Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways

Shaista Naqvi[a,1], Changfu Zhu[a,1], Gemma Farre[a], Koreen Ramessar[a], Ludovic Bassie[a], Jürgen Breitenbach[b], Dario Perez Conesa[c], Gaspar Ros[c], Gerhard Sandmann[b], Teresa Capell[a], and Paul Christou[a,d,2]

[a]Departament de Producció Vegetal i Ciència Forestal, Universitat de Lleida, 25198 Lleida, Spain; [b]Biosynthesis Group, Molecular Biosciences, Johann Wolfgang Goethe Universität, D-60054 Frankfurt, Germany; [c]Departamento de Technologia de Alimentos, Nutricion y Bromatologia, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain; and [d]Institucio Catalana de Recerca i Estudis Avancats, 08010 Barcelona, Spain

Communicated by Gurdev S. Khush, University of California, Davis, CA, February 10, 2009 (received for review November 24, 2008)

Vitamin deficiency affects up to 50% of the world's population, disproportionately impacting on developing countries where populations endure monotonous, cereal-rich diets. Transgenic plants offer an effective way to increase the vitamin content of staple crops, but thus far it has only been possible to enhance individual vitamins. We created elite inbred South African transgenic corn plants in which the levels of 3 vitamins were increased specifically in the endosperm through the simultaneous modification of 3 separate metabolic pathways. The transgenic kernels contained 169-fold the normal amount of β-carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate. Levels of engineered vitamins remained stable at least through to the T3 homozygous generation. This achievement, which vastly exceeds any realized thus far by conventional breeding alone, opens the way for the development of nutritionally complete cereals to benefit the world's poorest people.

folic acid | metabolic engineering | transgenic maize | vitamin A fortification | vitamin C

Micronutrient deficiency is a major challenge to health organizations and governments throughout the world, with an estimated 40–50% of the world's population suffering at any one time from diseases caused by a lack of essential minerals and vitamins (1, 2). In industrialized societies, micronutrient deficiency is addressed by ensuring that fresh fruits and vegetables are included in the diet, along with supplementation and fortification programs to enhance the nutritional value of staple foods. Developing countries lack similar provisions, and because most of their populations subsist on a monotonous diet of cereal grains that lack essential vitamins and minerals, micronutrient deficiency is rife and contributes significantly to the poor socioeconomic conditions prevalent in such regions (3).

Biofortification programs based on conventional breeding have met with only marginal success. For example, 4 polymorphisms at the *lcye* locus in corn were recently shown to alter the flux between the α-carotene and β-carotene branches of the carotenoid pathway, potentially allowing breeding for enhanced β-carotene levels (4). However, such a quantitative trait locus (QTL)-based approach would require years of conventional breeding to achieve significant enhancement in locally adapted varieties grown by subsistence farmers in the developing world. Success achieved by using this approach depends on the number of QTLs affecting β-carotene levels, the impact of each QTL on the nutritional phenotype, the ability to map each QTL accurately to facilitate breeding, the stability of QTL-dependent phenotypes in different genetic backgrounds and environments, and whether the same effects are exerted in relevant breeding germplasm. The complexity of such breeding programs would be increased each time additional vitamins and minerals were taken into consideration (if conventional breeding were practical at all), making the goal of "nutritionally complete" cereals next to impossible.

The absence of key vitamins in cereal grains reflects the fact that the corresponding metabolic pathways are absent, truncated, or inhibited in the endosperm. Therefore, a suitable strategy to enhance these pathways is to introduce genes encoding key enzymes free from feedback control (5–7). Several examples of nutritional engineering have received widespread coverage in the scientific literature as well as the general media, including rice and potato with enhanced β-carotene levels, lysine-rich corn, iron-rich lettuce, and lycopene-enhanced tomatoes (reviewed in ref. 8). Although all these studies have proven successful, they still address only individual deficiencies and if deployed successfully in developing countries would only serve to shift the focus onto the remaining deficiency diseases. Here we have gone beyond the current state of the art in vitamin enhancement by simultaneously increasing the levels of β-carotene, ascorbate, and folate in corn endosperm. These 3 vitamins represent 3 entirely different metabolic pathways, and the only way to achieve such a radical change in the nutritional properties of an elite breeding variety of corn so rapidly is to take advantage of multigene engineering via direct DNA transfer (9). Currently, only direct DNA transfer has the potential to facilitate the transfer of multiple genes to plants routinely and reliably, and only direct DNA transfer is versatile enough to achieve the direct transformation of commercially important germplasm.

