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Improving the nutritional value of Golden Rice through increased pro-vitamin A content

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'Golden Rice' is a variety of rice engineered to produce β-carotene (pro-vitamin A) to help combat vitamin A deficiency[1], and it has been predicted that its contribution to alleviating vitamin A deficiency would be substantially improved through even higher β-carotene content[2]. We hypothesized that the daffodil gene encoding phytoene synthase (*psy*), one of the two genes used to develop Golden Rice, was the limiting step in β-carotene accumulation. Through systematic testing of other plant *psy*s, we identified a *psy* from maize that substantially increased carotenoid accumulation in a model plant system. We went on to develop 'Golden Rice 2' introducing this *psy* in combination with the *Erwinia uredovora* carotene desaturase (*crtI*) used to generate the original Golden Rice[1]. We observed an increase in total carotenoids of up to 23-fold (maximum 37 μg/g) compared to the original Golden Rice and a preferential accumulation of β-carotene.

Carotenoids are a group of plant pigments important in the human diet as the only precursors of vitamin A. Certain carotenoids, most importantly β-carotene, are cleaved to vitamin A within the body and are referred to as pro-vitamin A[3]. Vitamin A deficiency, a major problem in parts of the developing world, can result in permanent blindness and increase the incidence and severity of infectious diseases[4]. In Asia, vitamin A deficiency is associated with the poverty-related predominant consumption of rice, which lacks pro-vitamin A in the edible part of the grain (endosperm). Providing pro-vitamin A in a staple food such as rice could be a simple and effective complement to supplementation programs[5] because, through farming, it would be ubiquitous and self-sustaining.

Golden Rice is the name coined to describe the genetically modified rice[1] that produces carotenoids in the endosperm of the grain, giving rise to a characteristic yellow color. In this pioneering work, a maximum level of 1.6 μg/g total carotenoids was achieved and has

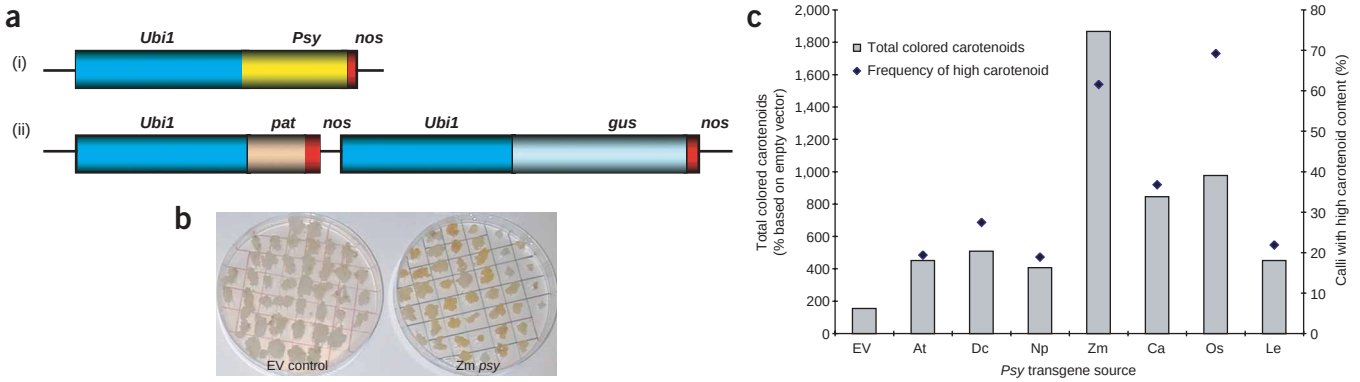

Figure 1 Expression of a *psy* transgene increases the carotenoid content of maize callus. (**a**) Schematic diagram of the gene cassettes in the two plasmids used to cotransform maize callus. Both contain the maize polyubiquitin1 promoter (*Ubi1*) and the *nos* terminator (*nos*). (i) The seven similar plasmids constructed with the phytoene synthase-coding region (*psy*) from each of the species listed below. (ii) The phosphoino N-acetyl transferase (*pat*) selectable marker and beta-glucuronidase (*gus*) gene cassettes. (**b**) Photograph showing individual maize calli cotransformed with the plasmid containing the maize *psy* (right, Zm *psy*) and an empty vector (EV) control (left). (**c**) Histogram showing the total colored carotenoid content of maize calli transformed with a given *psy* gene (from *Arabidopsis thaliana* (At), *Daucus carota* (Dc), *Narcissus pseudonarcissus* (Np), *Zea mays* (Zm), *Capsicum annuum* (Ca), *Oryza sativa* (Os) or *Lycopersicon esculentum* (Le)). Data shown represents the 75th percentile for each population of transgenic calli expressed as a percentage of the median empty vector (EV) control value. The second *y*-axis (diamonds) shows the percentage of calli from each population with a carotenoid content more than fivefold that of the EV median.

