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High-level Tryptophan Accumulation in Seeds of Transgenic Rice and Its Limited Effects on Agronomic Traits and Seed Metabolite Profile*

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ABSTRACT

Metabolic manipulation of plants to improve their nutritional quality is an important goal of plant biotechnology. Expression in rice (*Oryza sativa* L.) of a transgene (*OASA1D*) encoding a feedback-insensitive α subunit of rice anthranilate synthase results in the accumulation of tryptophan (Trp) in calli and leaves. It is shown here that the amount of free Trp in the seeds of such plants is increased by about two orders of magnitude compared with that in the seeds of wild-type plants. The total Trp content in the seeds of the transgenic plants was also increased. Two homozygous lines, HW1 and HW5, of *OASA1D* transgenic rice were generated for

characterization of agronomic traits and aromatic metabolite profiling of seeds. The marked overproduction of Trp was stable in these lines under field conditions, although spikelet fertility and yield, as well as seed germination ability, were reduced compared with the wild type. These differences in agronomic traits were small, however, in HW5. In spite of the high Trp content in the seeds of the HW lines, metabolic profiling revealed no substantial changes in the amounts of other phenolic compounds. The amount of indole acetic acid was increased about 2-fold in the seeds of the transgenic lines. The establishment and characterization of these *OASA1D* transgenic lines have thus demonstrated the feasibility of increasing the Trp content in the seeds of rice (or of other crops) as a means of improving its nutritional value for human consumption or animal feed.

Key words: Amino acids; Anthranilate synthase; AS; Noncontainment greenhouse; IAA; Isolated field; 5MT; *OASA1D*; *Oryza sativa.*

INTRODUCTION

Metabolic manipulation of plants to improve their nutritional value is a primary goal of plant biotechnology. Essential amino acids such as Lys, Met, Thr, and Trp contribute substantially to the nutritional quality of plantbased foods for humans and domestic animals, but the amounts of such amino acids are limited in many crops. Dietary supplementation with Trp increases the growth rate of pigs and poultry (Subcommittee on Poultry Nutrition, 1994; Subcommittee on Swine Nutrition, 1998). An inadequate supply of Trp leads to a marked reduction in food intake in pigs as a result of the reduced synthesis of serotonin in the brain (Henry *et al.,* 1992; Se`ve, 1999; Eder *et al.,* 2001). Indeed, Trp has been used as a pharmaceutical agent in the treatment of depression (Massey *et al.,* 1998). The ability to increase the level of Trp in food crops by metabolic engineering is thus desirable from both nutritional and

clinical viewpoints. The achievement of an increase in the amino acid content of seeds by genetic engineering, however, has been limited to Lys (Falco *et al.,* 1995; Mazur *et al.,* 1999; Zhu and Galili, 2003) and Met (Movig *et al.,* 1997; Lai and Messing, 2002).

The accumulation of free Trp in plants has been achieved by the introduction of genes encoding feedback-insensitive α subunits of anthranilate synthase (AS), which catalyses the conversion of chorismate to anthranilate. This approach has thus yielded increased levels of Trp in the roots of the forage legume *Astragalus sinicus* (Cho *et al.,* 2000) and in the leaves of tobacco (Zhang *et al.,* 2001). A mutant *OASA1* gene, *OASA1D* [*formerly referred to as OASA1 (D323N)*], that encodes a feedback-insensitive α subunit of rice AS (Tozawa *et al.,* 2001) has previously been generated. Rice calli and leaves as well as potato plants and tubers that express *OASA1D* accumulate large amounts of free Trp (Tozawa *et al.,* 2001; Yamada *et al.,* 2004).

Manipulation of metabolic pathways in plants by genetic engineering thus has the potential to improve the nutritional value of crops and to allow the production of desired natural products in higher plants (DellaPenna, 2001; Morandini and Salamini, 2003). The proteins encoded by the introduced genes might affect not only the abundance of the target metabolite, however, but also, through changes in metabolic networks, that of related compounds. Moreover, such changes may influence the physiology or morphology of plants. Little is known, however, of the consequences of such manipulation for metabolite profiles and agronomic traits in plants.

Metabolism of Trp in plants is associated with the generation of a range of secondary compounds such as indole alkaloids and indole acetic acid (IAA). Manipulation of the Trp biosynthetic pathway might thus be expected to influence the synthesis of such metabolites and thereby elicit a pronounced change in metabolite profile. Indeed, overexpression of Trp decarboxylase, which catalyses the conversion of Trp to tryptamine, resulted in a marked reduction in the amount of indole glucosinolate in canola plants (Chavadej *et al.,* 1994) and a decreased abundance of Trp, Phe, and chlorogenic acid in potato (Yao *et al.,* 1995).

Evaluation of transgenic plants of improved nutritional value for effects of the transgene on agronomic traits and metabolite profiles is thus essential. However, the possible effects of the accumulation of Trp or of any other essential amino acid in seeds on agronomic traits and metabolite profiles have not been determined to date. Demonstration that Trp is the only major metabolite that accumulates in transgenic rice seeds, for example, would be likely to increase their acceptability for consumption by humans and farm animals.

The effects of *OASA1D* expression in rice both on the amounts of Trp and other amino acids in seeds and on agronomic traits of plants cultivated in isolated field trials have now been studied. The profile of phenolic compounds as well as the content of IAA in the transgenic seeds was also determined. Rice is one of the major crops in the world and its use for animal feed has recently been developed (Sakai et *al.,* 2003*a*). The results of the increase of Trp in transgenic rice prove the usefulness of the gene for improving the nutrition of other crops for animal feed.

MATERIALS AND METHODS

Plant materials and growth conditions

Seed calli of rice (*Oryza sativa* L. cv. Nipponbare) were transformed with the use of *Agrobacterium* as described by Hiei *et al.* (1994). The generation of rice plants transgenic for *OASA1D* or *OASA2*, with both genes controlled by the promoter of the maize ubiquitin gene, has also been described (Tozawa *et al.,* 2001; Yamada *et al.,* 2004). Rice plants transgenic for the β-glucuronidase gene (*GUS*) were also generated as described by Urushibara *et al.* (2001). All transgenic plants contained the hygromycin phosphotransferase gene (*hpt*) under the control of the 35S promoter of the cauliflower mosaic virus. Transgenic rice plants (R0 to R4) and non-transformed control plants (Nipponbare) were grown at 28℃ and 60% humidity under natural light conditions in pots (1/10 000a) containing podosol soil (Sumitomo Chemical, Osaka, Japan) in a containment greenhouse.

