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Enrichment of cereal protein lysine content by altered $tRNA^{lys}$ coding during protein synthesis

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Summary

The world's major crops are deficient in lysine and several other amino acids essential for human and animal nutrition. Increasing the content of these amino acids in cereals, our major source of dietary energy, can help feed a global population whose reliance upon dietary protein is growing faster than crop yields. Here we document the heritable expression in rice, the world's major cereal crop, of $tRNA^{lys}$ species that introduce lysine at alternative codons during protein synthesis, resulting in a significant enrichment of the lysine content of proteins in rice seeds without changing the types or quantities of the seed storage proteins.

Introduction

Plants provide most of the nutrients and energy in human diets, and consequently, their production must increase commensurately with human population growth. The past 30 years have witnessed remarkable increases in the yields of cereal crops; however, sustaining this progress as the world's population doubles in the next 30 years will be especially challenging, particularly as dietary preferences for animal protein increase (Cassman, 1999; Per-Pinstrup *et al.*, 1999; Tilman *et al.*, 2002). The requisite yield enhancement can be partially mitigated by increasing cereal protein quality, which helps to increase nutrient utilization, thereby reducing the need for animal and microbial supplementation of food and feeds, lessening use of non-renewable energy sources for food production and reducing the production of animal wastes because feeds are not nutritionally balanced.

Recent efforts to improve plant seed protein quality include selecting for plant germplasm with an altered abundance of seed proteins, to favour those having greater quantities of the essential amino acids; or expressing in seeds foreign proteins with high levels of the essential amino acids; or causing the accumulation of the essential amino acids in free form (Krebbers *et al.*, 1997; Mazur *et al.*, 1999). These approaches have met with varied success, and many have encountered problems that prevent their widespread acceptance. The approach to improving plant protein quality we describe below is very different from these, and does not alter the type or quantity of the seed storage proteins. It involves increasing the protein sequence variability that occurs as a result of the natural infidelity of translating information from mRNA into protein (Kurland *et al.*, 1997; Parker, 1992), so that nutritionally limiting amino acids are substituted for the abundant amino acids in seed storage proteins, or in place of chain termination. Seed storage proteins exhibit an extensive heterogeneity in sequence and length, and this new approach expands such natural heterogeneity for plant protein quality improvement.

Our prior work indicated that the expression of $tRNA^{lys}$ species with altered anticodons in cultured tobacco and carrot cells causes the incorporation of lysine into reporter proteins in place of other amino acids and in place of chain termination (Chen Z. *et al.* 1998). In this report, we demonstrate that these $tRNA^{lys}$ species can be introduced into rice plants and heritably expressed so as to increase the lysine content of proteins and seeds without obvious detriment to plant growth or reproduction. This approach may also be applied to enhance the quantity of lysine and other essential amino acids in the other cereals and legumes and forages that provide much of human and animal nutrition.

Results

Introduction of DNAs encoding $tRNA^{lys}$ with altered anticodons into rice (*Oryza sativa* L.) callus tissues

Previously, transient assays with tobacco and carrot cell cultures indicated that the introduction of DNAs encoding