Results and Discussion

Expression Vectors and Transgenic Plants. Gene transfer to plants provides an effective way to study and modify metabolic pathways precisely, and multigene engineering allows entire pathways to be reconstructed free of endogenous regulation (10). Such experiments in turn require strategies to introduce multiple transgenes into plants and ensure their coordinated expression over many generations (6). The stable expression of multiple transgenes is one of the most significant hurdles currently limiting progress in plant molecular biology (11, 12), because the chances of failure for at least 1 of the transgenes increases with the number of genes introduced, requiring the generation of very large populations to ensure complete pathway reconstruction. Alternative approaches, such as individual transformation fol-

Author contributions: C.Z., G.R., G.S., and P.C. designed research; S.N., C.Z., G.F., K.R., L.B., J.B., D.P.C., and T.C. performed research; S.N., C.Z., G.F., K.R., L.B., J.B., D.P.C., G.S., and T.C. contributed new reagents/analytic tools; S.N., C.Z., G.F., J.B., D.P.C., G.R., G.S., T.C., and P.C. analyzed data; and G.S. and P.C. wrote the paper.

The authors declare no conflict of interest.

[1]S.N. and C.Z. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: christou@pvcf.udl.es.

This article contains supporting information online at www.pnas.org/cgi/content/full/0901412106/DCSupplemental.

lowed by crossing to "stack" transgenes, are unworkable for large numbers of genes because of the time taken to stack all transgenes in 1 line and the likelihood that unlinked genes will segregate in later generations. As a way to circumvent this difficulty, Zhu et al. (10) devised a combinatorial transformation strategy in which multiple transgenes were introduced randomly into the same white corn variety described herein. The principle developed was that the population could be screened for metabolic variants, reflecting the expression of different combinations of transgenes. Some plants contained and expressed all input genes and recapitulated the entire pathway under investigation, whereas others expressed subsets of transgenes and displayed corresponding metabolic profiles.

We transformed 10–14-day-old immature zygotic embryos of the South African elite white corn variety M37W by bombarding them with metal particles coated with 5 constructs (Fig. S1): The selectable marker *bar* and 4 genes/cDNAs encoding enzymes in the metabolic pathways for the vitamins β-carotene, ascorbate, and folate. To increase β-carotene levels, we introduced corn (*Zea mays*) phytoene synthase (*psy1*) cDNA under the control of the wheat LMW glutenin promoter and the *Pantoea ananatis* (formerly *Erwinia uredovora*) *crtI* gene (encoding carotene desaturase) under the control of the barley D-hordein promoter. To increase ascorbate levels we introduced rice dehydroascorbate reductase (*dhar*) cDNA, and to increase folate levels we introduced the *E. coli folE* gene encoding GTP cyclohydrolase (GCH1), both under the control of the barley D-hordein promoter.

In this study, a population of ≈75 transgenic plants was regenerated and screened by genomic PCR to identify primary transformants containing all 5 input transgenes. The genomic PCR was carried out by using sets of 3 primers for each gene, generating overlapping products [supporting information (SI) Table S1]. This strategy was useful because multiple gene transfer experiments occasionally generate transgene fragments that can be identified by PCR but fail to express a product, meaning that transgene content does not necessarily predict expression profiles and metabolic characteristics. The triple-primer approach provides a good impression of whether integrated transgenes are intact, so that plants with obviously truncated or rearranged transgenes can be discarded early, leaving those with intact transgenes to undergo more detailed expression analysis (e.g., by Northern blot). In this study, transgene expression was verified by Northern blot, and a lead event (plant line L-1) carrying all 4 metabolic transgenes and expressing them at high levels (Fig. 1) was chosen for further in-depth analysis of vitamin content, as discussed below. Twenty-seven independent lines contained and expressed all input transgenes (at varying levels). Table S2 shows data from 6 of these lines, including line L-1, which expressed all of the input transgenes strongly at the mRNA level.

Analysis of β-Carotene Levels in Transgenic Plants. White corn is the predominant food corn used in sub-Saharan Africa, and the white color of the kernels is ample demonstration of the lack of carotenoids (which tend to give fruits and vegetables that contain them a range of yellow, orange, and red colors). The yellow corn more commonly consumed in the West has a higher carotenoid content owing to higher levels of lutein and zeaxanthin, but in sub-Saharan Africa it is used predominantly as animal feed. To address the nutritional limitations of white corn we attempted to increase the carotenoid content by expressing the corn *psy1* gene (which is not usually expressed in the endosperm of this variety) and the *Pantoea ananatis crtI* gene (to increase flux through the pathway). In doing so the endosperm of the L-1 transgenic corn line, as well as all other lines expressing these 2 transgenes, appeared deep orange owing to the accumulation of more carotenes than present even in normal yellow corn. We carried out quantitative profiling of the carotenoid content by HPLC and found that the total carotenoid level in transgenic corn endosperm increased up to 112-fold compared with WT M37W corn (Fig. 2 and Table 1). In the best-performing line, the endosperm accumulated ≈60 μg/g β-carotene, 23 μg/g lycopene, and 36 μg/g zeaxanthin, which compares favorably with the most successful previous studies (Table S3). The distribution of carotene species indicated that the overexpression of *Zmpsy1* and *PacrtI* promotes the desaturation of phytoene, whereas the additional endogenous lycopene β-cyclase activity in corn endosperm results in the accumulation of significant amounts of lycopene as well as β-carotene.