Two *OASA1D* transgenic lines, HW1 and HW5, were also grown in a non-containment greenhouse (R5) and an isolated field (R6). In 2002, seeds of the HW lines and Nipponbare were sown on 15 April and the seedlings were transferred individually to pots (1/10 000a) containing podosol soil on 15 May. The plants were maintained in the non-containment greenhouse under natural light and temperature conditions at the National Institute of Crop Science (NICS). No additional fertilizer was applied. In 2003, seeds of the HW lines and Nipponbare were sown on 14 May and the seedlings were transferred to an isolated paddy field (10 m x 1.2 m) at the National Institute for Agro-Environmental Sciences in Tsukuba on 4 June. For each line, 40 seedlings were planted in duplicate in an area of 15 cm x 30 cm per plant. The total amount of fertilizer applied m^{-2} included 6 g of N, 6 g of P_2O_5, and 6 g of K_2O and was added at planting. Other practices followed the cultivation standards of

Ibaraki Prefecture (Ibaraki Prefecture Standard Lowland Rice Cultivation, 1990).

Evaluation of agronomic traits

Agronomic traits, including heading date, culm length, and morphological characteristics, were evaluated by standard protocols (Ibaraki Prefecture Standard Lowland Rice Cultivation, 1990). Plants in the isolated field were harvested individually, air-dried, and analysed for yield and seed germination. All panicles were harvested from each plant, dried at 38 ℃ for 4 weeks, and then maintained in a freezer at -30 ℃ until analysis of amino acids and of IAA and metabolite profiling. For germination analysis, 15 or 20 seeds of individual lines were transferred to a filter paper that had been moistened with distilled water and placed in a Petri dish (6 cm in diameter); the analysis was performed in duplicate. The Petri dish was maintained at 35℃ for 15 d in the dark, and seed germination was assessed each day according to the modified standard method of NICS based on viviparity (Sakai *et al.,* 2003*b*).

Amino acid analysis and determination of total nitrogen content

Dehulled seeds were autoclaved individually with 50 μl of water in a 1.5 ml Eppendorf tube for 15 min, and extracts were prepared from each seed as previously described (Wakasa and Widholm, 1987). The amounts of free amino acids were then quantified with the use of the PICO.TAG analysis system (Waters, Milford, MA). For quantitation of total Trp, five dehulled grains were pulverized with a mortar and pestle and heated in 5 M NaOH for 28 h at 110 ℃; the hydrolysate was then acidified with 6 M HCl and subjected to analysis with an L-8800 High Speed Amino Acid Analyser (Hitachi High-Technologies, Tokyo, Japan).

Dehulled rice seeds were weighed and decomposed in concentrated sulphuric acid in the presence of salicylic acid and sodium thiosulphate. The ammonia formed was distilled, and was determined colorimetrically at 640 nm using the indophenol method.

Metabolite profiling

Four dehulled seeds were pulverized with a mortar and pestle and subjected to extraction for 1 h with 10 vols (v/w) of a mixture of water: methanol: acetic acid (249: 250: 1, by vol.). The extract was centrifuged at 16 000 g for 20 min, the resulting supernatant was passed through a SepPak C_{18} cartridge (Waters, Milford, MA), and the eluate was subjected to reversed-phase HPLC (LC-10Avp system; Shimadzu, Kyoto, Japan) with a Cadenza Column CD-C18 [250 mm x 4.6 mm (inner diameter); Imtakt, Kyoto, Japan). Elution was performed with a mixture of acetonitrile and 0.02% aqueous trifluoroacetic acid (3: 97 v/v, at 0 min; 30: 70 v/v at 40 min; 98: 2 v/v at 75 min) at a flow rate of 0.85 ml min^{-1} and a temperature of 40 ℃; it was monitored with a photodiode array detector (Shimadzu SPDM10Avp) over a wavelength range of 190-400 nm.

IAA analysis

Six dehulled seeds were pulverized with a mortar and pestle and then soaked for 3 h at 4℃ in 10 vol (v/w) of 80% acetone in water containing 2.5 mM diethyl dithiocarbamate. This extraction procedure was repeated three times. The combined extract was divided into three portions that were respectively subjected to quantification of free IAA, free plus ester forms of IAA and total IAA. The free IAA in the extract was partially purified by solid-phase extraction and quantified by liquid chomatography and tandem MS (LC-MS/MS) as previously described by Matsuda *et*

al. (2005). For determination of the amount of free plus ester forms of IAA, the original extract was acidified to pH 2 with aqueous HCl and then analysed as for free IAA. For determination of the amount of total IAA, the extract was subjected to hydrolysis with 7 M NaOH for 3 h at 100℃ under N_2 before analysis. [Phenyl-$^{13}C_6$]IAA was used as the internal standard in these analyses.

RESULTS AND DISCUSSION

Generation of rice plants expressing OASA1D

Transgenic rice plants that express *OASA1D* were generated and subjected to the analyses of Southern blot and northern blot as described previously (Tozawa *et al.,* 2001; Yamada *et al.,* 2004). Given that expression of *OASA1D* confers resistance to 0.3 mM 5-methyltryptophan (5MT), some transgenic plants were generated from calli selected with 5MT instead of with hygromycin (Yamada *et al.,* 2004). Transgenic lines selected by growth in the presence of 5MT or hygromycin are denoted by M or H, respectively.