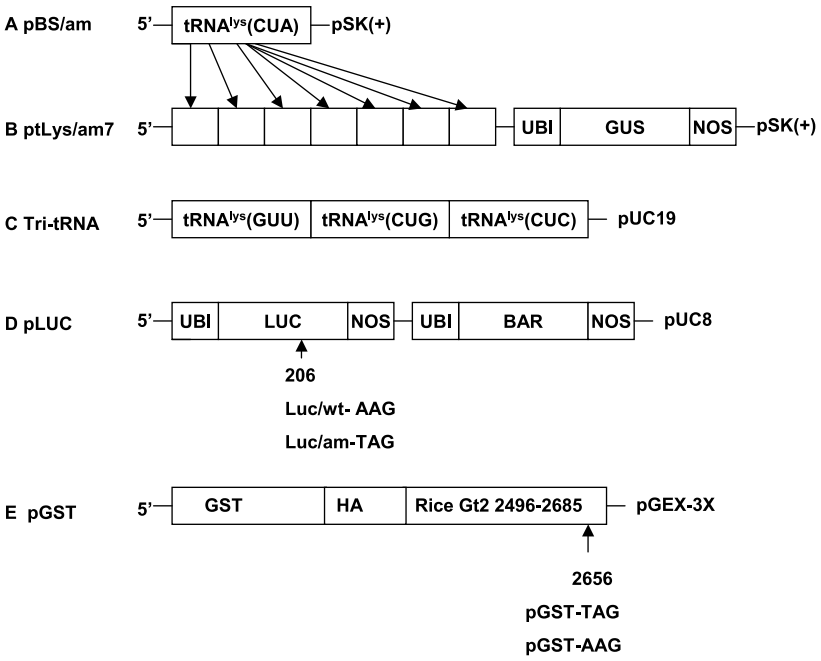

Figure 1 Gene constructs used for rice (*O. sativa*) transformation. tRNA^{lys} (CUA), *A. thaliana* tRNA^{lys} gene with anticodon sequence altered from CTT to CTA; UBI, maize ubiquitin promoter; GUS, β-glucuronidase gene; LUCam, firefly luciferase gene with TAG codon at position 206; BAR, Phosphinothricin acetyltransferase(PAT) gene; GST, Glutathione S-transferase gene; NOS, terminator of the nopaline synthase gene; HA, Haemagglutinin epitope coding sequence.

tRNA^{lys} with altered anticodons could significantly enhance the coding of lysine into proteins in place of chain termination and other amino acids (Chen Z. *et al*., 1998). Furthermore, tobacco cells stably expressing these altered tRNA^{lys} species grew in suspension as well as unmodified cells over many generations, indicating the expression of these tRNAs and enhancement of altered coding of lysine during protein synthesis was not deleterious to cell growth (unpublished data).

To assess whether cereal tissues would heritably express these tRNAs, plasmid DNAs with three tRNA^{lys} genes coding lysine in place of Gln, Asn and Glu, or single and multiple tRNA^{lys} (CUA) genes coding lysine at the UAG (amber) chain termination codon (Figure 1A–C) were introduced into rice callus by particle bombardment. The introduced DNAs were detected by PCR and hybridization with oligonucleotides specific for the altered tRNA^{lys} species. The altered coding of lysine in the rice tissues was sensitively detected by the measurement of active luciferase (LUC) expressed from co-bombarded DNAs containing the LUC gene with a lysine codon replaced by UAG (Figure 1D). The synthesis of the intact luciferase protein was confirmed by cross-reaction with antibodies in Western blots (data not shown).

Callus and plant tissues bombarded with DNAs encoding tRNA^{lys} (CUA) and the mutant LUC gene expressed luciferase activities ranging up to 300-fold over background. This was well above the levels observed with callus and tissues bombarded with only mutant LUC DNA, and nearly 10% of that observed for callus and tissues bombarded with wild-type LUC DNA (Table 1), indicating that the altered tRNA^{lys} species were expressed and were not detrimental to the growth of the rice callus and plant tissues.

Properties of rice plants expressing altered tRNA^{lys} species

Rice plants were regenerated from calli prepared by independent bombardments with the tRNA^{lys}(CUA) or tRNA^{lys} genes that code lysine in place of Gln, Asn or Glu. The R_0 rice plants with DNAs containing single copies of the tRNA^{lys} (CUA) genes exhibited normal vegetative growth, development of reproductive organs and seed production, and their seeds were of normal size (Table 1). In contrast, R_0 plants containing DNAs with multiple copies of the tRNA^{lys} gene generally exhibited abnormal spikelet and flower and seed development, and produced smaller seeds. However, the R_1 progeny of the latter plants displayed normal vegetative growth, flowering and seed setting, suggesting that these abnormalities were not heritable, nor caused by heritable expression of the tRNA^{lys} species (Table 1).