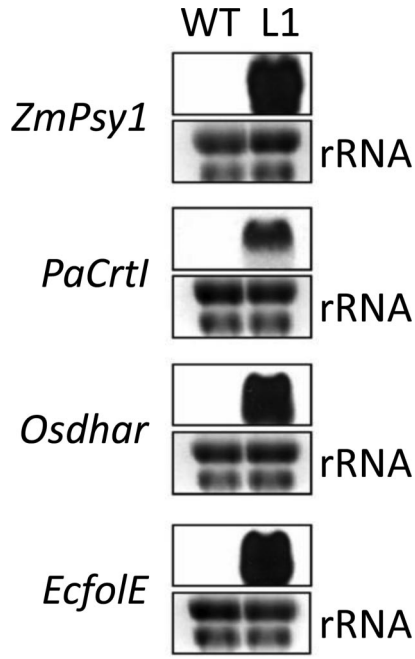

Fig. 1. Northern blot showing transgene expression in engineered (L-1) and wild-type (WT) M37W corn. We loaded 30 μg of total RNA in each lane and used rRNA (stained with ethidium bromide) as a loading control. *Zmpsy1*, corn phytoene synthase 1; *PacrtI*, *Pantoea ananatis* phytoene desaturase; *Osdhar*, rice dehydroascorbate reductase; *EcfolE*, *E. coli folE*.

In humans, vitamin A is synthesized from β-carotene (also known as provitamin A), which is why plants accumulating this compound are useful for the prevention of vitamin A deficiency (Fig. 3*A*). A number of reports have shown increases in the levels of β-carotene in transgenic staple food crops (e.g., in "Golden Rice") in which the pathway is extended beyond its normal termination point at the precursor geranylgeranyl diphosphate by the addition of *psy1*, *lycb*, and *crtI*. The total β-carotene levels in Golden Rice 1, which was transformed with daffodil *psy1* and *lycb* as well as bacterial *crtI* (13), increased to 1.6 μg/g dry weight (DW). In Golden Rice 2, in which corn *psy1* was used in place of the daffodil gene (14), the β-carotene levels reached 31 μg/g DW. Our corn line therefore contained twice the amount of β-carotene as the best-reported Golden Rice line and compares very favorably with similar studies in other crops (Table S3).

Analysis of Ascorbate Levels in Transgenic Plants. The expression of *dhar* cDNA in the L-1 transgenic corn endosperm enhanced ascorbate levels by up to 6-fold, achieving a total level of ≈110 μg/g DW. Ascorbate is synthesized in plants from the precursor D-glucose, through L-galactose and L-galactono-1,4-lactone (Fig. 3*B*) (15). The oxidation of ascorbate produces the short-lived radical monodehydroascorbate (MDHA), which can be converted to ascorbate by MDHA reductase, or otherwise it may disproportionate nonenzymatically to ascorbate and dehydroascorbate (DHA). DHA is either recycled to ascorbate by DHA reductase by using