Spikelet fertility of greenhouse-grown plants

Almost all regenerated plants of >120 transgenic lines grown in pots exhibited normal growth, with exceptional instances of dwarfism or slow growth presumably being attributable to somaclonal mutations (Phillips *et al.,* 1994). However, for plants grown in a greenhouse, the spikelet fertility of transgenic plants tended to be lower than that of the wild type (Table 1). The mean spikelet fertility of the transgenic plants was thus only 31%, compared with a value of 76% for seed-grown Nipponbare. Given that a decrease in spikelet fertility has previously been observed in transgenic rice plants generated by tissue culture (Hiei *et al.,* 1994; Urushibara *et al.,* 2001), part of the reduction in spikelet fertility apparent in *OASA1D*-expressing plants might be due to the regeneration process. However, transgenic rice plants that overexpress the

wild-type gene (*OASA2*) for another α subunit of rice AS but do not accumulate Trp in calli or leaves (Tozawa *et al.*, 2001) exhibited a spikelet fertility (61%) higher than that of plants that express *OASA1D*, but lower than that of Nipponbare, suggesting that accumulation of Trp might also contribute to the reduced spikelet fertility of the *OASA1D* transgenic plants. Analysis by genomic Southern hybridization of R0 (regenerated) plants of *OASA1D* transgenic lines with a spikelet fertility of >50% revealed that they had a relatively high copy number (three to seven) of the transgene (data not shown), suggesting that the copy number was not a principal cause of low spikelet fertility.

Table 1. Spikelet fertility of OASAID transgenic plants (R0) grown in a greenhouse

Transgene	Trp content	Spikelet fertility (%)						Total no. of plants
		≦20	21-40	41-60	61-80	81-100	Mean±SD	
OASAID	Increased	25	19	12	7	0	30.7±21.2	63
OASA2	Normal	5	4	6	14	11	61.4±27.2	40
NB [a]	Normal	0	1	2	3	6	76.2±19.7	12

[a] Seed grown Nipponbare plants.

Trp content of seeds produced by greenhouse-grown plants

Seeds of 12 *OASA1D* transgenic lines with a spikelet fertility of >50% were analysed for free and total Trp contents in the second or third generation (R2 or R3 seeds) of plants grown in a greenhouse. All lines showed a marked increase in the amount of free Trp, with the mean free Trp content ranging from 3037 to 23 705 nmol g^{-1} of dry seed weight (Table 2); these values correspond to increases of 55- to 431-fold compared with the free Trp content of non-transgenic Nipponbare seeds (55 nmol g^{-1}). The amount of free Trp as a percentage of total Trp in the transgenic seeds (34–87%) was also greatly increased compared with that in wild-type seeds (1.3%).

Given that seeds of a transgenic line expressing *GUS*, which encodes β -glucuronidase, exhibited a Trp content similar to that of Nipponbare seeds, it was unlikely that the Trp accumulation apparent in seeds of *OASA1D* transgenic plants resulted from an abnormality caused by the transformation process.

The transgenic seeds analysed for Trp content were a mixture of those homozygous or heterozygous for *OASA1D*. To examine the possible influence of genotype on Trp content, seeds from a single transgenic plant of the H17 line in the R1 generation were divided into two groups. One group of 26 seeds showed segregation of *OASA1D* by PCR analysis (14 positive, 12 negative). The other group of 32 seeds, which also should have been a mixture of transgene genotypes, all contained an increased level of free Trp (9134±3740 nmol g^{-1}). These results thus indicated that Trp accumulation in seeds is determined primarily by the genotype of the mother plant. However, given that the free Trp content of seeds of the H17 or M34 lines showed some variability (Table 2), the level of free Trp in seeds might also be influenced by seed genotype. Although the physiological conditions of plants and seeds also affect amino acid content, the increase in the amount of Trp in the seeds of transgenic rice expressing *OASA1D* was sufficiently high to be attributed to the activity of the transgene.

It is the total Trp content, including both free Trp and Trp in proteins, that is important for the nutritional value of seeds. The increase in the amount of free Trp in seeds of *OASA1D* transgenic plants was accompanied by an increase in the total Trp content (Table 2). The total Trp content of seeds of the various transgenic ines thus ranged from 8100 to 48 519 nmol g^{-1}, values that correspond to increases of 1.9- to 11.6-fold compared with that for Nipponbare (4188 nmol g^{-1}).

The weight of individual dehulled seeds tended to be s aller for *OASA1D* transgenic lines than for Nipponbare (Table 2). However, a reduced seed weight was also apparent for plants harbouring a *GUS* transgene, suggesting that this effect might be attributable to the transformation process. *OASA1D* transgenic line M34, whose seeds showed the highest Trp content, also manifested the lowest seed weight. However, the seeds of line M21, which exhibited a medium level of Trp accumulation, were also of low weight. No clear correlation between Trp content and seed weight was thus apparent.

Generation of homozygous OASA1D *transgenic rice lines for evaluation of agronomic traits*

Two *OASA1D* transgenic lines, M121 and H41, were advanced to obtain homozygotes for evaluation of agronomic traits. The resulting homozygous lines were designated HW1 for M121 and HW5 for H41. The early generations of these homozygous lines exhibited a spikelet fertility of >50% and normal morphological features. Genomic Southern blot analysis of selfed progenies and of F_2 plants of F_1 hybrids between either HW1 or HW5 and Nipponbare revealed that HW1 contained three copies of the transgene and HW5 harboured four copies. No segregation of hybridized bands was observed in F_2 plants, indicating that the transgenes were integrated at one locus (data not shown).

Table 2. Total and free Trp contents of seeds from *OASA1D* transgenic plants grown in a greenhouse.

Transgenis Line	Seed generation	Seed Weight (mg)	Total Trp (nmol g^{-1})	Free Trp (nmol g^{-1}) Means±SD
Nipponbare		18.6	4188	55.0±21.7
GUS 16 [a]	R2	14.2	5173	41.0±8.0
M25	R2	11.7	8100	
M121	R3	14.9	8810	3036.6±233.4
M31	R2	17.1	9237	
M21	R2	9.9	9264	
H29	R2	14.1	11 320	
H36	R2	17.0	14 425	7642.3±830.3
H17	R3	16.2	14 178	12 309.0±5764.9
H37	R2	14.0	15 619	
H42	R2	11.9	16 967	
H40	R2	12.7	17 322	
M45	R2	12.7	20 830	14 495.3±3528.0
M34	R2	7.7	48 519	23 705.3±11 681.2

[a] Transgenic line harbouring *GUS* and hph (*OASA1D* lines contain *OASA1D* and *hph*).

Five seeds of one panicle from each transgenic line were used for measurement of total Trp content and of average seed weight. Total Trp content and seed weight of Nipponbare were determined as average values for two plants. Free Trp was measured in three to five single seeds, and values presented are means ±SD. Trp content is shown as nmol Trp g^{-1} dry seed weight.