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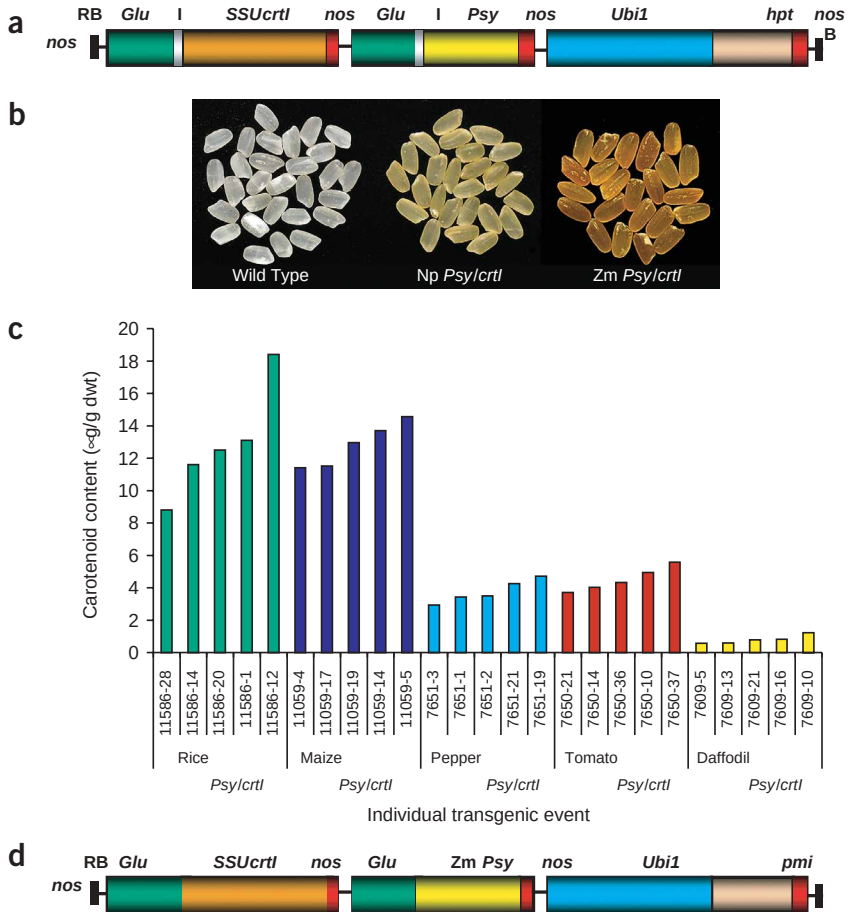

Figure 2 Carotenoid enhancement of the rice endosperm by transformation with *psy* orthologues and *crtI*. (**a**) Schematic diagram of the T-DNAs used to generate transgenic rice plants. The T-DNA comprised the rice glutelin promoter (*Glu*) and the first intron of the catalase gene from castor bean (I), *E. uredovora crtI* functionally fused to the pea RUBISCO chloroplast transit peptide (*SSUcrtI*) and a phytoene synthase from each of five plant species (*psy*), with a *nos* terminator, as well as a selectable marker cassette comprising the maize polyubiquitin (*Ubi1*) promoter with intron, hygromycin resistance (*hpt*) and *nos* terminator. (**b**) Photograph of polished wild-type and transgenic rice grains containing the T-DNA (as above) with the daffodil *psy* (Np) or maize *psy* (Zm) showing altered color due to carotenoid accumulation. (**c**) Histogram showing the total carotenoid content of T_1 rice seed containing a T-DNA (as above) with the *psy* gene from either rice, maize, pepper, tomato or daffodil from the five events with the highest carotenoid content for each T-DNA. Measurement error tended to be proportional to absolute carotenoid content and pooling across all 25 transformants resulted in a measurement standard error of $\pm$ 6.3% approximately. dwt, dry weight. (**d**) Schematic diagram of the T-DNA in pSYN12424 used to create Golden Rice 2. The T-DNA components were as described above with a selectable marker cassette comprising the maize polyubiquitin (*Ubi1*) promoter with intron, phosphomannose isomerase gene (*pmi*) and *nos* terminator. The use of an intron was abandoned because it was shown to have no effect on carotenoid accumulation (data not shown).

not been surpassed in subsequent experiments using alternative rice varieties[6,7]. The limited production of pro-vitamin A in Golden Rice is cited in the media as the major hurdle to the success of this particular solution for vitamin A deficiency.

Phytoene synthase is thought to be the limiting step for carotenoid biosynthesis in some wild-type tissues and is viewed as a major regulatory step[8–10] (a pathway diagram is shown in **Supplementary Fig. 1** online). This was the case in canola seed, where sole expression of *crtB* (the gene encoding a bacterial phytoene synthase) led to a substantial increase in carotenoid accumulation[11]. In wild-type rice endosperm, the first barriers to carotenoid biosynthesis are both phytoene synthase and carotene desaturase, which are provided by the daffodil *psy* and *crtI* transgenes in Golden Rice[1]. It is unknown what limits the further accumulation of carotenoid in Golden Rice. As no phytoene was accumulated (P. Beyer, University of Freiburg, personal communication), it appears that the desaturation of phytoene to lycopene is proceeding efficiently using the *crtI* gene product. We hypothesized that PSY may still be the limiting factor in this transgenic tissue. Daffodil PSY protein is known to be present at similarly high levels in both the Golden Rice endosperm and the daffodil petal[12] which suggests either that it is insufficiently active or that an alternative PSY functionality is required to overcome it being the rate-limiting step in the transgenic material.

All tissues that accumulate high levels of carotenoid have a mechanism for carotenoid sequestration including crystallization, oil deposition, membrane proliferation or protein-lipid sequestration[13]. The noncarotenogenic starchy rice endosperm is very low in lipid and apparently lacks any such means for carotenoid deposition. This in itself may cap the carotenoid content of Golden Rice at its low level regardless of transgenic pathway capability[14]. Another restriction in Golden Rice could be precursor supply. We chose not to investigate these hypotheses initially and in the course of our work duly demonstrated that they are of no immediate concern.