Rice accumulate glutelins as the major seed storage proteins, while in wheat, maize and many other cereals, prolamins constitute about half of the total grain protein. The glutelins contain small amounts of lysine, which is virtually absent from the prolamins (Lásztity, 1999). Both classes of proteins contain abundant amounts of glutamine, asparagine and glutamic acid, hydrophilic amino acids that are frequently surface-exposed, as is lysine. As a consequence, the infrequent substitution of these amino acids by lysine at the surface-exposed sites should be well tolerated (Bowie *et al*., 1990).

Table 1 Summary of rice transformants

Bombarded DNAs	Independent transformants		Luciferase activity	Plant phenotypes	
	Callus	Plants		R_0	R_1
Luc/wt	30	12	1~3000x background	Normal	ND
Luc/am	20	20	1~6x background	Normal	ND
Luc/am plus pBS/am	20	7	1~19x background	Normal	Normal
Luc/am plus ptLys/am7	30	8	1~300x background	Normal vegetative growth, abnormal flower development	Normal
Luc/wt plus Tri-tRNA	11	5	ND	Normal vegetative growth, abnormal flower development	Normal

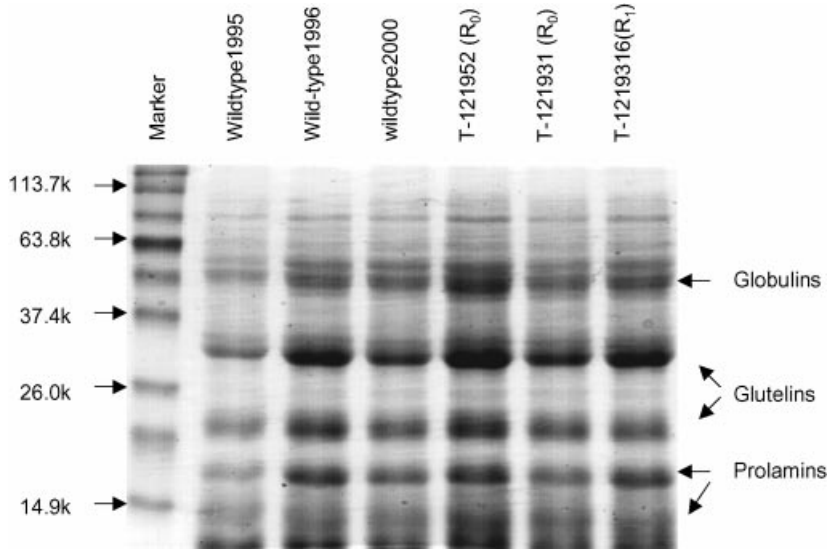

Figure 2 SDS-PAGE analysis of total rice seed storage proteins. Proteins from wild-type seed harvested from plants raised in year indicated. Independent transgenic lines T121931 and T121952 were derived from callus bombarded with Tri-tRNA DNAs.

Seed storage proteins of a given class are heterogeneous in sequence and length, but still maintain tissue-specific expression and function, suggesting that they might be particularly amenable to substitution. Sequence variability between members of these multigenic families is responsible for much of this heterogeneity (Clarke and Appels, 1999), and to a limited extent, so might be natural errors in protein synthesis (Beier and Grimm, 2001; Entwistel *et al*., 1991).

The SDS-PAGE patterns of the total proteins of seeds of plants containing the $tRNA^{lys}$ genes were indistinguishable from the patterns of seed of wild-type plants (Figures 2 and 3A), indicating that any enhanced substitution of lysine into these proteins that does occur as a result of expression of the altered $tRNA^{lys}$ species does not dramatically alter the type or quantity of the major classes of seed storage proteins. More refined analyses, such as by 2D PAGE accompanied by Western blotting with protein-specific antibodies will be required to assess heterogeneity in minor seed proteins.

