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Reburnishing Golden Rice

The revelation last month that Syngenta has mistakenly distributed an unapproved variety of GM corn containing an ampicillin resistance marker over the past four years reveals a monumental foul up. It's ironic then that the same company deserves credit for a classic piece of industrial development applied to a public good. Syngenta has brought the world 'Golden Rice 2,' an improved version of transgenic rice engineered to produce β-carotene (provitamin A). The company has developed the crop to the point where it might now fulfill its promise as a remedy for certain forms of malnutrition that principally affect people in developing countries.

Golden Rice first burst onto the scene five years ago. It was heralded then by some as biotech's solution to a staggering human health crisis: vitamin A deficiency, which is responsible for 3,000 deaths per day and 500,000 cases of infant blindness per year. The problem is that Golden Rice was miscast as a panacea for the world's poor. In fact, it is one of many solutions that need to be developed in tandem, including educational initiatives to promote consumption of fruit, vegetables and animal products, local efforts to fortify existing food staples with vitamin A and international programs to distribute dietary supplements in developing countries.

Low-tech solutions by themselves, however, can only do so much. The poorer the family, the less likely they are to receive a balanced diet, particularly in times of famine when fruit and vegetables are in short supply. And the more rural the family, the smaller the chance they will get to hear about educational programs or benefit from vitamin A–fortified foods or supplements distributed by aid programs.

Ingo Potrykus, one of the codevelopers of the original Golden Rice strain, understood this. He developed β-carotene–enriched rice as a biological solution to the same problem, one that is much simpler. A single Golden Rice grain potentially allows a subsistence farmer to produce 1,000 grains of rice, from which might be produced 1,000,000 seeds, and so on. From one kernel, a farmer could grow 20,000 tons of rice in two years after four generations. And Golden Rice has fewer cost and aid implications: educational programs and vitamin supplements need annual budgets, networks for delivery, and they foster dependency. Rice seed, on the other hand, can be replanted each year at no extra cost to the farmer.

This argument would be compelling were it not for the fact that even the best lines of the original Golden Rice accumulated β-carotene to levels that supply only 15–20% of the recommended dietary allowance (RDA) for vitamin A. Biotech opponents, such as Greenpeace, have seized on this to claim that Golden Rice is 'a technical failure' because malnourished children would need to consume kilograms of rice to attain any tangible benefit (a position that conveniently ignores the reality that most people are only partly deficient in vitamin A and require only a small supplement to their daily carotenoid intake). Even last month, Greenpeace claimed in a press release that Golden Rice would "exacerbate malnutrition and undermine food security because it encourages a diet based on a single industrial staple food."

What Syngenta has now done in Golden Rice 2 is to replace the daffodil phytoene synthase gene with the equivalent gene from maize (p. 482). The consequence is that the new strain accumulates levels of the provitamin A that are more than 20-fold higher than those of the original. Syngenta scientists estimate that Golden Rice 2 could provide 50% of the RDA for vitamin A, although overall bioavailability would depend on the presence of dietary oils and proteins.

This is just the sort of thing that happens when you set goal-focused industrial R&D on a problem: they get on it and solve it. It might not always be pretty science. It might not always offer huge mechanistic insights or fundamental understanding. But it works and it usually works quite quickly. Unlike Golden Rice, which was the product of an academic collaboration between the groups of Potrykus and Peter Beyer with funding from the Rockefeller Foundation, the new rice strain is entirely a product of Syngenta's corporate R&D funding. Does that mean that the company aims to monopolize on its valuable product through exhorbitant licenses or sales? Actually no, not at all.

Syngenta is a member of the Humanitarian Golden Rice Network, which has obtained free licenses for humanitarian use of the necessary technology from more than 32 different companies and universities. The company will work with breeders in the public rice research institutions in Bangladesh, China, India, Indonesia, South Africa, the Philippines and Vietnam to make locally adapted varieties of Golden Rice 2 freely available to small-scale farmers with incomes less than $10,000. Once approved for release, varieties directly bred from Syngenta's rice will become the farmer's property, which they will be able to resow year after year without payment.