Trp content of seeds produced by field-grown OASA1D *transgenic plants*

The free and total Trp contents as well as the nitrogen content of R5 and R6 seeds of the HW lines grown in a greenhouse were higher than those of Nipponbare seeds (Table 3), and all of these values were slightly higher for HW1 than for HW5. The levels of free and total Trp in seeds were stable during growth of the HW lines in a greenhouse for 2 years (data not shown). In the field condition, the free Trp content of seeds of the HW lines was increased about 2-fold compared with the corresponding values for seeds of greenhouse-grown plants (Table 3). This increased

accumulation of free Trp in the seeds of fieldgrown plants was not accompanied by a similar increase in the total Trp content. The level of total Trp in seeds of the HW lines was thus stable under different growth conditions. The nitrogen content of seeds of field-grown HW lines and Nipponbare was lower than the corresponding values for seeds of greenhouse-grown plants. The amounts of free and total Trp in Nipponbare seeds were also lower in the field condition than in the greenhouse, with the result that the relative values to Nipponbare for seeds of the fieldgrown HW lines were increased accordingly (Table 3). The levels of Trp in the seeds of Nipponbare grown in another field were similarly low (data not shown).

Table 3. Nitrogen and Trp contents of seeds produced by HW lines under field or greenhouse conditions

Growth condition	Line	N content (%)	Free Trp		Total Trp	
			(nmol g^{-1})	R.V. [a]	(nmol g^{-1})	R.V. [a]
Greenhouse	HW1	1.39±0.12 (2)	3403±283.4 a (6)	55.9	7883±151.2 a (3)	1.9
	HW5	1.30±0.10 (2)	1762±333.3 b (6)	41.0	6949±387.6 b (3)	1.7
	NB	1.24±0.04 (2)	43±15.7 c (6)	1	4118±237.0 c (3)	1
Field	HW1	1.13±0.03 (2)	5294±2672.6 a (6)	311.0	8477±1139.0 a (4)	14.1
	HW5	1.04±0.01 (2)	3288±1366.1 a (6)	193.0	5684±1818.0 b (4)	9.4
	NB	0.96±0.13 (2)	17±9.4 b (9)	1	602±413.0 c (3)	1

[a] Relative value compared with Nipponbare.

Five seeds of Nipponbare (NB) or HW lines were analysed for nitrogen content (percentage of dry seed weight) and total Trp content (nmol g^{-1} dry seed weight) in each of the indicated (in parentheses) number of experiments, and data presented are means ±SD. Values for free Trp content (nmol g^{-1} dry seed weight) are presented as means ±SD for the numbers of seeds indicated in parentheses. Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

The opposite effects of field growth on the free Trp content of seeds of the HW lines and of Nipponbare seeds might be attributable, in part, to a response of the transgene promoter to the cooler temperatures or to the difference in temperature between night and day in the field. The *OASA1D* gene was driven by the ubiquitin gene promoter, which is responsive to stress (Takimoto *et al.*, 1994) and might therefore be activated by low temperatures, resulting in increased expression of *OASA1D* and a greater accumulation of free Trp.

Amino acid composition of seeds produced by field-grown* OASA1D *transgenic plants

The increased Trp content of rice calli expressing *OASA1D* does not result in substantial changes in the amounts of other amino acids (Tozawa *et al.*, 2001). The marked accumulation of free Trp in the seeds of the HW1 and HW5 lines grown under field conditions was accompanied by an increase in the amounts of other amino acids to some extent (Table 4). Their increases were relatively small compared with that of Trp. The total amount of free amino acids was increased 4.2- and 2.9-fold in HW1 and HW5, respectively, compared with the value for Nipponbare, with the maximal change in the content of any one amino acid (other than Trp) being limited to a 5.2-fold increase. However, with the exception of Ala (and Trp), the ratio of the amount of each amino acid to the total amount of free amino acids was reduced or remained virtually the same in the transgenic lines compared with Nipponbare.

Table 4. Free amino acid composition of seeds produced by field-grown HW lines.

Amino acid	Free amino acid content(nmol g^{-1})							
	NB		HW1			HW5		
	Mean±SD	(%)[a]	Mean±SD	(%)[a]	Relative value[b]	Mean±SD	(%)[a]	Relative value[b]
Gly	51±29 a	1.9	153±95 b	1.4	3.0	142±26 b	1.8	2.8
Ala	154±95 a	5.8	794±581 b	7.3	5.2	608±315 b	7.9	3.9
Val	32±19 a	1.2	84±37 b	0.8	2.6	68±16 b	0.9	2.1
Leu	13±7 a	0.5	36±20 b	0.3	2.7	32±8 b	0.4	2.5
Ile	8±5 a	0.3	28±15 b	0.3	3.5	26±9 b	0.3	3.3
Ser	172±95 a	6.5	288±107 a	2.6	1.7	277±68 a	3.6	1.6
Thr	37±30 a	1.4	70±37 a	0.6	1.9	57±31 a	0.7	1.5
Met	5±4 a	0.2	8±2 a	0.1	1.6	6±2 a	0.1	1.2
Asp	575±333 a	21.9	1285±727 a,b	11.7	2.2	1136±139 b	14.7	2.0
Asn	595±760 a	22.6	1269±616 a	11.6	2.1	746±296 a	9.7	1.3
Glu	660±359 a	25.1	810±303 a	7.4	1.2	739±165 a	9.6	1.1
Gln	51±31 a	1.9	47±13 a	0.4	0.9	54±8 a	0.7	1.1
Arg	67±71 a	2.5	239±123 b	2.2	3.6	102±48 a	1.3	1.5
Lys	16±7 a	0.6	65±34 b	0.6	4.1	48±14 b	0.6	3.0
His	28±17 a	1.1	115±48 b	1.1	4.1	116±75 b	1.5	4.1
Phe	7±4 a	0.3	30±15 b	0.3	4.3	25±7 b	0.3	3.6
Tyr	91±55 a	3.5	208±74 b	1.9	2.3	171±28 b	2.2	1.9
Trp	20±9 a	0.8	5294±2673 b	48.4	264.7	3288±1366 b	42.5	164.4
Pro	49±31 a	1.9	125±60 b	1.2	2.6	96±17 b	1.2	2.0
Total	2629±2001 a	100	10 947±4484 b	100	4.2	7734±1810 b	100	2.9

[a] Amount of each amino acid as a percentage of total amino acids.
[b] Amount of each amino acid relative to that in Nipponbare.