Heritable introduction of lysine into glutelin proteins by read-through of chain termination codons

Coding of lysine by the UAG chain terminating codon in rice glutelin mRNAs was assessed using an antibody directed against a synthetic polypeptide having the sequence of the wild-type carboxyl terminal three amino acids (ESS) encoded by the rice Gt2 (GluA-1) (Okita *et al*., 1989) gene, followed by a lysine and the eight read-though amino acids predicted from the gene sequence (ESSKVGLRIKNN). The glutelin proteins were extracted and fractionated by SDS-PAGE, and analysed by Western blotting with this antibody (Figure 3). A very small amount of cross-reactive protein was detected in wild-type seeds, probably due to natural read-through of the UAG chain terminating codon (Beier and Grimm, 2001; Entwistel *et al*., 1991). Much greater amounts of cross-reactive protein were detected in the seeds of plants containing altered $tRNA^{lys}$ (CUA) genes (Figure 3B). The seed of the R_1 plants

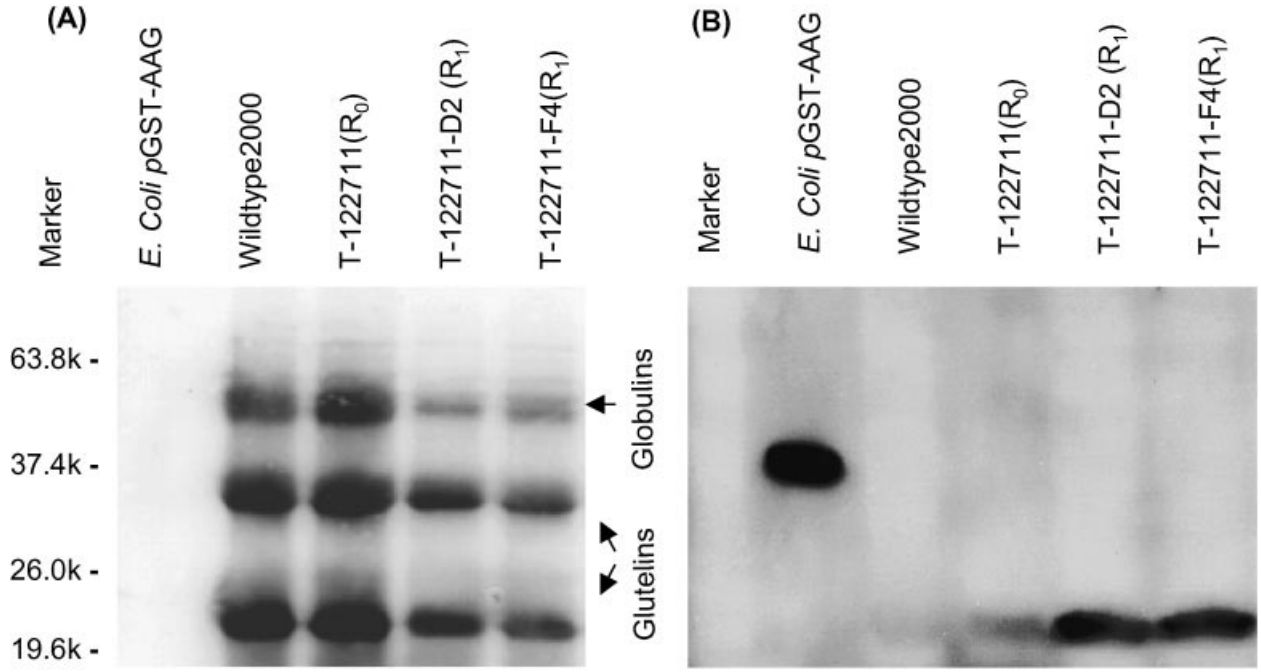

Figure 3 SDS-PAGE of rice glutelin proteins. (A) Stained before Western blotting. (B) Western blot with antibody directed against glutelin-2 read-through protein. *E. coli* expressed Gt2 read-through protein (34 kDa) was used to estimate the amount of Gt2 protein in seeds. Independent transgenic line T122711 was derived from callus bombarded with Luc/am plus pBS/am.

had more read-through protein than the seed of R_0 plants, perhaps because the R_0 plants were chimeras. No significant difference in the amounts of read-through Gt2 proteins was noted in seeds of R_1 plants homozygous for the $tRNA^{lys}$ gene (T-122711-F4) and heterozygous (T-122711-D2) plants.

