble fraction of the seed proteins is increased by ≤7-fold, which can improve Fe uptake in the intestine (Lucca *et al*. 2002). Feeding trials can only be performed, however, when sufficient material from field tests is available.

Cassava

Cassava is a tropical and subtropical crop producing large starch-rich storage roots that feeds about 800 million

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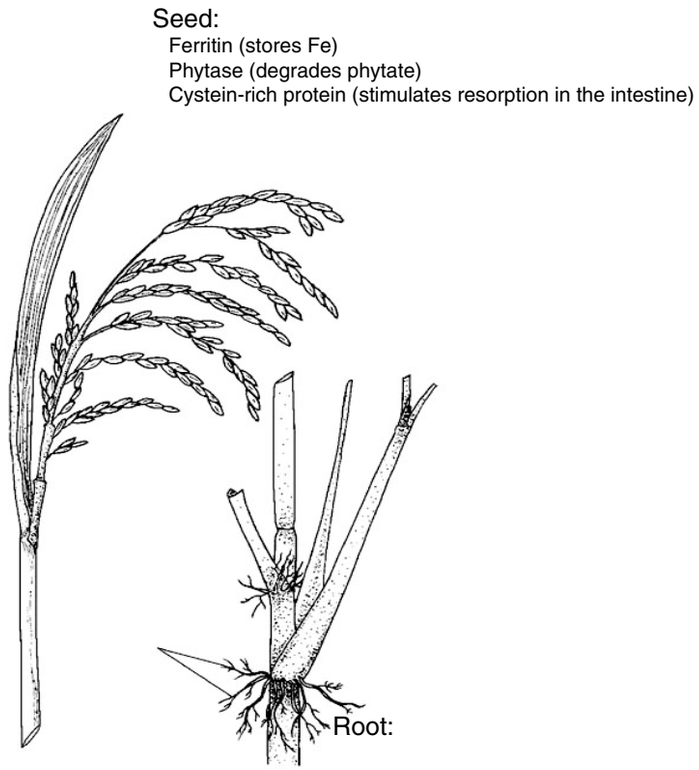

Fig. 4. Approach to iron fortification of rice.

among the world's poorest populations. Cassava grows in poor soils and is resistant to Al, a common element in tropical soils. During drought conditions cassava loses its leaves, but when conditions become sufficiently humid it produces new leaves. The roots can remain in the soil until they are consumed, so that storage facilities are not required. Part of the stem of the harvested plant is transplanted in the soil, where it roots and produces new plants.

Cassava also has disadvantages; it is a member of the *Euphorbiaceaen* family and thus contains cyanogenic glycoproteins. These compounds produce cyanide in the event of an injury to the plant, when the normal compartmentalization is disturbed. Thus, cassava has to be either boiled for 3 h or otherwise processed (e.g. fermented) in order to obtain pure starch. As the roots contain only low levels of protein, the processed product is protein-free. Consequently, individuals consuming exclusively or predominantly cassava usually suffer from protein-deficiency symptoms. Thus, fortification of cassava is primarily a question of amino acids and only secondarily of trace elements.

Protein fortification

An artificial protein, artificial storage protein 1 consisting of four identical subunits, has been constructed with the maximum possible content of essential amino acids (Fig. 5). The gene for this protein has been transferred together with a root-specific promoter into cassava (Zhang *et al*. 2003), and using serological methods the protein is detectable in the transgenic plants. Thus, this approach has potential for increasing the protein content of cassava roots. Whether the expression of an additional protein also increases the amount of essential amino acids is not known.

In rice this approach only redistributes a constant amount of S-rich amino acids and does not increase the total amount (Hagan *et al*. 2003). In other cases an accumulation of methionine has been reported (Lai & Messing, 2003), which does, however, require manipulation of the mRNA stability. It is possible that the equilibrium of the amino acids also has to be adapted, e.g. with codon-modified tRNA (Wu *et al*. 2003), which has been demonstrated to increase lysine content in cereals.

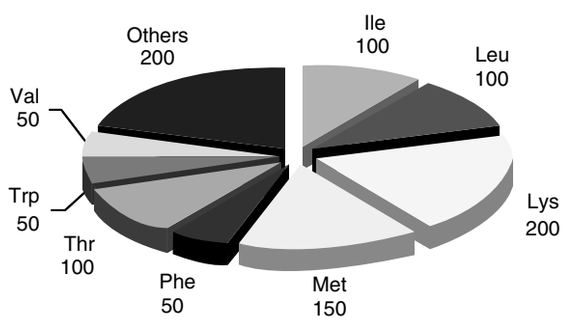

Fig. 5. Amino acid composition (mg/g) of artificial storage protein 1.

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Prospects

In addition to vitamin A, other important vitamins that could be enriched in plants using gene technology are vitamin E and folic acid.

Tocopherol occurs in plants in four different forms, α-, β-, γ- and δ-tocopherol, which differ in the position of a methyl group on the phenol ring (Kanwischer *et al*. 2005), but only α-tocopherol can substitute for vitamin E as an additive to food (Leonard *et al*. 2004). Thus, the accumulation of α-tocopherol has been suggested as a means of vitamin E fortification of plants (Hofius & Sonnewald, 2003). Currently, attempts are underway to promote the accumulation of α-tocopherol in soyabean by gene technology (Sattler *et al*. 2004).

Folic acid is an essential cofactor for reactions in which a C moiety is transported, and such reactions are important in both animals and plants (Sattler *et al*. 2004). While man is unable to synthesize folic acid, plants synthesize tetrahydrofolate in plastids from chorismate via p-aminobenzoate and from 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate derived from GTP in the cytosol. Tetrahydrofolate is the substrate for all other folic acids (Hanson & Gregory, 2002). Currently, attempts are being made to promote the accumulation of folic acid in tomato and other crop plants (Díaz de la Garza *et al*. 2004; Hossain *et al*. 2004). It has been shown that the expression of a mammalian GTP cyclohydrolase, which is not regulated by a feedback mechanism in plants, could lead to the accumulation of the intermediate product pterin by ≤ 1250-fold, which could increase folic acid production by 10-fold (Díaz de la Garza *et al*. 2004). This impressive example shows how the homeostasis of specific biochemical pathways can be positively modulated by gene technology, an outcome that has little possibility of being achieved using classical breeding (Poletti & Sautter, 2005).

Zn is another important trace element that is important not only for the consumer but also for the plant itself. When the Fe content is increased, the content of Zn is often also improved in plants because Zn, also a divalent metal ion, is taken up by the plant and transported similarly to Fe^{2+}.

The first generation of GM plants had benefits primarily for the farmers and for production, but also for the environment. The new generation of GM plants offers targetted improvements in the nutritional quality and the health of the consumer. 'Golden Rice' has paved the way for this new generation of GM plants and its development is already well advanced. This progress increases confidence that other health-promoting fortifications with essential amino acids will also be made available in the future. A prerequisite for success, particularly in the poorest developing countries, is that the public are open-minded and there is a readiness to apply gene technology in agronomy together with a willingness to provide sufficient financial resources for this promising field of research.

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