Data are expressed as nmol of amino acid g^{-1} dry seed weight and are means ±SD of values from six seeds of Nipponbare (NB) or HW lines. Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

The levels of Lys and Phe were low in Nipponbare seeds but their absolute amounts were increased in the HW seeds, with the result that their percentage contributions to the total amount of free amino acids were the same in the HW lines and in the wild type. Given that Gln is the amino donor for the synthesis of anthranilate, its abundance might have been expected to be changed in the seeds of the transgenic lines. Its absolute amount in seeds of the HW lines was similar to that in Nipponbare seeds, however. Serine is also a precursor for Trp synthesis, but its amount in HW seeds was not significantly increased compared with that in Nipponbare seeds.

The increases in the absolute amounts of free amino acids in the seeds of the transgenic lines suggest the existence of regulatory mechanisms that increase amino acid synthesis in response to Trp accumulation. The *opaque-2* mutation in maize (Oh545*o2*) is associated with an increased level of free amino acids in mature endosperm (Wang and Larkins, 2001). Genetic analysis suggests that the gene for aspartate kinase 2 is the gene responsible for the effect of this mutation on free amino acid content (Wang *et al.*, 2001). A mutation in a transcriptional regulator of AS genes also renders Trp biosynthesis insensitive to Trp concentration (Bender and Fink, 1998). Our results therefore suggest that Trp accumulation in rice seeds might increase the transcription of genes that encode enzymes responsible for amino acid synthesis.

Agronomic traits of field-grown* OASA1D *transgenic plants

HW lines grown in a greenhouse appeared similar to Nipponbare with regard to most agronomic traits analysed (Table 5). For plants grown under field conditions, however, differences in traits related to seed productivity were apparent between the transgenic lines (especially HW1) and the wild type. Both HW lines grown in the field thus exhibited a spikelet fertility lower than that of Nipponbare, although pollen

fertility (Table 5) and anther size (reflecting the number of pollen grains) (data not shown) for the transgenic lines were similar to those for Nipponbare. Moreover, the average spikelet number per panicle was significantly smaller for the HW lines than for Nipponbare. The reduction in the number of spikelets per panicle and the low spikelet fertility likely contributed to an observed decrease in harvested seed weight for HW1 and HW5 to 52.5% and 69.6% of the value for Nipponbare, respectively. The harvested plant weight was similar for the HW lines and Nipponbare (data not shown). Whereas the individual brown seed weight varied among transgenic lines in early generations (Table 2), it did not differ markedly among HW lines and Nipponbare under field conditions (Table 5).

The high concentration of Trp in the HW lines is likely to be the primary cause of the differences in agronomic traits between these lines and Nipponbare grown under field conditions. Spikelet number per panicle is determined at an early stage of development of the inflorescence apex and is influenced by several conditions, such as nitrogen and carbon availability as well as temperature (Takeoka *et al.,* 1993). Accumulation of Trp might increase the sensitivity of plants of the HW lines to environmental stress and thereby reduce spikelet number and fertility in the field condition.

The culm length of HW lines grown in the field was smaller than that of Nipponbare, although this difference was statistically significant only for HW1 (Table 5). No difference in plant height was observed between HW lines and Nipponbare (data not shown). A short culm length has often been observed in plants regenerated from tissue culture (Phillips *et al.,* 1994), suggesting that this characteristic of the HW lines might be attributable to somaclonal mutation.

Table 5. Agronomic traits of HW1 and HW5 lines

Growth condition (year)	Line	*n* [a]	Day to Heading ±SD	Culm length (cm)	Panicle length (cm)	No.of tillers plant^{-1}	Spikelet fertility (%) ±SD[b]	No.of spilelets panicle^{-1}	Mean dry matter of harvested seeds (30 plants g^{-1}) ±SD[c]	Pollen fertility (%)[d]	Brown seed weight (1000 grains g^{-1})[e]
Green house (2002)	HW1	10	129±0.64	78.1 a	16.1 a	13.7 a	79.5±6.84			98.3	20.1 a
	HW5	10	132±2.04	74.9 a	15.7 a	13.1 a	88.3±5.19			97.5	20.1 a
	NB	23	130±1.13	80.1 a	17.0 a	11.6 a	88.2±6.65			98.9	20.1 a
Field (2003)	HW1	2	100±0.00	73.3 b	18.1 a	15.5 a	65.0±18.21	71.6 b	443.6±35.64	97.6	20.2 ab
	HW5	2	101±0.50	75.7 ab	17.8 a	13.2 a	81.3±11.00	85.6 b	588.4±50.77	98.5	21.0 a
	NB	2	100±0.00	81.3 a	20.1 a	12.2 a	91.6±2.91	122.3 a	844.8±27.86	99.4	20.0 b

[a] n means repetition. The number of plants or plots for the greenhouse and field conditions, respectively. Heading date, culm length, panicle length, and the number of tillers were recorded for each of 10 or 23 plants in the greenhouse condition and for each of 15 plants per plot in the field condition.

[b] Spikelet fertility was recorded for all panicles of 10 plants in 2002 and for three panicles of main culms of 60 plants in 2003.

[c] Mean dry matter of harvested seeds which were air-dried (assumed moisture content of seeds was approximately 10–15%).

[d] Pollen fertility was recorded for three spikelets of each of three randomly selected plants. It was estimated from microscopic analysis of the shape and color of the pollen stained with I-KI solution (1%).

[e] The weight of 1000 seeds was determined from that of 20 or 100 grains in the greenhouse and field conditions, respectively.

Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

Germination of seeds from field-grown OASA1D *transgenic plants*

The growth condition markedly influenced seed germination in HW lines. Whereas the germination percentage for seeds of greenhouse-grown plants was similar for HW lines and Nipponbare, it was greatly reduced for seeds of fieldgrown HW1 plants compared with that for field-grown HW5 or Nipponbare plants (Table 6). The time to germination was also increased for seeds from both HW lines grown under field conditions, as well as for seeds from HW1 plants grown in the greenhouse.

Table 6. Germination percentage and time to seedling establishment for HW lines.