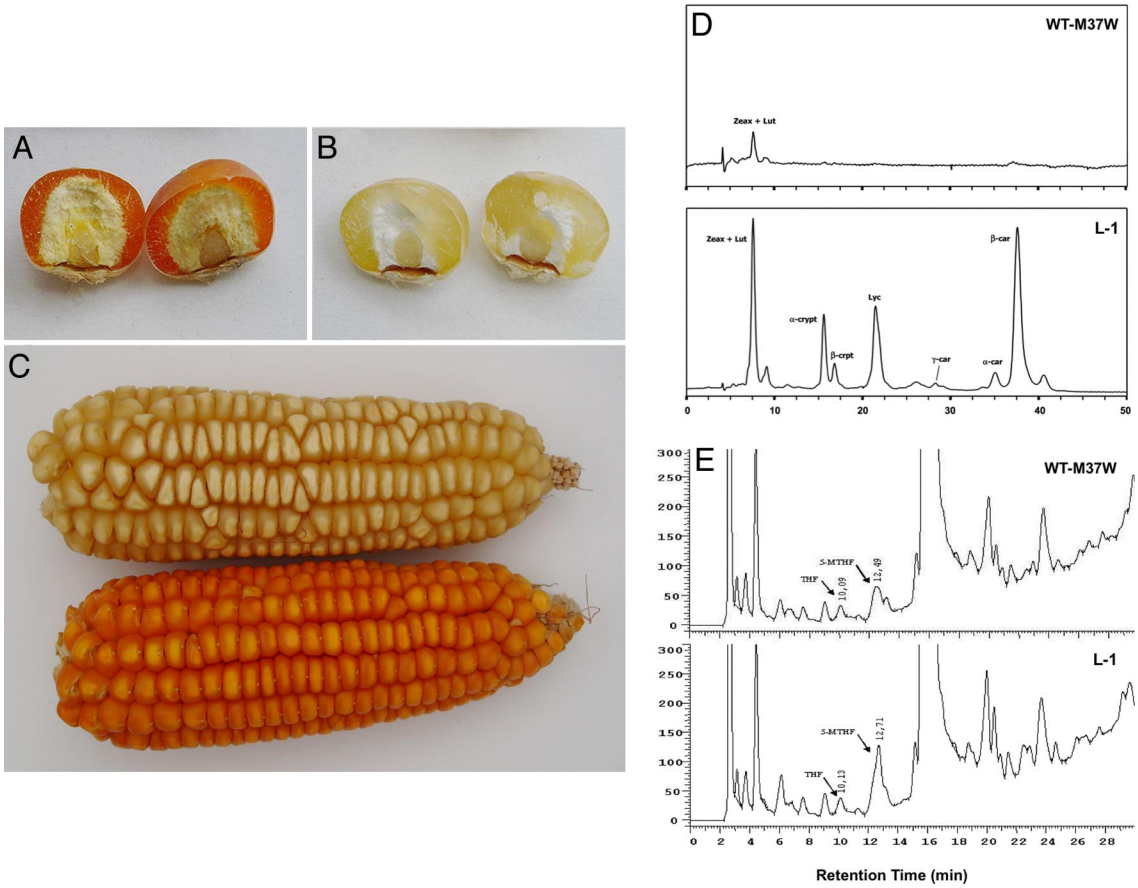

Fig. 2. Accumulation of carotenes in the endosperm of transgenic corn line L-1. (*A*) Orange-yellow phenotype of the transgenic endosperm. (*B*) Normal phenotype of the WT M37W endosperm. (*C*) Comparison of WT and transgenic cobs, showing significant increases in the levels of key carotenoids in the transgenic cobs. (*D*) HPLC analysis of carotenoid content in WT M37W and transgenic corn; *x* axis shows retention time, and *y* axis shows intensity (mV). (*E*) HPLC analysis of folate content in WT M37W and transgenic corn; *x* axis shows retention time, *y* axis shows intensity (mV).

glutathione as the reductant, or undergoes an irreversible hydrolysis to generate 2,3-diketogulonic acid (Fig. 3*B*). Because at least 3 separate metabolic pathways converge on ascorbate in plants in addition to the recycling of oxidation products, numerous strategies have been used to enhance its synthesis and regeneration, but enhancing ascorbate regeneration has been among the most successful (15) (Table S3). The constitutive overexpression of wheat *dhar* cDNA in corn in a previous study increased ascorbate levels up to 4-fold (16), but our use of the endosperm-specific barley D-hordein promoter resulted in a 6-fold increase and more than twice the amount of total ascorbate as previously achieved. The increase is likely to reflect the greater amount of steady-state *dhar* mRNA present in the endosperm in our corn plants.

Analysis of Folate Levels in Transgenic Plants. The expression of *Escherichia coli folE* doubled the folate levels in the L-1 transgenic corn endosperm to 1.94 μg/g DW, which is similar to the levels we observed in WT yellow corn. However, the result represents a

Table 1. Comparison of levels of carotenoids and other vitamins in WT M37W corn and transgenic line L-1

Corn line	M37W	L-1	Conventionally bred corn (4)	Golden Rice 2 (14)
Lyc	0	22.78 ± 2.56	ND	ND
γ-Car	0.09 ± 0.02	4.79 ± 1.08	ND	ND
α-Car	0.12 ± 0.05	7.26 ± 0.87	ND	ND
β-Car	0.35 ± 0.06	59.32 ± 3.65	1.65	31
α-Cryptox	ND	13.42 ± 2.0	ND	ND
β-Cryptox	ND	5.28 ± 0.84	ND	ND
Lut	0.57 ± 0.18	14.68 ± 2.16	11.36	ND
Zeax	0.32 ± 0.05	35.76 ± 4.35	8.187	ND
CAR	1.45 ± 0.21	163.29 ± 8.61	23.06	37
Asc	17.53 ± 2.90	106.94 ± 7.56	—	—
Fol	0.93 ± 0.32	1.94 ± 0.17	—	—

Values are micrograms per gram DW ± SD (n = 3–5 mature T3 seeds). Also compared are a corn line bred conventionally (4) and genetically engineered Golden Rice 2, expressing *Zmpsy1* and *Pacrtl* (14). Lyc, lycopene; γ-Car, γ-carotene; α-Car, α-carotene; β-Car, β-carotene; α-Cryptox, α-cryptoxanthin; β-Cryptox, β-cryptoxanthin; Lut, lutein and lutein epoxide; Zeax, zeaxanthin; CAR, total carotenoids; Asc, ascorbate; Fol, folate; ND, not determined.