We systematically tested *psy* cDNAs from alternative plant sources, particularly carotenoid-rich sources, with the aim of increasing the carotenoid content of Golden Rice. In an attempt to rank the suitability of the *psy*s for use in rice, each was stably transformed into inherently carotenogenic maize callus[15] (**Fig. 1a**). Marked differences in performance of the various *psy*s were obvious in terms of both the absolute amounts of carotenoid produced and in the proportion of highly colored calli (**Fig. 1b,c**). Both of these measures were judged to be indicative of potential transgene efficacy. The most efficacious were maize *psy*[16], with a high carotenoid content, and a novel rice *psy* (AJ715786, cloned for this work) with a high proportion of highly colored calli. Carrot *psy* (AB032797), tomato *psy1*[17], bell pepper *psy1*[18] and *Arabidopsis thaliana psy*[19] were intermediate in efficacy. Daffodil *psy*[20] performed least well.

Based on our *psy* rank obtained from the callus experiments, the maize, tomato, pepper and rice *psy* cDNAs were individually used to transform rice, each with the *crtI* gene (**Fig. 2a**). Daffodil *psy* was included as a reference. Transgenic T_1 rice grains containing any of the five *psy* cDNAs (with *crtI*) were visibly yellow when polished, some with a distinctly orange hue (**Fig. 2b**). Polished, nontransgenic seeds were white. The amounts of carotenoid produced by the different *psy*

transgenes varied (**Fig. 2c**) and were in general agreement with the grain color. Consistent with the rankings observed in callus (**Fig. 1**), the highest carotenoid content in the T_1 seed was achieved using either the maize (14 μg/g) or rice (18 μg/g) *psy* cDNAs with the *crtI* gene (**Table 1**). The presence of the pepper or tomato *psy* cDNAs resulted in intermediate pigment content, whereas daffodil *psy* gave the lowest levels (1.2 μg/g). A number of events showing a 3:1 segregation of colored from white grain were progressed to the next generation (excluding rice and daffodil *psy*). Analysis of the T_2 seed showed that the carotenogenic ability was stable and heritable for all *psy* cDNAs (**Table 1**), and high levels of carotenoids were again observed in seed from homozygous progeny containing the maize *psy/crtI* transgenes (over 16 μg/g). In some higher colored events, some seeds were more intensely colored than others. Plant phenotype, the weight of one hundred T_1 seed and germination rates were similar for the transgenic events to those of the wild-type control plants for each of the different *psy/crtI* combinations (data not shown). There was no correlation between the number of T-DNA insertion sites (assessed by segregation ratio) and the carotenoid content. The presence of maize PSY and CRTI proteins was assessed by western blot analysis of transgenic endosperm and both were confirmed to be of the predicted size (**Supplementary Fig. 2** online).

Altering the source of the *psy* transgene was shown to have a major impact on the callus carotenoid content as well as that of transgenic rice grains. All *psy* cDNAs proved more efficacious than the daffodil *psy* that was used in the original Golden Rice[1] despite the latter's involvement in extremely high carotenoid accumulation in its natural context and being from a monocot source. This proves the hypothesis that daffodil PSY itself is the barrier to even higher levels of carotenoid accumulation in Golden Rice. There was apparently no shortage of the precursor geranyl geranyl diphosphate and no problem with product sequestration. The limitation was overcome by providing PSY proteins from different species, which presumably have slightly differential functionality. Maybe the same would hold true in transgenic canola[12], whereby switching the phytoene synthase source may result in even higher levels of carotenoid in the oil. Phytoene was not detected in the endosperm of any transgenic plants indicating that the *crtI* gene product is capable of desaturating all of the phytoene produced, even by the most efficacious PSYs. There is no evidence to suggest that the bacterial desaturase (CRTI) is rate-limiting for carotenoid biosynthesis in any of the transgenic rice produced in this study—this was the case only in callus (**Supplementary Table 1** online).

The reasons for the differing efficacy of *psy* cDNAs from alternative sources are not obvious and further study would be necessary to satisfactorily explain this. Differences in transgene transcription are unlikely to be a primary factor because all the *psy* transgenes were expressed under the control of the same promoter in both maize callus and rice. Transgene expression itself may be influenced by the evolutionary relationships of the transgene source species. Since the ranking of the PSYs was maintained in both transgenic systems, an inherent property of the enzymes' catalytic ability (e.g., k_{cat}, K_M) could be implicated. Given the high sequence similarity between these PSYs, any structural differences that account for varying efficacy are likely to be subtle. Perhaps not surprisingly, the two best performing PSY proteins (from rice and maize) are more similar to each other in primary sequence (89% identical) than to any of the other PSYs used in the study (and to other putative rice PSYs in the databases). Structure-function modeling based on the known structure of the related squalene synthase protein[21] did not reveal convincing reasons for the differences (unpublished data, R. Vine, C.S., R.D.). It seems unlikely that cofactors are differentially limiting PSY function because any deficiencies would have to be mirrored in maize callus and rice endosperm. The maize *psy* gene is known to be involved in carotenoid generation in maize endosperm plastids[22]. Perhaps the similarity in organellar environment with rice endosperm amyloplasts provides this particular PSY enzyme with an optimal setting.