Line	Greenhouse		Field	
	Germination (%)	Time to establish seedlings (d)	Germination (%)	Time to establish seedlings (d)
HW1	95	6.3	53.3	10.1
HW5	100	3.1	93.3	5.3
NB	100	3	100	2.8

The germination test was performed with 20 seeds from greenhouse grown plants and 15 seeds from field-grown plants. Data are means of values from two experiments.

Poor seed germination has been observed in other plants with increased levels of an essential amino acid. Transgenic soybean with a large increase in free Lys content thus manifested reduced seed viability (Falco *et al.,* 1995). Seeds of transgenic *Arabidopsis thaliana* with a high Lys content also exhibited retarded germination and seed establishment (Zhu and Galili, 2003). The seeds of HW1 plants grown in the field showed the lowest frequency of and greatest delay in germination as well as the highest content of free Trp compared with seeds of HW1 or HW5 plants grown in the greenhouse and HW5 plants grown in the field. These observations suggest that an increase in Trp content over a certain threshold level might substantially influence germination. The importance of amino acid metabolism such as Gln, Lys, and Met in maize germination efficiency has been shown (Limami *et al.,* 2002; Anzala e*t al.,* 2006). Trp has been shown to be an endogenous inhibitor of embryo germination in white wheat (Morris *et al.,* 1988).

It might prove possible to ameliorate the unfavourable traits observed in the HW lines grown under field conditions by controlling the extent and tissue distribution of Trp accumulation with the use of a different promoter to drive *OASA1D* expression.

Potential promoters for this purpose include those of embryo-specific genes or of genes that are not responsive to stress.

Metabolite profile of seeds from field-grown* OASA1D *transgenic plants

Changes in the composition of aromatic components in the seeds of field-grown HW1 and HW5 plants were analyzed by reversed-phase HPLC. Elution was monitored with a photodiode array detector over a wavelength range of 190–400 nm. Typical chromatograms obtained at 280 nm, the most effective wavelength for detection of changes in the composition of anthranilate-related metabolites, are shown in Fig. 1. The aromatic metabolite profiles of dehulled seeds revealed no apparent marked accumulation of components other than Trp in the transgenic seeds (Fig. 1, inset). Magnification of the chromatograms revealed small differences between HW lines and Nipponbare (Fig. 1). A peak with a retention time of 10 min, for example, was specifically detected in both transgenic lines. Essentially, identical results were obtained by monitoring elution at wavelengths other than 280 nm (data not shown). These results are surprising given the high levels of Trp in the transgenic seeds and that the Trp biosynthetic pathway gives rise to various secondary metabolites, such as the indole alkaloids and indole glucosinolates, in many plants. To date, no remarkable secondary metabolites of Trp origin have been reported in rice plants and the present study suggested that the absence of such Trp-derived secondary metabolites was probably not due to a shortage of Trp supply, but to a very low capability of relevant Trp utilization in rice. Furthermore, Trp decarboxylase probably plays more important role in the divergence of Trprelated carbon flow into the secondary metabolism, as has been observed in transgenic plants overexpressing Trp decarboxylase (Chavadej *et al.,* 1994; Yao *et al.,* 1995).

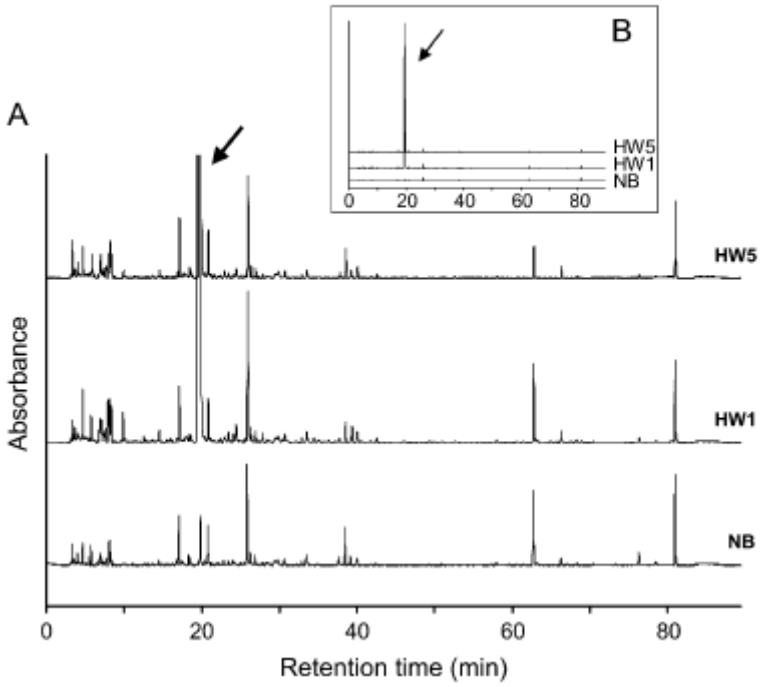

Fig. 1. Aromatic metabolite profiles of seeds derived from field-grown HW lines. Seed extracts of HW1, HW5, and Nipponbare were analysed by reversed-phase HPLC with detection at 280 nm. The traces in the inset are shown at higher magnification in the main panel. The arrow indicates the peak corresponding to Trp.

It was specifically investigated whether the accumulation of Trp in the seeds of the field-grown HW lines affected the amount of IAA, given the close relations between the Trp biosynthetic pathway and IAA production. Therefore the levels of free IAA, of free IAA plus its ester conjugates, and of total IAA (including amide conjugates) were measured in the seeds of the HW lines and Nipponbare (Fig. 2A). The amounts of free and conjugated forms of IAA were each increased about 2-fold in the seeds of both HW lines compared with those in Nipponbare seeds. The increase in the level of

free IAA in seeds was consistent with our previous demonstration of IAA accumulation in rice calli expressing *OASA1D* (Morino *et al.,* 2005). Increased auxin content has been associated with Trp accumulation in cultured carrot and potato cells resistant to 5MT (Widholm, 1977; Sung, 1979). The level of IAA conjugates was also found to be increased in the *Arabidopsis* mutant *Amt1*, which expresses a feedback-insensitive AS and accumulates Trp (Kreps and Town, 1992; Ludwig-Muller *et al.,* 1993). In addition, 5MT-resistant mutants of *Lemna gibba* showed an approximately 3-fold increase in the amount of free IAA (Tam *et al.,* 1995). Seeds of rice and maize normally contain higher concentrations of IAA than do other organs of these plants (Bandurski and Schulze, 1977). The increase in the amounts of free and total IAA apparent in the seeds of the HW lines thus suggests that rice seeds are able to accumulate IAA to especially high levels.