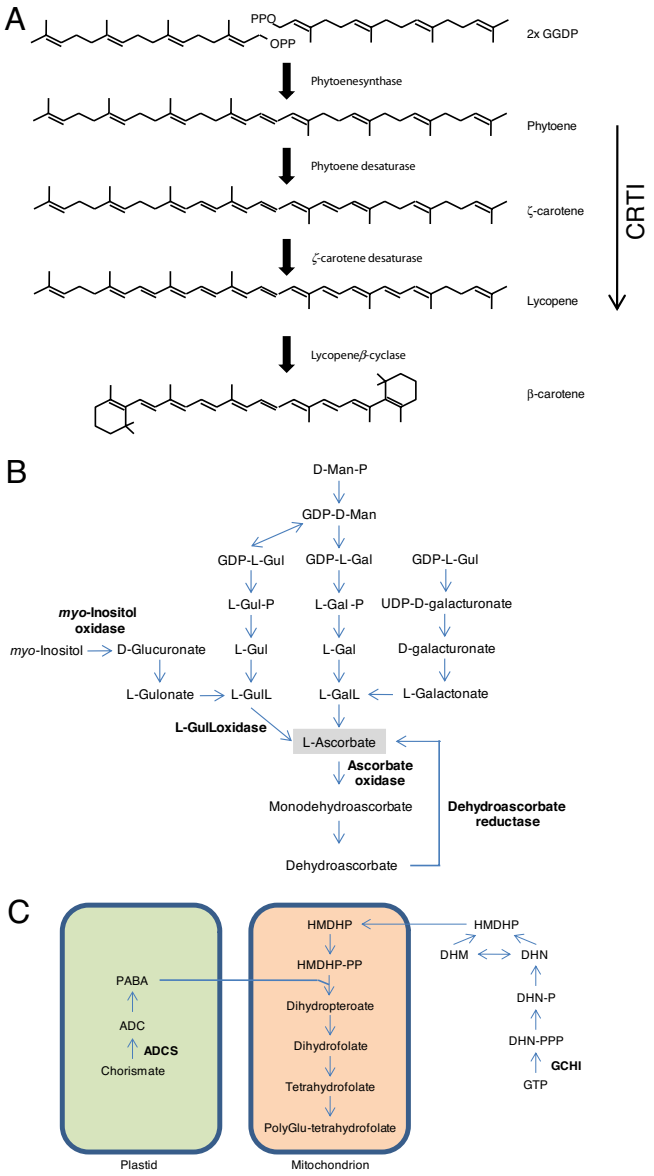

Fig. 3. Metabolic pathways engineered in this investigation. (*A*) Enzymatic steps and metabolic products in the β-carotene biosynthesis pathway that are missing in cereal grains. The synthesis of carotenes in plants is a branch of the isoprenoid pathway, and the first committed step is the joining of 2 geranylgeranyl diphosphate (GGPP) molecules to form the precursor phytoene. The conversion of phytoene into β-carotene requires 3 additional enzyme activities: Phytoene desaturase, β-carotene desaturase, and lycopene β-cyclase. Rice and other cereal grains accumulate GGPP but lack the subsequent enzymes in the pathway, so the genes for all 3 enzymes are required. (*B*) The network of proposed biosynthetic pathways for ascorbate in plants. L-Gal, L-galactose; L-GalL, L-galactono-1,4-lactone; L-Gul, L-gulose; L-GulL, L-gulono-1,4-lactone; D-Man, D-mannose; UDP, uridine diphosphate. (*C*) The plant folate biosynthesis pathway. Folates are tripartite molecules consisting of pteridine, PABA, and glutamate moieties, with pteridines synthesized in the cytosol and PABA in the plastids. These moieties are then transported to the mitochondria, where they condense to form dihydropteroate and are conjugated to glutamate. DHN, dihydroneopterin; -P/-PP/-PPP, mono/di/triphosphate; DHM, dihydromonapterin; HMDHP, hydroxymethyldihydropterin.