Table 1 Carotenoid content and composition of transgenic rice endosperm

			Colored carotenoid composition, % of total in T_1 (T_2[a]) seed				
psy Source	Event identity (number of T_1 transgenic plants analyzed)	Total carotenoid content in T_1 (T_2[a]) seed (μg/g dry weight)	β-carotene	α-carotene	β-cryptoxanthin	Zeaxanthin	Lutein
Maize	11059–5 (6)	14.5 (14.4)	89.0 (83.3)	9.7 (10.4)	0.6 (2.6)	0.3 (1.9)	0.4 (1.7)
	11059–11 (6)	9.8 (14.2)	85.8 (84.7)	10.4 (9.5)	1.7 (2.9)	1.0 (1.6)	1.0 (1.3)
	11059–14 (5)	13.7 (16.0)	87.1 (86.0)	11.0 (9.3)	1.2 (2.3)	0.3 (1.3)	0.4 (1.1)
	11059–16 (6)	10.1 (11.8)	85.6 (85.8)	10.5 (8.9)	1.7 (2.7)	1.2 (1.5)	1.0 (1.1)
	11059–17 (6)	11.5 (16.5)	86.7 (85.0)	10.4 (9.1)	1.5 (2.6)	0.9 (1.8)	0.5 (1.5)
Pepper	7651–3 (5)	2.9 (2.1)	80.5 (72.7)	9.8 (11.2)	2.7 (4.9)	3.6 (4.9)	3.5 (6.2)
	7651–19 (5)	4.7 (5.2)	77.9 (76.6)	12.4 (11.9)	2.6 (4.5)	4.0 (4.9)	3.1 (2.1)
	7651–21 (5)	4.2 (4.9)	77.8 (78.8)	12.6 (9.9)	2.6 (5.0)	4.1 (4.0)	2.8 (2.2)
Tomato	7650–4 (5)	1.1 (2.2)	64.3 (65.9)	15.5 (9.9)	3.7 (4.7)	5.1 (9.0)	11.4 (10.6)
	7650–8 (4)	0.9 (1.3)	61.5 (58.9)	15.7 (9.8)	4.8 (6.8)	5.6 (12.3)	12.4 (12.2)
	7650–11 (2)	1.2 (2.0)	68.0 (68.4)	13.8 (11.9)	4.9 (6.7)	4.7 (6.8)	8.7 (6.2)
Rice	11586–1	13.1	81.2	13.6	1.7	1.2	2.2
	11586–12	18.4	85.0	12.2	1.0	0.7	1.0
	11586–14	11.6	86.4	9.6	1.9	1.0	1.0
	11586–20	12.5	78.4	16.1	2.2	1.3	1.9
	11586–28	8.8	84.4	10.2	2.5	1.3	1.5
Daffodil	7609–10	1.2	68.5	11.6	6.2	6.8	7.0
	7609–16	0.8	58.5	10.8	4.6	9.4	15.0
	7609–21	0.8	65.8	10.5	4.7	7.8	10.4

[a]The number given represents the average carotenoid content of the homozygous T_2 grain analyzed.

A very high proportion of β-carotene (80–90%) in the transgenic rice endosperm is associated here with the highest levels of carotenoid production. The increase in total carotenoid content brought about by the more highly effective *psy* genes is largely due to a preferential increase in β-carotene rather than a proportional increase in all carotenoids (**Table 1**). The same trend was observed in the callus model (**Supplementary Table 1** online) and a similar phenomenon was seen in transgenic canola using *crtB*[11]. In contrast, increases in the amount of β-carotene in transgenic tomato were associated with a reduced total carotenoid content possibly because of feedback inhibition at the level of phytoene synthase activity[23]. A possible explanation for the high β-carotene levels we observed (**Tables 1** and **2**) might be that the downstream processing of carotenes to xanthophylls (**Supplementary Fig. 1** online) does not keep pace with the rate of flux through the pathway when an efficacious PSY is expressed and as a consequence β-carotene accumulates. A further explanation is that the pathway endpoint may be influenced by sequestration, perhaps rendering β-carotene inaccessible to downstream hydroxylases. Lycopene (the product of *crtI* phytoene desaturase activity) was not observed upon analysis of the endosperm of any of the *psy*/*crtI* transgenic lines.

Table 2 Carotenoid content and composition of Golden Rice 2 T_2 endosperm

Event identity (number of T_1 transgenic plants analyzed)	Average total carotenoid content in T_2 seed (μg/g dry weight)	Colored carotenoid composition, % of total		
		β-carotene	α-carotene	β–cryptoxanthin
SGR2A1 (3)	8.8	80.2	14.4	3.3
SGR2B1 (3)	10.9	84.7	10.5	2.6
SGR2C1 (3)	11.0	79.7	16.0	2.5
SGR2D1 (3)	11.3	79.1	13.4	3.9
SGR2E1 (3)	11.4	80.9	13.4	3.2
SGR2F1 (3)	12.1	81.5	17.2	1.4
SGR2G1 (3)	12.8	75.5	19.3	2.9
SGR2H1 (3)	13.4	84.8	15.2	**
SGR2J1 (3)	13.4	79.8	14.3	2.8
SGR2K1 (4)	13.7	80.4	14.2	2.9
SGR2L1 (3)	13.8	80.1	14.8	3.0
SGR2M1 (2)	14.3	83.4	16.6	**
SGR2N1 (5)	15.8	83.2	12.2	2.8
SGR2P1 (3)	17.4	79.0	14.9	3.3
SGR2Q1 (3)	18.8	81.3	15.6	**
SGR2R1 (3)	19.7	81.1	15.0	2.1
SGR2S1 (2)	20.4	75.5	18.9	2.4
SGR2T1 (3)	23.0	76.4	19.3	2.4
SGR2V1 (3)	25.1	80.9	14.6	1.9
SGR2W1 (3)	25.4	82.7	14.1	1.9
SGR2X1 (3)	25.5	82.6	14.6	1.5
SGR2Y1 (3)	31.8	80.9	16.2	1.2
SGR2Z1 (3)	36.7	84.1	13.2	1.0