Greenpeace, Friends of the Earth and their political allies in European governments and nongovernmental organizations will not welcome Golden Rice 2. They will continue to reject and stall biotech products at the mere hint of a transgene, no matter what the humanitarian value of the crop and no matter how spurious the environmental concerns. But there comes a time when arguments against a GM product that could help prevent blindness in hundreds of thousands and death in millions each year should be seen for what they are: ideological bigotry.

Golden Rice is an exception to the rule that we don't give away gold or grain for free. It cannot change the way the world works. And it cannot reverse all the health or economic inequities that exist around the globe. But it can change for the better the plight of the world's malnourished, if only those rigidly opposed to GM crops would let it.

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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

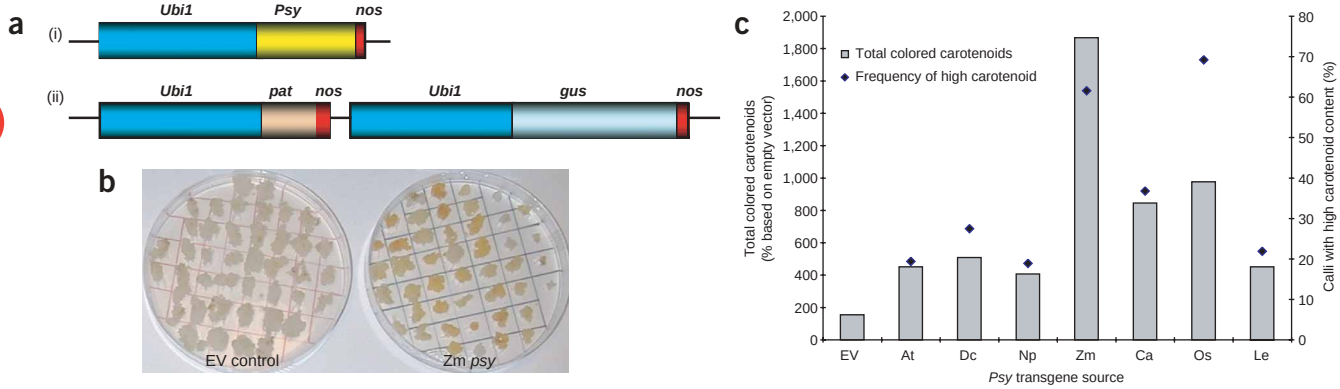

Figure 1 Expression of a *psy* transgene increases the carotenoid content of maize callus. (**a**) Schematic diagram of the gene cassettes in the two plasmids used to cotransform maize callus. Both contain the maize polyubiquitin1 promoter (*Ubi1*) and the *nos* terminator (*nos*). (i) The seven similar plasmids constructed with the phytoene synthase-coding region (*psy*) from each of the species listed below. (ii) The phosphino 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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Published online 27 March 2005; doi:10.1038/nbt1082