5–10-fold increase over the folate levels for yellow corn reported in the literature (17) and probably reflects our detection methodology (see *Methods*), which involves the use of α-amylase in combination with a protease and a conjugase, followed by measurement by HPLC (18). This improved methodology is more sensitive, hence our data agree with more recent reports of folate measurements by using the same procedure (19).

There is much greater scope for improvement in folate levels, given that folate biosynthesis in plants takes place in 3 different cell compartments: the cytosol, plastids, and mitochondria (Fig. 3*C*), and increased GCH1 activity only affects the cytosolic (pterin) branch. In previous studies, the overexpression of GCH1 has resulted in massive increases in pterin levels but only a doubling of folate levels, presumably because the plastidial *p*-aminobenzoate (PABA) branch was depleted (20, 21). Similar limitations affected plants with the PABA branch enhanced by overexpression of aminodeoxychorismate (ADC) synthase (ADCS), reflecting depletion of the pterin branch (22). However when tomato plants separately enhanced for these pathways were crossed, the double-transgenic fruit accumulated up to 25-fold more folate than controls (22). In similar studies, expression of *Arabidopsis thaliana gch1* and *adcs* cDNAs in rice endosperm increased folate levels up to 100-fold compared with WT grains (23). Because only the pterin branch was modified in our plants, and this modification achieved similar enhancements to those seen in tomato transformed with the same gene (21), we assume similar limitations are in place and that better folate levels could be achieved through simultaneous manipulation of the PABA branch of the folate synthesis pathway.

Potential Nutritional Impact of Multivitamin Biofortified Corn. We have demonstrated that transgenic corn can be engineered to enhance the products of at least 3 separate metabolic pathways simultaneously, leading to 3 nutritionally valuable groups of compounds. Previous studies (summarized in Table S3) have succeeded in increasing the levels of 1 major vitamin, and in some cases vitamin production has been enhanced together with the more efficient accumulation of minerals such as iron; here, multiple pathways have been manipulated to broadly increase the nutritional value of a plant. The level of different carotenoids, for example, vastly exceeds the best levels achieved by conventional breeding (Table 1), with ≈36-fold more β-carotene, a 20–30% increase in lutein, a greater than 4-fold increase in zeaxanthin, and the accumulation of a large amount (23 μg/g) of lycopene. No improvement in lycopene content has been achieved in conventional breeding programs (4). The levels of vitamins we achieved compare favorably with their recommended daily intakes (RDI), indicating that 100–200 g of grain (a typical portion) would provide the full RDI of β-carotene (as a sole source of vitamin A), an adequate intake of folate, and approximately 20% of the RDI of ascorbate. Although a further source of ascorbate would be necessary to reach the RDI, our approach opens the door for similar experiments in which an even greater number of pathways can be engineered, and the levels of each vitamin can be standardized to RDI values. This process could lead to the development of transgenic cereals loaded with vitamins, minerals, essential amino acids, and long-chain polyunsaturated fatty acids, providing a nutritionally complete meal without the need for artificial supplementation. One matter that needs to be considered is the bioavailability of the enhanced vitamins. In most micronutrient-enhanced plants reported to date, assumptions about whether the nutrients can be readily absorbed by humans have not been tested (24), although there are examples in which absorption studies have been carried out with mice and humans to show the direct nutritional benefits of mineral-enhanced crops (25).

In assessing strategies to deal with micronutrient deficiency, the provision of a varied diet with fresh fruit, vegetables, and fish would be ideal. However, where this varied diet is impossible because of poverty and poor governance, superenhanced, nutritionally complete cereals could provide a durable solution to improve the health and general well-being of impoverished populations. The social and economic impacts of nutritionally

enhanced transgenic plants must be addressed, such as the cost-effectiveness of adapting local varieties, the social acceptance of the strategy, and the overarching regulatory policy for producing such crops on an agronomic scale (26). Even so, there is no doubt that the nutritional qualities of plants can be enhanced by genetic engineering and that the results outstrip those achieved through conventional breeding. Breeding is rarely an adequate solution on its own because of the characteristics of the plant species itself or the nutrient of interest (7). Therefore, the best biofortification strategies will likely involve genetic engineering in conjunction with conventional breeding, particularly when the direct enhancement of local elite breeding varieties is required. The adoption of nutritionally improved corn will help to improve the health and well-being of the world's poorest people, but this advancement will only be possible if political differences over the development and use of transgenic crops are set aside and their deployment and cultivation is regulated according to robust, science-based criteria.