The quantities of read-through glutelin-2 protein present in R_1 seeds were estimated by comparison with the antibody staining of these proteins and the staining of known amounts of *Escherichia coli* expressed read-through glutelin-2 protein (Figure 3B), as described in the Materials and Methods. These calculations indicate that 2–3% of the Gt2 proteins in the rice seed contained additional lysines as a result of translational read-through. This level resembles that of translational read-through of the LUC(am) gene (i.e. functional luciferase) expressed by these plants.

Heritable enhancement of lysine in rice prolamins and rice seed

Enrichment of lysine in the prolamin seed proteins and in the rice seed was determined by amino acid analyses. Based upon multiple analyses of the seed of R_1 plants, the expression of $tRNA^{lys}$(CUA) enhanced the lysine content of the prolamin fraction by ≈ 43% (Table 2). R_1 plants containing DNAs coding for $tRNA^{lys}$ species pairing with CAG (Gln), AAC (Asn) and GAG (Glu) codons expressed prolamin proteins having ≈ 75% additional lysine (Table 2). The lysine content of the rice seeds of these plants was enriched by ≈ 0.9% and 6.6%, respectively (Table 2). These increases in lysine content were not related to the size of the seeds, nor to alterations in the content or type of proteins.

Discussion

The greatest need for high quality protein occurs in Asia, where 80% of the world's children affected by protein-calorie malnutrition live, and where rice is the major source of food (De Onis *et al*., 2000). Inadequate variation in the lysine content among rice cultivars has hindered the development of breeding programmes to increase the lysine content (Coffman and Juliano, 1987). As one approach to increasing the lysine content of rice, lines have been derived from callus cultures selected to have alterations in lysine metabolism (Schaeffer and Sharp, 1987) but the grains are chalky, and most are smaller and of reduced fertility (Krishnan, 1999; Schaeffer and Sharp, 1990; Schaeffer *et al*., 1989, 1994). Alternatively, the soybean glycinin gene has been introduced into rice, causing a 20% increase in protein content (Momma *et al*., 1999), but the lysine content relative to total protein (and thus quality) is reduced. The approach we describe here is the first to substantively enhance the lysine content of rice without obvious detriment to seed quality.

Table 2 Lysine content of prolamins and rice grain

Sample	Weight (g) of 100 seeds	Prolamins		Rice grain	
		Lysine content (%) ± SE	Per cent increase	Lysine content (%) ± SE	Per cent increase
Taipei 309 Wild-type*	2.20	0.81 ± 0.07	0	4.27 ± 0.13	0
T32811 (R_1)	2.10	1.16 ± 0.08	43.2	4.31 ± 0.06	0.9
T121931 (R_0)	1.52	1.31§	61.7	4.51 ± 0.08	6.1
T1219316 (R_1)	2.02	1.42 ± 0.20	75.3	4.55 ± 0.17	6.6

*Seed harvested from wild-type plants regenerated from callus.
†Line T32811 was derived from callus bombarded with Luc/am plus ptLys/am7.
‡Line T121931 was derived from callus bombarded with Luc/wt plus Tri-tRNA.
§Due to abnormal seed development in R_0 plants, only one sample was analysed.