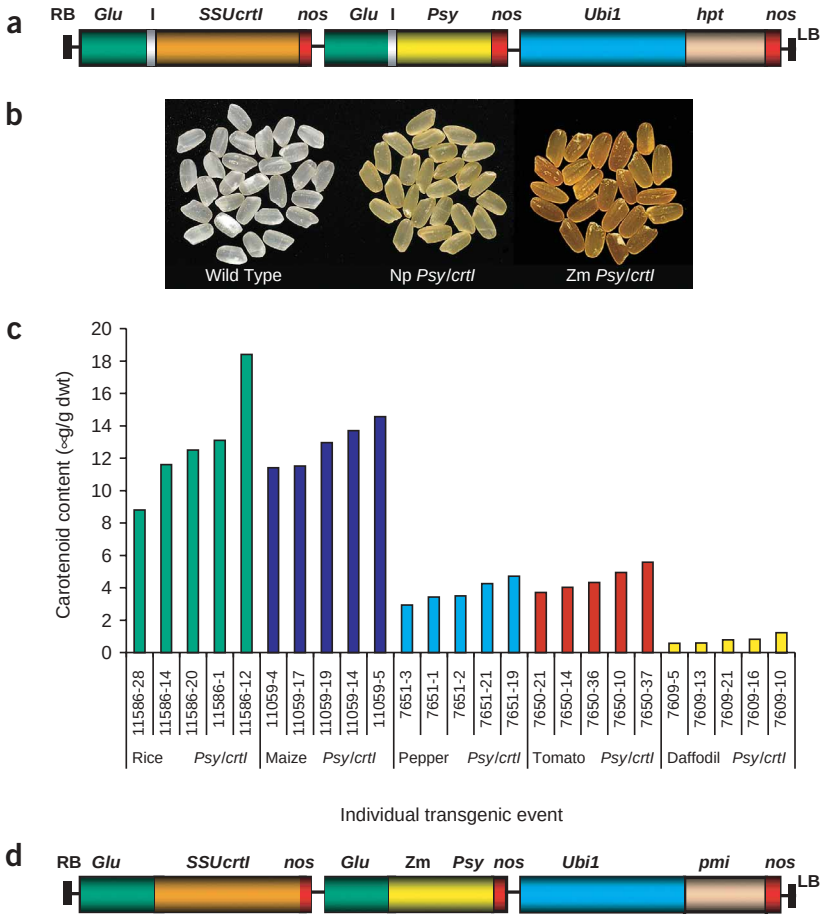

Figure 2 Carotenoid enhancement of the rice endosperm by transformation with *psy* orthologues and *crtI*. (**a**) Schematic diagram of the T-DNAs used to generate transgenic rice plants. The T-DNA comprised the rice glutelin promoter (*Glu*) and the first intron of the catalase gene from castor bean (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.

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

Table 1 Carotenoid content and composition of transgenic rice endosperm

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)	Colored carotenoid composition, % of total in T_1 (T_2[a]) seed				
			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.

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 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. 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 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, and in constructs for rice transformation except Golden Rice 2, the addition of GCGGCCGCC (*Not*I site) immediately downstream of the ATG start codon), 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.

ACKNOWLEDGMENTS

The authors would like to thank Will Parish, Erik Dunder, Dong Fang Chen and Annalisa Tiozzo for tissue culture, Karen Bacon and Fasica Woldeyes for plant growth, Melanie Watkins for plant assessment, Elek Bolygo for analytical advice, Ebun Eno-Amooquaye for western blot analysis, Keith Ward for advice on statistics and others who gave technical support to the research. We would also like to thank Peter Beyer, Lu Liu and John Ray for plasmids. We thank Peter Beyer for discussion on the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 30 July 2004; accepted 15 February 2005
Published online at http://www.nature.com/naturebiotechnology/