Methods

Expression Construct Design. *Zea mays psy1* cDNA was cloned from corn inbred line B73 by RT-PCR by using forward primer 5′-AGG ATC CAT GGC CAT CAT ACT CGT ACG AG-3′ and reverse primer 5′-AGA ATT CTA GGT CTG GCC ATT TCT CAA TG-3′. The cDNA was inserted into plasmid p326 under the control of the LMW glutenin promoter (27). The *Pantoea ananatis* (formerly *Erwinia uredovora*) *crtI* gene fused in frame with the transit peptide signal from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase (28) was amplified from its source plasmid by PCR by using forward primer 5′-ATC TAG AAT GGC TTC TAT GAT ATC CTC TTC-3′ and reverse primer 5′-AGA ATT CTC AAA TCA GAT CCT CCA GCA TCA-3′. The cDNA was inserted into plasmid pHor-P under the control of the barley D-hordein promoter (29). *Oryza sativa dhar* cDNA and the *E. coli folE* gene were cloned from rice and *E. coli*, respectively, by using forward and reverse primers based on sequence information in GenBank and inserted into the pHor-P plasmid described above.

Corn Transformation. Corn plants (*Zea mays* L., cv. M37W) were grown in the greenhouse and growth room at 28/20 °C day/night temperature with a 10-h photoperiod and 60–90% relative humidity for the first 50 days, followed by maintenance at 21/18 °C day/night temperature with a 16-h photoperiod thereafter. Immature zygotic embryos were excised at 10–14 days after pollination and cultured on N6 medium. After a further 5 days, the embryos were transferred to N6 medium containing high osmoticum (0.2 M mannitol, 0.2 M sorbitol) for 5–6 h before bombardment, then bombarded with 10 mg of coated gold particles, as previously described (30).

Bombarded callus was selected on phosphinothricin-supplemented medium (31), and transgenic plantlets were regenerated and hardened off in soil. Independent transgenic events were identified and characterized by PCR (see below) and then either pollinated with nontransformed white corn (M37W) or self-pollinated to produce T1 seeds. Nontransformed control plants were regenerated from the same batch of callus material and grown under the same conditions as the transgenic lines. Homozygous T2 and subsequent T3 generations were derived through selfing.

DNA Analysis of Transgenic Plants. Transgenic corn lines were characterized by PCR by using 3 primer sets for each transgene, amplifying segments of the 5′ end, middle, and 3′ end of the transgene (Table S1). The corresponding plasmids were used as positive controls. PCRs were carried out under standard conditions by using 100 ng of genomic DNA and 0.5 units of GoTaq DNA polymerase in a 20-μL reaction volume. The reactants were denatured at 95 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min.

mRNA Analysis. Total RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen), and 30-μg aliquots were fractionated on a denaturing 1.2% (wt/vol) agarose gel containing formaldehyde before blotting. The membrane was probed with digoxigenin-labeled partial cDNAs at 50 °C overnight by using DIG Easy Hyb (Roche Diagnostics). After washing and immunologic detection with anti-DIG-AP (Fab-Fragments Diagnostics) according to the manufacturer's instructions, CSPD chemiluminescence (Roche) was detected on Kodak BioMax light film (Sigma-Aldrich).

Carotenoid Analysis. Total carotenoids were extracted from freeze-dried endosperm in 20 mL 50/50 (vol/vol) tetrahydrofuran and methanol at 60 °C for 15–20 min, and total carotenoids were quantified by measuring absorbance at 465 nm. For HPLC separation, the solvent was evaporated under a stream of N_2 gas at 37 °C, redissolved in 50 μL acetone, and a 20-μL aliquot was injected immediately. Samples were separated on a Nucleosil C18 3-μ column with the same mobile phase at 25 °C. In each case zeaxanthin and lutein were separated in parallel runs on a C18 Vydac 218TP54 column, with methanol as the mobile phase (32). Samples were monitored with a Kontron DAD 440 photodiode array detector with on-line registration of the spectra. All carotenoids were identified by cochromatography with authentic reference compounds and comparison of their spectra. Those standards were also used for quantitation in combination with the extinction coefficients (33).

Ascorbate and Folate Analysis. Ascorbate was measured in transgenic corn endosperm as previously described (34). Folates were extracted and quantified as previously described (16) according to protocols developed by Pfeiffer et al. (35) and Vahteristo et al. (36).

ACKNOWLEDGMENTS. This study was supported by the Ministerio de Educación y Ciencia, Spain (BFU2007-61413); the Ramon Y Cajal program, Spain; and the Juan de la Cierva program, Spain. S.N. is the recipient of a Ph.D. fellowship from Ministerio de Educación y Ciencia, Spain (BES-2005-9161).