Protein synthesis naturally incurs errors, at frequencies for particular codons approaching 1% (Kurland *et al*., 1997; Parker, 1992). As has been extensively studied in prokaryotes and to a limited extent in yeasts and animal cells, the expression of altered tRNAs in plant cells and plants heightens a natural lack of fidelity of protein synthesis (Betzner *et al*., 1997; Carneiro *et al*., 1993; Chen Z. *et al*., 1998; Choisne *et al*., 1997; Franklin *et al*., 1992; Ulmasov and Folk, 1995). We have expressed altered $tRNA^{lys}$ species in rice to increase the level of lysine in proteins, with no apparent loss of fertility or change in the type or quantity of seed storage proteins.

Still greater improvement in protein quality can most likely be achieved with plants having both the $tRNA^{lys}$(CUA) that introduces lysine at chain terminating codons as well as $tRNA^{lys}$ species that introduce lysine in place of other amino acids. Furthermore, as the changes in the anticodons of these $tRNA^{lys}$ species reduce the efficiency of tRNA amino-acylation by the plant lysyl tRNA synthetase (Folk *et al.*, unpublished data), it might be possible to selectively enhance the amino-acylation of these tRNAs and to target their utilization during protein synthesis by expressing high levels of free lysine and/or the lysyl tRNA synthetase in endosperm. As one of the problems associated with expressing high levels of free lysine in plant seeds is the production of lysine catabolites from the excess lysine (Galili, 2002; Mazur *et al*., 1999) enhancing lysine incorporation into proteins might provide a shunt and alleviate this problem.

A very high level of infidelity in protein synthesis can lead to an overall reduction in fitness (Kurland *et al*., 1997; Parker, 1992), but this depends upon the particular type of error, and for each organism, different protein targets will be vulnerable. Targeting amino acid substitutions to residues that are frequently surface-exposed minimizes the effect of the substitution. It appears from these studies that modest levels of substitution by lysine are not particularly deleterious to plant vegetative growth and reproduction. Further studies are required, however, to fully assess the agronomic properties of the plants expressing these altered tRNAs.

The infrequent introduction in a stochastic manner of additional lysine by this method might produce many changed proteins, but there should only be a very small amount of each, and such changes should therefore have little or no effect upon the safety of food or feed, based upon what is known to cause proteins to be allergens, nor to cause these proteins to acquire novel traits that might prove to be harmful to humans or animals. The abundance of any single protein with a particular alteration is likely to be very low, and its stability to digestion is unlikely to be dramatically increased. As enhancing the lysine content of plants by this method only requires the use of plant genes, issues relating to public acceptance should be minimized.

Maize prolamins also are deficient in lysine and constitute the majority of the proteins in maize kernels (Coleman *et al*., 1997). All of the highly expressed α-zein and δ-zein genes (Woo *et al*., 2001) utilize UAG chain termination codons, and some of the zein genes that are poorly expressed have internal UAG codons. Altered coding by $tRNA^{lys}$(CUA) at the levels observed in rice could increase the lysine content of maize very significantly, thereby relieving one of the major nutritional limitations of this important crop. Also, of course, coding of lysine in place of the abundant, hydrophilic amino acids in the prolamins should significantly improve protein nutritive quality.

Experimental procedures

Plasmid constructions

The *A. thaliana* $tRNA^{lys}$ gene with an anticodon sequence altered from CTT to CTA was amplified from ptLys/am