**denotes less than 1% of total carotenoid. Zeaxanthin and lutein were less than 1% of the total in all cases.

To develop a second generation Golden Rice (Golden Rice 2) that might be suitable for practical use, maize *psy* and *crtI* were transformed again on a larger scale into a different variety using an alternative to antibiotic selection (**Fig. 2d**). We selected events showing a high endosperm color and yellow:white T_1 seed in a typical mendelian ratio of 3:1, indicative of a single T-DNA insertion locus. The carotenoid content in the polished T_2 seed of homozygous plants from these events ranged from 9 to 37 μg/g (**Table 2**), a range of phenotype being usual in a population of transgenic plants. This was even higher than had been seen in the earlier experiment (up to 16 μg/g) with the same very high proportion of β-carotene (see discussion above) and exceptionally low levels of xanthophylls (**Table 2**). The fact that several hundred primary transformants were generated, compared to 21 in the earlier experiment, will have increased the probability of seeing strong phenotypes. The difference in rice variety and growing environment could also contribute to differences in performance. It is, however, the carotenoid content achieved in Golden Rice 2 plants under field conditions, when the transgenes have been introduced by backcrossing into locally adapted varieties that is the ultimate determining factor in their contribution to the alleviation of vitamin A deficiency. Further research and development activities are required before these events could be released from regulations. As before, there was no evidence to suggest that plant phenotype, seed weight or germination was affected by the presence of the transgenes (data not shown). Again in some plants, several seeds were more highly colored than others, perhaps containing an estimated 2–3 times more carotenoid.

The Golden Rice 2 reported here has up to 37 μg/g carotenoid of which 31 μg/g is β-carotene. This increase in total carotenoid and proportion of β-carotene over the original Golden Rice promises a greater impact on vitamin A deficiency and related health issues. A value of 30 μg/g (25 μg/g β-carotene), however, is used here for calculations of impact for vitamin A deficiency, this number being chosen at this early stage as a moderate prediction of future performance. Definitive statements on the benefit of Golden Rice for the alleviation of vitamin A deficiency cannot be made. The vitamin A delivered and its impact on the body depends on several unquantified factors, including β-carotene uptake and conversion to vitamin A, as well as the amount of rice consumed by the individual. These factors are under rigorous investigation at present but for the time being only estimates are available. The symptoms and effects of vitamin A deficiency are most dramatic in children. Therefore, for the purpose of this estimate we have used the US recommended dietary allowance (RDA) for 1- to 3-year-old children (300 μg vitamin A[24]). It would also be reasonable to assume that an individual receives at least some vitamin A from the current diet. Based on a retinol equivalency ratio for β-carotene of 12:1 (ref. 24), 50% of the children's RDA is delivered by 72 g of dry Golden Rice 2. This is likely to be an underestimate because β-carotene may prove to be more bioavailable from rice, a comparatively simple food matrix, than it is from fruits and vegetables upon which the equivalency ratio is based[24]. A typical child's portion is about 60 g of rice and more than one portion is frequently consumed per day in regions where rice is a staple food.

To the author's knowledge, this direct comparison of gene orthologues is unique in plant metabolic engineering. We used it to identify an effective means of increasing the pro-vitamin A content of Golden Rice. The Golden Rice 2 reported here should have a substantially improved impact on the alleviation of vitamin A deficiency. Our results support a new hypothesis that even in Golden

Rice 2, expressing the efficacious maize *psy*/*crtI* transgene combination, phytoene synthase is still the limitation to yet higher levels of carotenoid.

METHODS

Humanitarian Project for Golden Rice. Syngenta has no commercial interest in Golden Rice. The reported transgenic rice events are experimental. Consistent with Syngenta's support of the Humanitarian Project for Golden Rice, Golden Rice 2 transgenic events will be donated for further research and development through license under certain conditions. Such conditions include being governed by the strategic direction of the Golden Rice Humanitarian Board and full regulatory compliance. Please direct requests to Adrian Dubock in the first instance (adrian.dubock@syngenta.com).