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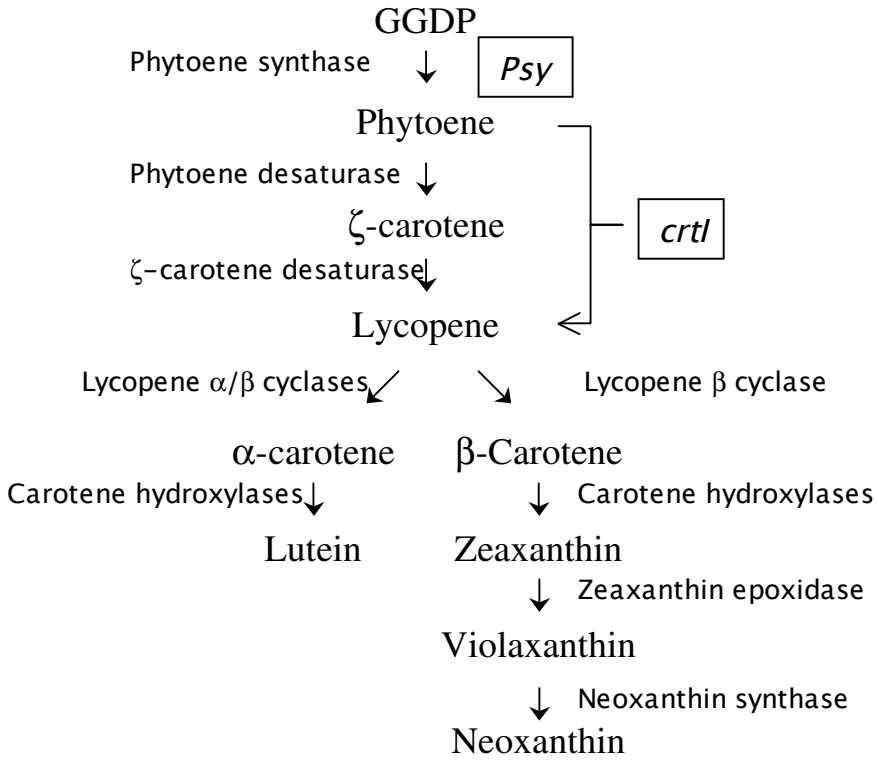
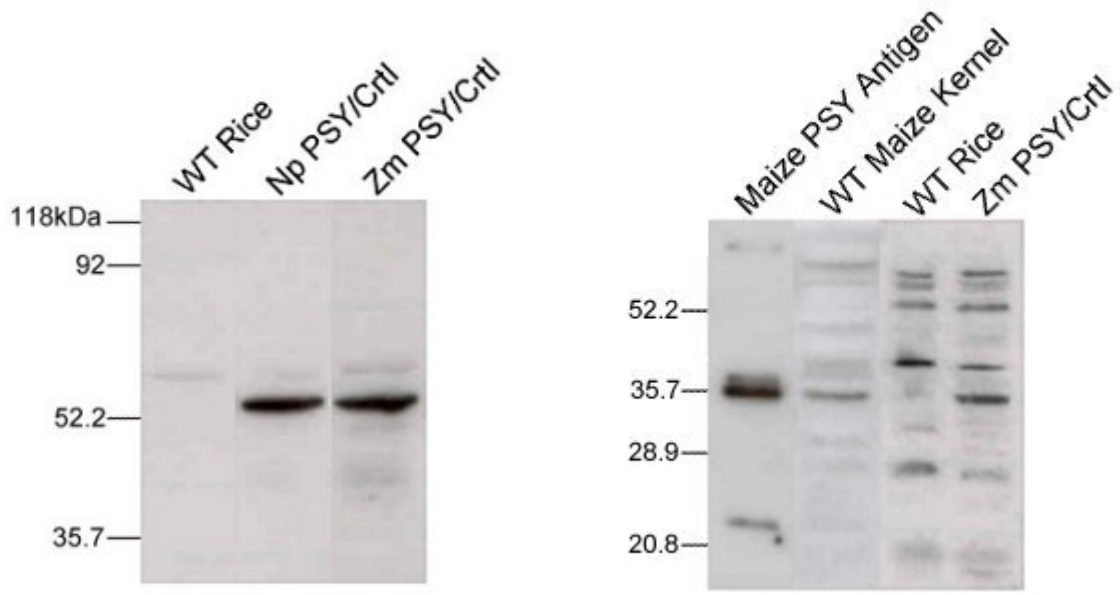
Supplementary Figure 1 Carotenoid Biosynthesis in plants



The isoprenoid precursor for carotenoid biosynthesis is geranyl geranyl diphosphate (GGDP). The transgenes present in Golden Rice are boxed. These encode the enzymes phytoene synthase (*Psy*) and the multifunctional carotene desaturase (*crtI*). The enzyme names of the plant biosynthetic pathway are shown in a simplified form. Carotenoids below α/β-carotene are xanthophylls.