1. Graham RD, Welch RM, Bouis HE (2001) Assessing micronutrient malnutrition through enhancing the nutritional quality of staple foods: Principles, perspectives and knowledge gaps. *Adv Agron* 70:77–142.
2. Food and Agriculture Organization of the United Nations (2006) *State of Food Insecurity in the World* (Food and Agriculture Organization of the United Nations, Rome).
3. Timmer CP (2003) Biotechnology and food systems in developing countries. *J Nutr* 133:3319–3322.
4. Harjes CE, et al. (2008) Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. *Science* 319:330–333.
5. Christou P, Twyman RM (2004) The potential of genetically enhanced plants to address food insecurity. *Nutr Res Rev* 17:23–42.
6. Capell T, Christou P (2004) Progress in plant metabolic engineering. *Curr Opin Biotechnol* 15:148–154.
7. Zhu C, et al. (2007) Transgenic strategies for the nutritional enhancement of plants. *Trends Plants Sci* 12:548–555.
8. Davies KM (2007) Genetic modification of plant metabolism for human health benefits. *Mutat Res* 622:122–137.
9. Altpeter F, et al. (2005) Particle bombardment and the genetic enhancement of crops: Myths and realities. *Mol Breeding* 15:305–327.
10. Zhu C, et al. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc Natl Acad Sci USA* 105:18232–18237.
11. Halpin C (2005) Gene stacking in transgenic plants—the challenge for 21st century plant biotechnology. *Plant Biotechnol J* 3:141–155.
12. Dafny-Yelin M, Tzfira T (2007) Delivery of multiple transgenes to plant cells. *Plant Physiol* 145:1118–1128.
13. Ye X, et al. (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287:303–305.
14. Paine JA, et al. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat Biotechnol* 23:482–487.
15. Ishikawa T, Dowdle J, Smirnoff N (2006) Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. *Physiol Plant* 126:343–355.
16. Chen Z, Young TE, Ling J, Chang SC, Gallie DR (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proc Natl Acad Sci USA* 100:3525–3530.
17. Bekaert S, et al. (2008) Folate biofortification in food plants. *Trends Plants Sci* 13:28–35.
18. Konings EJ (1999) A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. *J AOAC Int* 82:119–127.
19. Poo-Prieto R, et al. (2006) Use of the affinity/HPLC method for quantitative estimation of folic acid in enriched cereal-grain products. *J Nutr* 136:3079–3083.
20. Hossain T, et al. (2004) Enhancement of folates in plants through metabolic engineering. *Proc Natl Acad Sci USA* 101:5158–5163.
21. Díaz de la Garza RI, et al. (2004) Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. *Proc Natl Acad Sci USA* 101:13720–13725.
22. Díaz de la Garza RI, Gregory JF, Hanson AD (2007) Folate biofortification of tomato fruit. *Proc Natl Acad Sci USA* 104:4218–4222.
23. Storozhenko S, et al. (2007) Folate fortification of rice by metabolic engineering. *Nat Biotechnol* 25:1277–1279.
24. Jeong J, Guerinot ML (2008) Biofortified and bioavailable: The gold standard for plant-based diets. *Proc Natl Acad Sci USA* 105:1777–1778.
25. Morris J, Hawthorne KM, Hotze T, Abrams SA, Hirschi KD (2008) Nutritional impact of elevated calcium transport activity in carrots. *Proc Natl Acad Sci USA* 105:1431–1435.

26. Ramessar K, Capell T, Twyman RM, Quemada H, Christou P (2008) Trace and traceability—a call for regulatory harmony. *Nat Biotechnol* 26:975–978.
27. Colot V, Robert LR, Kavanagh TA, Bevan MW, Thompson RD (1987) Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco. *EMBO J* 6:3559–3564.
28. Misawa N, et al. (1993) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of beta-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J* 4:833–840.
29. Sorensen MB, Muller M, Skerritt J, Simpson D (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. *Mol Gen Genet* 250:750–760.
30. Ramessar K, et al. (2008) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proc Natl Acad Sci USA* 105:3727–3732.
31. Drakakaki G, et al. (2005) Endosperm specific co-expression of recombinant soybean ferritin and *Aspergillus* phytase in maize results in significant increases in the levels of bioavailable iron. *Plant Mol Biol* 59:869–880.
32. Davies BH (1976) Carotenoids. *Chemistry of Plant Pigments*, ed Goodwin TW (Academic, London).
33. Gillespie KM, Ainsworth EA (2007) Measurement of reduced, oxidized and total ascorbate content in plants. *Nat Protoc* 2:871–874.
34. Jain AK, Nessler CL (2000) Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. *Mol Breeding* 6:73–78.
35. Pfeiffer CM, Rogers LM, Gregory JF, III (1997) Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. *J Agric Food Chem* 45:407–413.
36. Vahteristo L, et al. (1996) Third EU MAT intercomparison study on food folate analysis using HPLC procedures. *Food Chem* 57:109–111.