Cloning a novel rice *psy* gene. A TBlastX similarity search against the rice genome[25] using the *Arabidopsis thaliana psy* and rice *psy* (AY024351) genes identified genomic sequences of similarity in which genes were predicted using FGENESH algorithm with the monocot training set. Putative rice PSY sequences were aligned to several known plant PSY proteins using the CLUSTALW algorithm and one likely candidate was selected (now known as AJ715786). Total RNA was extracted from rice leaves (Asanohikari) using an RNeasy Mini kit (Qiagen) and polyA$^+$ mRNA was purified using an Oligotex kit (Qiagen). RT-PCR of the rice *psy* was performed (Qiagen Omniscript RT kit) using the primers 5′-CTGTCCATGGCGGCCATCACGCTCCT-3′ and 5′-CGTCGGCCTGCATGGCCCTACTTCTGGCTATTTCTCAGTG-3′. Alignment of putative mature PSY proteins was performed using T-COFFEE Version_1.37 with default parameters, BLOSUM62 matrices and Gendoc 2.6.002 program, after removal of a putative transit peptide from each sequence ending with the amino acid aligning with Leu67 in the daffodil PSY protein.

Overexpression of *psy* genes in callus. The accession numbers of all nucleotide sequences are given at the end of the Methods and any deviations in sequences noted. The *psy* coding sequences of pepper, daffodil, *A. thaliana* and tomato were obtained by PCR or restriction digestion from plasmid DNA. The carrot and maize *psy* coding sequences were obtained by RT-PCR and PCR. In all cases, the coding sequence was cloned without any untranslated regions. Using primers, an *Nco*I or *Kpn*I site was added at the 5′ end (incorporating or adjacent to the start codon, respectively) and a *Sfi*I site at the 3′ end. Each *psy* was transferred into a pUC-based vector containing the maize polyubiquitin (*Ubi-1*) promoter with intron and *nos* terminator. Cloning into the *Nco*I or *Kpn*I, and *Sfi*I site of the vector placed the coding sequence within 6 nucleotides downstream of the *Ubi-1* promoter and upstream of *nos*. *SSUcrtI* is a functional fusion of the pea RUBISCO small subunit plastid transit peptide and with *Erwinia uredovora crtI*[26]. The *SSUcrtI* expression plasmid was obtained by replacing the CaMV *35S* promoter from pUCET4[1] with the *Ubi-1* promoter at the *Hin*dIII/*Xba*I sites. A separate pUC-based vector contained the *pat* selectable marker gene (phosphino N-acetyl transferase) and the reporter gene *gus* (to assess cell viability), each under the control of the *Ubi-1* promoter and *nos* terminator.

Transient expression in suspension cultures, protoplasts, endosperm, epidermis and leaf material from tobacco, maize, onion, rice and wheat failed to alter carotenoid accumulation within the time frame of the experiment. These experiments were abandoned in favor of maize callus, which relies on stable integration of the transgenes.

Black Mexican Sweetcorn callus was cotransformed with a *psy* and *pat* construct (**Fig. 1a**) in at least two separate experiments essentially as described[15]. The suspension cell medium had 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) without asparagine or thiamine. Transformed cells were selected on 1 mg/l Bialaphos (Duchefa) for one week, 5 mg/l for a further four weeks, and then 2 mg/l Bialaphos for ~3 weeks until calli were large enough to analyze. PCR was used to identify surviving calli containing the desired carotenoid transgene(s). The numbers of transgenic calli analyzed for carotenoid content were 95, 51, 105, 112, 57, 36, 13 and 272 for daffodil, carrot, tomato, maize, pepper, *A. thaliana* and rice *psy* and the empty vector (pBluescript II SK–), respectively. Each transgenic callus was an individual transformation event. Cotransformation of *psy* with the *crtI* gene had no effect on phytoene, or carotenoid content or composition.

Constructs for plant transformation. The *SSUcrtI* and *psy* coding sequences were cloned between the intron and terminator of a pUC-based plasmid containing the rice glutelin *Glut01* (*Glu*) promoter (nucleotides 1568–2406), castor bean catalase intron[27] and *nos* terminator. The resulting *Glu::intron::SSUcrtI::nos* cassette was transferred to a Bin19-based binary vector containing the *hpt* marker gene (pJH0104h) under the control of the *Ubi-1* promoter and *nos* terminator to create pJH0104hcrtI. *SSUcrtI* contained GCGGCCGCC (*Not*I site) immediately downstream of the *crtI* start codon. The *Glu::intron::psy::nos* cassettes were then transferred to pJH0104hcrtI. Short linker sequences were used between the cassette components to facilitate cloning.

For Golden Rice 2, a *Glu::SSUcrtI::nos* cassette (without the additional GCGGCCGCC) was transferred into a Bin19-based binary vector containing the *E. coli* phospho-mannose isomerase (PMI) marker gene[28] under the control of the *Ubi-1* promoter and *nos* terminator to create pNOV2115crtI. A *Glu::psy::nos* cassette with maize *psy* coding sequence was then transferred to pNOV2115crtI generating pSYN12424.

Rice transformation. Rice transformation for the test of various *psy*s was based on previous protocols[29,30] using cultivar Asanohikari with the following modifications. Embryogenic calli of 3–4 mm were incubated with *Agrobacterium tumefaciens*, spread onto R2COMAS (R2 Micro salts, 1/2 R2 Macro salts, B5 vitamins, 20 g/l sucrose, 10 g/l glucose, 1 g/l casein hydrolysate, 2 mg/l 2,4-D, 100 μM acetosyringone, pH 5.2) and placed in the dark at 26 °C. After selection, surviving embryogenic calli were transferred to regeneration medium (1/2 N6 Macro, N6 Micro and vitamins, AA amino acids, 20 g/l sucrose, 1 g/l casein hydrolysate, 0.2 mg/l naphthyleneacetic acid, 1 mg//l kinetin, 50 mg/l hygromycin B, pH 5.8, gelrite 6 g/l) to form transgenic plantlets. The numbers of transgenic plants analyzed for seed color were 32, 37, 21, 36 and 31 for daffodil, tomato, maize, pepper and rice *psy*, respectively.