(Chen Z. *et al*., 1998) using tRNA 5′(CCGCATGCATGTATAAGTGTGTCGGAACTGGG) and tRNA 3′(TGCTAGCAGGTTTGACTAACTAACGGGGTTGTTG) primers, and the products were inserted into pBluescript SK(+) to generate pBS/am (Figure 1A). Seven copies of the $tRNA^{lys}$(CUA) gene were inserted into the pCR-scriptSK(+) plasmid in the *Srf*I site (by annealing and ligation in the presence of 40% polyethylene glycol), and the *Hin*dIII fragment of pAHC25 (provided by Dr Peter H. Quail) containing the maize ubiquitin promoter (UBI-1) with 5′ untranslated sequence, exon, intron, GUS gene and NOS transcription termination sequence, was introduced into the *Hin*dIII site to generate ptLys/am7 (Figure 1B). The $tRNA^{lys}$(GUU) (Asn), $tRNA^{lys}$(CUG)(Gln) and $tRNA^{lys}$(CUC)(Glu) genes were amplified from ptLys/Asn1, ptLys/Gln and ptLys/Glu DNAs (Chen Z *et al*., 1998) using Mul-tRNA5′ (ATAGAATTCAGATCTATGTATAAGTGTGTCGGAACTG) and Mul-tRNA-3′ (ATAAAGCTTGGATCCGTTTGACTAACTAACGGGGT TG) primers, and the products were inserted into pUC19 to generate plasmid Tri-tRNA (Figure 1C). The reporter pLUC/wt(am)-BAR (Figure 1D) contains the firefly luciferase gene with a UAG codon at position 206 (Chen Z. *et al*., 1998). To express the last exon of the rice Gt2 gene in *E. coli*, primers 5′Gt2-F2496 (ACATATGATCTTCCGTGCTCTCCCAA) and 3′Gt2-rev (TGAATTCTTAGTTATTCTTTATCCGCAATCC) were used to amplify a fragment containing the Gt2 gene between nt2496 to nt2685 from rice genomic DNA. Another 3′ primer Gt2-mut (TGAATTCTTAGTTATTCTTTATCCGCAATCCGACCTTAGAG) was used to alter the chain termination codon TAG to AAG. PCR products of these constructs were inserted into pBluescript SK(+), generating vectors pBS-Gt2-TAG and pBS-Gt2-AAG. The *Nde*I–*Eco*RI restriction fragments were subcloned into pGEX 3X-A.t. H4 c5 (provided by Dr A. Kenzior) to generate vectors pGSTGt2-TAG and pGSTGt2-AAG (Figure 1E).

Rice transformation

Callus from *O. sativa* cv. variety Taipei 309 was used for particle bombardment following the protocol described by Chen L. *et al*., 1998), except that 1–3 mg/L bialaphos was used in the culture medium instead of 50 mg/L hygromycin B.

Luciferase assay and bialaphos testing

About 50 mg callus or leaves from each sample were homogenized in Cell Culture Lysis Reaction Buffer (Promega) using a polypropylene pellet pestle. The supernatant was assayed for luciferase activity as previously reported (Ulmasov and Folk, 1995) for 10 s. The protein content of the cell lysate was determined by Bradford assay (Bio-Rad Laboratory). To test the regenerated plants for their response to bialaphos, young fully expanded leaves were painted or sprayed with a 1% Herbiace® solution.

Polymerase chain reaction (PCR) analysis

Transgenic DNA was detected in rice leaf samples using the REDExtract-N-Amp™ Plant PCR Kit (Sigma). PCR analyses were carried out with the following primers: for tRNAlys-am, tRNA 5′(CCG CAT GCA TGT ATA AGT GTG TCG GAA CTG GG) and tRNA-3′(TGC TGC AGG TTT GAC TAA CTA ACG GGG TTG TTG); for the luciferase gene, 5′ luc (GTA CAC GTT CGT CAC ATC TC) and Luc-3′(TGG AGC TCT TAC AAT TTG GAC TTT CCG CCC TT). Thermal cycling for 45 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C was performed for 28 cycles.

Preparation of antibodies against the read-through product of the Gt2 gene and synthesis of this product in *E. coli*

The polypeptide ESSKVGLRIKNN, corresponding to the carboxyl terminal three amino acids of rice glutelin-2 (ESS) followed by a lysine and the additional eight amino acids predicted to be added by read-through of the UAG codon in the rice Gt2 gene was synthesized and used to raise and to affinity purify rabbit anti-Gt2 read-through antibody by BioSource International, MA. *E. coli* BL21 DE3 containing plasmid pGST-TAG or pGST-AAG were grown in 100 mL of LB medium at 30 °C to an OD_{600} of 0.5–0.6; 0.5 M isopropyl β-D-thiogalactopyranoside (IPTG) was added and the cell pellets were harvested after continued incubation for an additional 3 h. The GST fusion proteins were purified over Glutathione Sepharose 4B (Pharmacia Biotech) by the manufacturer's protocol. The purified protein was used to test and select the rabbit serum samples produced by Biosource International, and to estimate the amount of the Gt2 read-through product in rice seeds.