Given that IAA affects multiple aspects of plant growth, the low spikelet fertility and density as well as the impaired seed germination of the HW lines might reflect the increased abundance of IAA rather than that of Trp. IAA markedly inhibited the germination of wheat embryos excised from caryopses that were highly dormant (Ramaih *et al.,* 2003); Trp, the precursor of IAA, was shown to be equally inhibitory in this instance, however. In the case of these HW lines, the impairment in seed agronomic traits was greater for HW1 than for HW5, whereas the amount of total IAA was slightly higher in the seeds of HW5 than in those of HW1. The impairment thus appeared to be more correlated with Trp content than with total IAA (Fig. 2). Regardless, the growth of HW1 and HW5 seedlings after germination overtook that of Nipponbare and no differences in plant growth at the harvesting stage were detected between Nipponbare and the HW lines (data not shown).

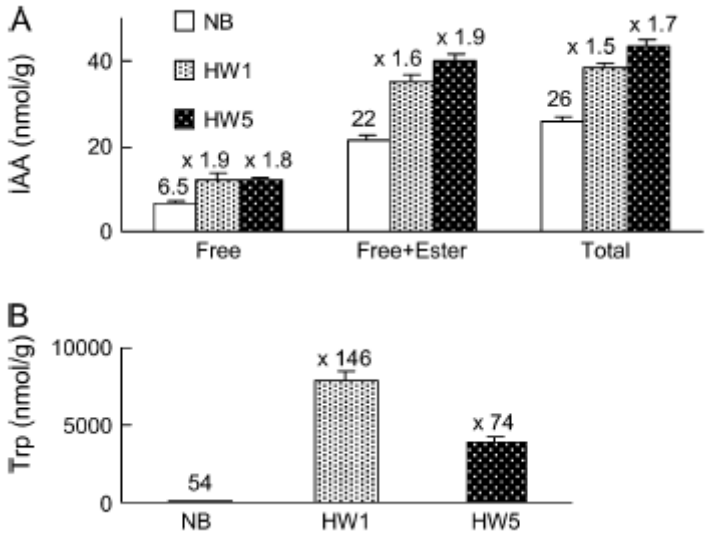

Fig. 2. IAA and Trp contents of seeds derived from field-grown HW lines. The amounts of IAA (A) and free Trp (B) were quantitated in the same seeds of HW1, HW5, or Nipponbare (NB) plants. Data are expressed as nanomoles of analyte per gram of dry seed weight and are means ±SD of values determined from three groups of six seeds.

CONCLUSION

These results have revealed that the seeds of rice plants expressing the *OASA1D* transgene accumulate free Trp to high levels and in a stable manner. This accumulation of Trp was not accompanied by substantial changes in the amounts of other phenolic compounds. The growth of *OASA1D* plants under the field conditions used here revealed a reduction in spikelet fertility and in the number of spikelets per panicle, as well as in the efficiency of seed germination compared with Nipponbare. However, these differences with Nipponbare were more prominent in the HW1 line than in the HW5 line, the latter being largely similar to the wild type with regard to many agronomic traits.

For use as human food, it is preferable that transgenic crops do not contain antibiotic resistance genes. An advantage of *OASA1D* transgenic rice is that the transgene confers sufficient resistance to 5MT to allow the selection of transformed cells with this agent, thereby obviating the need for another gene as a selectable marker (Yamada *et al.,* 2004). Although rice is primarily grown for human consumption, its use for animal feed has been recently developed (Sakai *et al.,* 2003*a*). An increased Trp content of rice seed would thus also prove beneficial for animal nutrition.

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REFERENCES

Anzala F., Paven M.M., Fournier S., Rondeau D., and Limami A.M. 2006. Physiological and molecular aspects of aspartate-derived amino acid metabolism during germination and post-germination growth in two maize genotypes differing in germination efficiency. Journal of Experimental Botany 57, 645–653.

Bender J., Fink G.R. 1998. A myb homologue, ATR1, activates tryptophan gene expression in *Arabidopsis.* Proceedings of the National Academy of Sciences, USA 95, 5655–5660.

Bandurski R.S. and Schulze A. 1977. Concentration of indole-3-acetic acid and its derivatives in plants. Plant Physiology 60, 211–213.

Chavadej S., Brisson N., McNeil J.N., and De Luca V. 1994. Redirection of

tryptophan leads to production of low indole glucosinolate canola. Proceedings of the National Academy of Sciences, USA 91, 2166–2170.

Cho H.J., Brotherton J.E., Song H.S., and Widholm J.M. 2000. Increasing tryptophan synthesis in a forage legume Astragalus sinicus by expressing the tobacco feedback-insensitive anthranilate synthase (ASA2) gene. Plant Physiology 123, 1069–1076.

DellaPenna D. 2001. Plant metabolic engineering. Plant Physiology 125, 160–163.

Eder K., Peganova S., and Kluge H.. 2001. Studies on the tryptophan requirement of piglets. Archiv fu¨r Tierena¨hung 55, 281–297.

Falco S.C., Guida T., Locke M., Mauvais J., Sanders C., Ward R.T., and Webber P. 1995. Transgenic canola and soybean seeds with increased lysine. Biotechnology 13, 577–582.

Henry Y., Se`ve B., Colle´aux Y., Ganier P., Saligaut C., and Je´go P. 1992. Interactive effects of dietary levels of tryptophan and protein on voluntary feed intake and growth performance in pigs, in relation to plasma free amino acids and hypothalamic serotonin. Journal of Animal Science 70, 1873–1887.

Hiei Y., Ohta S., Komari T., and Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant Journal 6, 271–282.

Ibaraki Prefecture Standard Lowland Rice Cultivation. 1990. In: Standard cultivation of crops in Ibaraki Prefecture. Ibaraki Prefecture, 1–12.

Kreps J.A., and Town C.D. 1992. Isolation and characterization of a mutant of *Arabidopsis thaliana* resistant to α-methyltryptophan. Plant Physiology 99, 269–275.

Lai J., and Messing J. 2002. Increasing maize seed methionine by mRNA stability. The Plant Journal 30, 395–402.