Transformation of cultivar Kaybonnet with pSYN12424 was performed as above with the following modifications. Embryogenic cultures were established from mature embryos on MS-CIM (4.3 g/l MS salts, 5 ml/l B5 vitamins 1×; 30 g/l sucrose, 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein hydrolysate, 2 mg/l 2,4-D, pH 5.8, 3 g/l Phytagel). Inoculated calli were incubated at 22 °C for 2 d, transferred to MS-CIM with ticarcillin (400 mg/l) for 7 d, and then to mannose selection (MS-CIM with 17.5 g/l mannose, 5 g/l sucrose, 300 mg/l ticarcillin) for 5 weeks in the dark. Proliferating colonies were transferred to regeneration medium (MS-CIM with 0.5 mg/l IAA, 1 mg/l zeatin, 200 mg/l ticarcillin, 20 g/l mannose, 30 g/l sorbitol, no sucrose), grown in the dark for 14 d and then moved to light at 30 °C for 14 d. Shoots were transferred to Murashige & Skoog medium with 20 g/l sorbitol for 2 weeks and then to soil.

Rice cultivation and analysis. For the experimental comparison of *psy* cDNAs from various sources, plants were glasshouse grown in the UK using supplementary lighting at 70% relative humidity, with a 16-h day, and day/night temperatures of 27/21 °C. Flowering was initiated by a short day (10 h) treatment for 3 weeks at 8 weeks after planting. Seed was harvested from fully ripened panicles and dried for 3 days at 30 °C before threshing (Wintersteiger Stationary Thresher LD350).

'Golden Rice 2' plants were glasshouse grown in the US using supplementary lighting at >50% relative humidity, with a 13-h day, and day/night temperatures of 29/23 °C. Seed was dried as above at 38 °C.

The number of Golden Rice 2 primary transformants created with pSYN12424 was 619. Using a series of quantitative PCR analyses (data not shown), we retained events that were highly likely to contain a single-copy of the T-DNA although owing to inherent inaccuracies with this method a small proportion will have been incorrectly categorized. Of the retained events, 103 produced at least 100 seeds.

Carotenoid extraction and analysis. All samples were analyzed in low light or darkness and on ice where possible. Rice seed were dehusked (TR-200 Electromotion rice husker, Kett) and polished for 1 min (Pearlest polisher, Kett). The yellow seed was homogenized to a fine powder using a Glen Creston 8000 Mixer/Mill (Spex Certiprep) equipped with a hardened tool steel vial set with a 9-mm stainless steel ball. Before organic extraction, a known amount of astaxanthin (Sigma) was added as an internal standard. Homogenized samples (approximately 0.5 g) were rehydrated using 1 ml water, agitated on a vortex for

3 s followed by a 10-min incubation period. Carotenoids were extracted twice in 6 ml acetone and once in 2 ml *tert*-butylmethylether (TBME) by 30 s agitation, 5 min incubation and centrifugation at 1,370*g* for 5 min. Callus samples were freeze-dried, weighed and extracted twice with 1 ml acetone using centrifugation at 16,060*g* and a 10-min incubation. Pooled supernatants were evaporated to dryness with a stream of nitrogen gas and then redissolved in 75 µl ethyl acetate. High performance liquid chromatography analysis was performed using an YMC C30 column (3.0 µm, Fisher Scientific) and a 6% min^{-1} gradient from methanol/H_2O/TBME, 1.3 mM NH_4 acetate (70:25:5 vol/vol) to methanol/H_2O/TBME, 1.3mM NH_4 acetate (7:3:90 vol/vol). Elution of colored carotenoids was followed at 472 nm. Given the condensed run-time it was not possible to resolve phytoene and phytofluene. These two were followed together at 286 nm and are referred to in the text as phytoene. An acceptance criterion of recoveries for the internal standard was between 70% and 110% and a coefficient variation percentage of maximum 20% was used.

Accession numbers. Accession numbers with any nucleotide substitutions and the coordinates used in this study follow: *psy* sequences: *Arabidopsis thaliana* (AF009954), *Daucus carota* (AB032797, nucleotide changes t642c, c1030t, a1059g, a1065g), *Narcissus pseudonarcissus* (X78814), *Capsicum annuum* (X68017), *Oryza sativa* (AJ715786), *Lycopersicon esculentum* (M84744), *Zea mays psy* (U32636 with nucleotide changes g117c, gc195cg, g372a, c529a, t753a, a769t, a798g, g819a, t927c, a1031c, or for pSYN12424 U32636, B73 allele a1031c). Other sequences: rice glutelin promoter (D00584 1568–2406), transit peptide of pea RUBISCO SSU (X00806), *Erwinia uredovora crtI* (D90087 with change a3992g), phosphino N-acetyl transferase (X17220), *gus* (nucleotides 115–2115, X84105), *hpt* (V01499 with additional GGATCCGTCGACCTGCA GATCGTTCAAACATTTGGCAATAAAGTTTCTTAA at 3′ end), PMI (M15380), maize polyubiquitin Ubi–1 promoter with intron (nucleotides 2–1993, S94464, with changes a160g, addition of c at 813, deletion of c at 1012), castor bean catalase intron (nucleotides 679–867, D21161, with nucleotide changes at 791–795 to CGTGT, at 847–860 to TTGATCATCTTGATA) and terminator regions of *nos* (nucleotides 1848–2100, V00087).