Protein extraction

Total seed protein and crude glutelins from samples of 10 seeds were extracted according the method of Luthe (1983) with these minor modifications: dehulled mature rice seeds were ground with a pestle and mortar; the flour was defatted by votexing in five volumes of cyclohexane for 2 h; the mixture was centrifuged at 27 000 ***g*** for 20 min at 4 °C, and the supernatant fluid was discarded and the flour was dried by air or by gentle vacuum. The total storage protein fraction

was extracted with buffer (0.5% SDS, 1% β-mercaptoethanol, 10 mM Tris-HCl, 1 mM PMSF pH 7.5) by vortexing (200 mg flour/1 mL solvent) for 2 h at room temperature. After centrifugation at 27 000 ***g*** for 20 min at 4 °C, the clear supernatant fluid was collected. Crude glutelins were obtained by the same procedure, but the prolamins were first treated with buffer (70% ethanol, 10 mM Tris-Cl, pH 7.5) for 2 h at room temperature.

Western blot analysis

Aliquots of protein were combined with 2x SDS sample buffer and boiled for 5 min. Crude rice glutelin proteins and *E. coli*-expressed Gt2 read-through protein were fractionated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 10% (v/v) methanol. Protein blots were incubated at room temperature for 1 h in blocking solution (5% instant non-fat dry milk in 1x PBS with 0.01% Tween 20). The blots were probed with the purified rabbit anti-Gt2 read-through antibody (1.0 μg/ml) for 1–2 h at room temperature and then incubated with anti-rabbit HRP (3 : 10 000 dilution) for 1 h at room temperature and developed with Supersignal Chemiluminescent substrate (Pierce). After or before Western blot analysis, the protein blots were stained in 40% methanol, 1% acetic acid and 0.1% Coomassie Blue and destained in 50% methanol.

Estimate of lysine introduced into seed proteins

The frequency of translational read-through of the Gt2 UAG codon was estimated using the following calculation:

$$SupL = \frac{Mbgt2 \times \dfrac{Ipgt2 \times MWpgt2}{Ipgt2 \times MWbgt2}}{Mgt2} \times 100\%$$

where *SupL* – suppression level; *Mbgt2* – amount of *E. coli* expressed read-through protein loaded on to gel; *Ipgt2* – Intensity of rice Gt2 protein from exposed film; *Ibgt2* – Intensity of *E. coli* expressed Gt2 read-through protein from exposed film; *MWpgt2* – molecular weight of rice Gt2 β-subunit (21 kDa); *MWbgt2* – molecular weight of *E. coli* expressed Gt2 read-through protein (34 kDa); *Mgt2* – estimated amount of Gt2 β-subunit, calculated as follows:

$$Mgt2 \approx Mp \times \frac{I\beta}{IT} \times 10\%$$

where *Mp* – amount of glutelin protein fraction loaded on to gel; *I*β – Intensity of glutelin β-subunit from stained membrane; *IT* – Intensity of rice proteins from stained membrane; 10% – Estimated Gt2 protein fraction of glutelin proteins based upon gene copy number (Takaiwa, 1999).

Amino acid analyses

Standard protein hydrolysates of whole ground rice seed or the prolamin fraction extracted with 55% isopropanol from 250 mg samples were analysed by the University of Missouri Experiment Station Chemical Laboratories. Composition and quantification were determined with post-column derivatization on Beckman 6300 amino acid analysers with full computer integration

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