Limami A.M., Rouillon C., Glevarec G., Gallais A., and Hirel B. 2002. Genetic and physiological analysis of germination efficiency in maize in relation to nitrogen metabolism reveals the importance of cytosolic glutamine synthetase. Plant Physiology 130, 1860–1870.

Ludwig-Muller J., Sass S., Sutter E.G., Wodner M., and Epstein E. 1993. Indole-3-butyric acid in Arabidopsis thaliana. I. Identification and quantification.

Plant Growth Regulation 13, 179–187.

Massey K.A., Blakeslee C.H., and Pitkow H.P. 1998. A review of physiological and metabolic effects of essential amino acids. Amino Acids 14, 271–300.

Matsuda F., Miyazawa H., Wakasa K., and Miyagawa H. 2005. Quantification of indole-3-acetic acid and amino acid conjugates in rice by liquid chromatography-electrospray ionization-tandem mass spectrometry. Bioscience, Biotechnology, and Biochemistry 69, 778–783.

Mazur B., Krebbers E., and Tingey S. 1999. Gene discovery and product development for grain quality traits. Science 285, 372–375.

Morandini P. and Salamini F. 2003. Plant biotechnology and breeding: allied for years to come. Trends in Plant Science 8, 70–75.

Morino K., Matsuda F., Miyazawa H., Sukegawa A., Miyagawa M., and Wakasa K. 2005. Metabolic profiling of tryptophanoverproducing rice calli that express a feedback-insensitiveαsubunit of anthranilate synthase. Plant and Cell Physiology 46, 514–521.

Morris C.F., Mueller D.D., Faubin J.M., and Paulsen G.M. 1988. Identification of L-tryptophan as an endogenous inhibitor of embryo germination in white wheat. Plant Physiology 88, 435–440.

Movig L., Tabe L.M., Eggum B.O., Moore A.E., Craig S., Spencer D., and Higgins T.J.V. 1997. Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. Proceedings of the National Academy of Sciences, USA 94, 8393–8398.

Phillips R.L., Kaeppler S.M., and Olhoft P. 1994. Genetic instability of plant tissue cultures: Breakdown of normal controls. Proceedings of the National Academy of Sciences, USA 91, 5222–5226.

Ramaih S., Guedira M., and Paulsen G.M. 2003. Relationship of indole acetic acid and tryptophan to dormancy and preharvest sprouting of wheat. Functional Plant Biology 30, 939–945.

Sakai M., Iida S., Maeda H., Sunohara Y., Nemoto H., and Imbe T. 2003*a*. New varieties for whole crop silage use in Japan. Breeding Science 53, 271–275.

Sakai M., Imbe T., Nemoto H., *et al*. 2003*b*. A new rice variety for whole-crop silage

‘Kusahonami’. Bulletin of National Institute of Crop Science 4, 1–15.

Se`ve B. 1999. Physiological roles of tryptophan in pig nutrition. Advances in Experimental Medicine and Biology 467, 729–741.

Subcommittee on Poultry Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council. 1994. Nutrient requirements of chickens. In: Nutrient requirements of poultry, 9th edn. National Academy Press, 19–34.

Subcommittee on Swine Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council. 1998. Nutrient requirement tables. In: Nutrient requirements of swine, 10th edn. National Academy Press, 110–123.

Sung Z.R. 1979. Relationships of indole-3-acetic acid and tryptophan concentrations in normal and 5-methyltryptophan-resistant cell lines of wild carrots. Planta 145, 339–345.

Takeoka Y., Shimizu M., and Wada T. 1993. Panicles. In: Matsuo T, Hoshikawa K, eds. Science of the rice plant, Vol. 1. Morphology. Food and Agriculture Policy Research Center, 295–338.

Takimoto I., Christensen A.H., Quail P.H., Uchimiya H., and Toki S. 1994. Non-systemic expression of a stress-responsive maize polyubiquitin gene (*Ubi-1*) in transgenic rice plants. Plant Molecular Biology 26, 1007–1012.

Tam Y.Y., Slovin J.P., and Cohen J.D. 1995. Selection and characterization of α -methyltryptophan-resistant lines of Lemna gibba showing a rapid rate of indole-3-acetic acid turnover. Plant Physiology 107, 77–85.

Tozawa Y., Hasegawa H., Terakawa T., and Wakasa K. 2001. Characterization of rice anthranilate synthase α-subunit genes *OASA1* and *OASA2*. Tryptophan accumulation in transgenic rice expressing a feedback-insensitive mutant of OASA1. Plant Physiology 126, 1493–1506.

Urushibara S., Tozawa Y., Kawagishi-Kobayashi M., and Wakasa K. 2001. Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium tumefaciens.* Breeding Science 51, 33–38.

Wakasa K. and Widholm J. 1987. A 5-methyltryptophan resistant rice mutant, MTR1, selected in tissue culture. Theoretical and AppliedGenetics 74, 49–54.

Wang X. and Larkins A. 2001. Genetic analysis of amino acid accumulation in opaque-2 maize endosperm. Plant Physiology 125, 1766–1777.

Wang X., Stumpf D.K., and Larkins A. 2001. Aspartate kinase 2. A candidate gene of a quantitative trait locus influencing free amino acid content in maize endosperm. Plant Physiology 125, 1778–1787.

Widholm J. 1977. Relation between auxin autotrophy and tryptophan accumulation in cultured plant cells. Planta 134, 103–108.

Yamada T., Tozawa Y., Hasegawa H., Terakawa T., Ohkawa Y., and Wakasa K. 2004. Use of a feedback-insensitive α subunit of anthranilate synthase as a selectable marker for transformation of rice and potato. Molecular Breeding 14, 363–373.

Yao K., De Luca V., and Brisson N. 1995. Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans.* The Plant Cell 7, 1787–1799.

Zhang X.H., Brotherton J.E., Widholm J.M., and Portis Jr A.R. 2001. Targeting a nuclear anthranilate synthase α-subunit gene to the tobacco plastid genome results in enhanced tryptophan biosynthesis. Return of a gene to its pre-endosymbiotic origin. Plant Physiology 127, 131–141.

Zhu X. and Galili G. 2003. Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also transregulates the metabolism of other amino acids in *Arabidopsis* seeds. The Plant Cell 15, 845–853.