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Ye, X. *et al.* Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287**, 303–305 (2000).
2. Zimmerman, R. & Qaim, M. Potential health benefits of Golden Rice: a Philippines case study. *Food Policy* **29**, 147–168 (2004).
3. Yeum, K.J. & Russell, R.M. Carotenoid bioavailability and bioconversion. *Ann. Rev. Nutr.* **22**, 483–504 (2002).
4. West, K.P. Jr. & Darnton-Hill, I. Vitamin A deficiency. in *Nutrition and health in developing countries* (eds. Semba, R.D. & Bloem, M.W.) 267–306 (Humana Press, Totowa, NJ, 2001).
5. Dawe, D., Robertson, R. & Unnevehr, L. Golden Rice: what role could it play in alleviation of VAD? *Food Policy* **27**, 541–560 (2002).
6. Datta, K. *et al.* Bioengineered 'golden' indica rice cultivars with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems. *Plant Biotechnol. J.* **1**, 81–90 (2003).
7. Hoa, T.T.C., Al-Babili, S., Schaub, P., Potrykus, I. & Beyer, P. Golden Indica and Japonica rice lines amenable to deregulation. *Plant Physiol.* **113**, 161–169 (2003).
8. Fraser, P.D., Truesdale, M., Bird, C.R., Schuch, W. & Bramley, P.M. Carotenoid biosynthesis during tomato fruit development. *Plant Physiol.* **105**, 405–413 (1994).
9. Ronen, G., Cohen, M., Zamir, D. & Hirschberg, J. Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant. *Delta. Plant J.* **17**, 341–351 (1999).
10. Fraser, P.D. *et al.* Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proc. Natl. Acad. Sci. USA* **99**, 1092–1097 (2002).
11. Shewmaker, C.K., Sheehy, J.A., Daley, M., Colburn, S. & Yang Ke, D. Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* **20**, 401–412 (1999).
12. Burkhardt, P.K. *et al.* Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J.* **11**, 1071–1078 (1997).
13. Camara, B., Hugueney, P., Bouvier, F., Kuntz, M. & Moneger, R. Biochemistry and molecular biology of chromoplast development. *Int. Rev. Cytol.* **163**, 175–247 (1995).
14. Rabbani, S., Beyer, P., Lintig, J., Hugueney, P. & Kleinig, H. Induced beta–carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant Physiol.* **116**, 1239–1248 (1998).
15. Keappler, H.F., Somers, D.A., Rines, H.W. & Cockburn, A.F. Silicon carbide fiber-mediated stable transformation of plant cells. *Theor. Appl. Genet.* **84**, 560–566 (1992).
16. Buckner, B., San Miguel, P., Janick-Buckner, D. & Bennetzen, J.L. The *y1* gene of maize codes for phytoene synthase. *Genetics* **143**, 479–488 (1996).
17. Bartley, G.E., Viitanen, P.V., Bacot, K.O. & Scolnik, P.A. A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. *J. Biol. Chem.* **267**, 5036–5039 (1992).
18. Romer, S., Hugueney, P., Bouvier, F., Camara, B. & Kuntz, M. Expression of the genes encoding the early carotenoid biosynthetic enzymes in *Capsicum annuum*. *Biochem. Biophys. Res. Commun.* **196**, 1414–1421 (1993).
19. Scolnik, P.A. & Bartley, G.E. Nucleotide sequence of an *Arabidopsis* cDNA for phytoene synthase. *Plant Physiol.* **104**, 1471–1472 (1994).
20. Schledz, M. *et al.* Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering. *Plant J.* **10**, 781–792 (1996).
21. Pandit, J. *et al.* Crystal structure of human squalene synthase. A key enzyme in cholesterol biosynthesis. *J. Biol. Chem.* **275**, 30610–30617 (2000).
22. Palaisa, K.A., Morgante, M., Williams, M. & Rafalski, A. Contrasting effects of selection on sequence diversity and linkage disequilibrium at two phytoene synthase loci. *Plant Cell* **15**, 1795–1806 (2003).
23. Romer, S. *et al.* Elevation of the provitamin A content of transgenic tomato plants. *Nat. Biotechnol.* **18**, 666–669 (2000).
24. Institute of Medicine Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc (National Academy Press, Washington, DC, 2001). Q3
25. Goff, S.A. *et al.* A draft sequence of the rice genome (*Oryza sativa L. ssp japonica*). *Science* **296**, 92–100 (2002).
26. Misawa, N. *et al.* Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* **4**, 833–40 (1993).
27. Tanaka, A. *et al.* Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. *Nuc. Acids Res.* **18**, 6767–6770 (1990).
28. Negrotto, D., Jolley, M., Beer, S., Wenck, A.R. & Hansen, G. The use of phosphomannose isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Reports* **19**, 798–803 (2000).
29. Hiei, Y., Ohta, S., Komari, T. & Kumashiro, T. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282 (1994).
30. Zhang, J., Xu, R.-J., Elliott, M.C. & Chen,, D.-F. *Agrobacterium*-mediated transformation of elite japonica and indica rice varieties. *Mol. Biotechnol.* **8**, 223–231 (1997).

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