Supplementary Figure 2 Expression of CRTI and maize PSY proteins in rice endosperm

a **b**



Immunoblots of grain protein extracts from wild-type (WT) and transgenic plants. The blots were immunodecorated with polyclonal antisera raised against the CRTI **(a)** or maize PSY **(b)** proteins. Analysis was performed on transgenic rice plants containing the daffodil *Psy* and *crtI* genes (Np PSY/crtI) and the maize *Psy* and *crtI* genes (Zm PSY/crtI). The maize PSY antigen and an extract from mature wild-type maize kernels were included in **(b)** as controls.

The CRTI antiserum gave a strong reaction with a protein of 57 kDa in endosperm containing the daffodil *Psy/crtI* and maize *Psy/crtI* gene pairs (Fig. 2a) with no equivalent band in the untransformed sample. The CRTI protein has a predicted molecular mass of 55 kDa assuming accurate processing of the RUBISCO small subunit plastid transit peptide. Despite cross-reacting bands, the maize PSY antiserum detected one protein of approximately 36 kDa which was unique to transgenic rice seed containing the maize *Psy* transgene (Fig. 3b). Given the limitation of SDS-PAGE for estimating protein size especially for hydrophobic proteins, it is likely that this protein band represents the processed maize PSY protein (predicted molecular mass, assuming transit peptide cleavage after Tyr62, of 39.8 kDa). A band of a very similar molecular mass was also detected in the protein extract of maize kernels most probably representing the endogenous maize PSY protein.

Methods for production of antigen, antibody and western blot analysis

The *crtI* coding sequence was inserted into the *XmnI/HindIII* site of a pBR322-based vector containing the *tac* promoter and the coding sequence for six His residues 5' to the *XmnI* cloning site resulting in an N-terminal tag on the CRTI protein. The tagged-CRTI was expressed in *E. coli* DH5α and purified from the supernatant on a Ni-NTA agarose column (Qiagen) according to the manufacturers protocol. The maize *Psy* coding sequence, lacking the first 354 bp from the 5' end of the coding sequence, was cloned into the *NcoI/BamHI* site of pET24a (Novagen) under control of the *T7*

promoter. The truncated maize PSY was expressed in *E. coli* BL21(DE3) RP cells and purified from pelleted inclusion bodies.

Protein (100 μg) in Freunds Complete Adjuvant was administered subcutaneously to New Zealand White rabbits. A further 100 μg of protein was administered in Freunds Incomplete Adjuvant 28, 56 and 84 days after the initial immunisation and antibody was collected at 98 days.

Polished rice endosperm was homogenised in Laemmli sample buffer. Protein was collected in the supernatant by centrifugation at 13 000 rpm for 30 min. SDS-PAGE[40] and western blotting[41] were performed using standard protocols. Gel lanes contained 100 ng maize PSY antigen, 100 μg maize kernel protein extract or 40 μg polished rice endosperm protein extract respectively. Detection of antibody-reactive proteins was carried out using the ECL system and manufacturers protocol (Amersham).

Supplementary Table 1 Carotenoid composition of maize callus expressing a *Psy* transgene

	Colored carotenoid composition, % of total[a]						
***Psy* source**	**β–carotene**	**α–carotene**	**lutein**	**violaxanthin**	**neoxanthin**	**minor xanthophylls**	**phytoene (g/g)[b]**
Maize	51.3	22.5	19.0	3.9	0.7	2.6	96
Rice	35.5	11.0	33.1	12.9	n.d.	7.4	59
Tomato	15.8	5.4	50.1	21.6	6.9	0.3	2.5
Pepper	10.1	3.3	41.9	43.3	1.4	n.d.	1.6
Carrot	8.6	2.5	51.0	25.7	11.9	0.2	3.7
Arabidopsis	21.1	10.5	44.1	20.4	2.4	1.6	0.4
Daffodil	15.9	4.7	49.5	21.2	6.5	2.1	0.1
None (empty vector)	5.4	1.4	47.0	36.9	7.4	1.9	1.0

[a] Colored carotenoid values are the average for the five calli containing the highest carotenoid content from each population (n.d. is not detected).
[b] For phytoene, the content of the 75th percentile sample is shown.