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Research Article

Production of Marker-free Transgenic Rice (*Oryza sativa* L.) with Improved Nutritive Quality Expressing *AmA*1

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Background: Rice seed proteins are lacking essential amino acids (EAAs). Genetic engineering offers a fast and sustainable method to solve this problem as it allows the specific expression of heterologous EAA-rich proteins. The use of selectable marker gene is essential for generation of transgenic crops, but might also lead to potential environmental and food safety problems. Therefore, the production of marker-free transgenic crops is becoming an extremely attractive alternative and could contribute to the public acceptance of transgenic crops.
Objectives: The present study was conducted to examine whether AmA1 can be expressed specifically in rice seeds, and generate marker-free transgenic rice with improved nutritive value.
Materials and Methods: *AmA1* was transferred into rice using *Agrobacterium*-mediated co-transformation system with a twin T-DNA binary vector and its integration in rice genome was confirmed by southern blot. Transcription of *AmA1* was analyzed by Real-Time PCR and its expression was verified by western analysis. Protein and amino acid content were measured by the Kjeldahl method and the high-speed amino acid analyzer, respectively.
Results: Five selectable marker-free homozygous transgenic lines were obtained from the progeny. The expression of recombinant AmA1 was confirmed by the observation of a 35 kDa band in SDS-PAGE and western blot. Compared to the wild-type control, the total protein contents in the seeds of five homozygous lines were increased by 1.06~12.87%. In addition, the content of several EAAs, including lysine, threonine, and valine was increased significantly in the best expressing line.
Conclusions: The results indicated that the amino acid composition of rice grain could be improved by seed-specific expression of *AmA1*.

Keywords: *AmA1* gene; Co-transformation; Essential amino acid; Selectable marker-free; Rice.

1. Background

Rice (*Oryza sativa* L.) is amongst important crops and the staple food for over half the world's population (1), and also main source of protein and energy of the people in South East Asia (1, 2). However, like other cereals, rice proteins are incomplete in nutrition value due to the deficiency in certain essential amino acids (EAAs), such as lysine, threonine and methionine (3-5). Due to limited availability of genetic resources for genetic breeding, and the fact that genetic traits for high contents of lysine, or methionine are generally associated with abnormal plant growth, attempts at improving the EAAs content of rice through conventional breeding had little success (4). Genetic engineering approaches seem to be more promising to improve the EAAs content of rice grains, as it allows the specific expression of certain EAA-rich proteins (4, 6, 7), such as pea lysine-rich legumin (8), winged bean lysine-rich protein WBLRP (9), potato glutamic acid-rich protein GARP (10), sunflower sulfur-rich albumin SFA8 (11), and sesame methionine-rich albumin S2AS (12).

The AmA1 seed albumin from the pseudo-cereal *Amaranthus hypochondriacus* is rich in all EAAs, and unlike Brazil nut 2S albumin, it is non-allergenic (13). Chakraborty *et al*. (14) expressed AmA1 in potato

tuber , resulting in a significant increase in most EAAs, such as methionine, lysine, cysteine and tyrosine, and the protein content was also increased by 35~45%, compared with the untransformed counterpart. Furthermore, the data on safety evaluation indicated that the *AmA1*-transgenic potato tubers are nontoxic, nonallergenic, and safe to consume (15). Thomas *et al*. (16) reported the expression of *AmA1* in wheat seeds, using a powerful wheat endosperm-specific promoter, and an increase in both total protein and EAA levels were also noted. To the best of our knowledge, there is limited information so far on AmA1 use in improving the EAA composition of staple rice.

Selectable marker genes (SMGs), such as antibiotic or herbicide resistance genes, are used in nearly every plant transformation protocol to efficiently distinguish transformed from non-transformed shoots (lines). However, once transgenic plants are regenerated, SMGs generally serve no useful purpose. On the contrary, the continued presence of SMGs in transgenic plants may raise public and regulatory concerns on their biosafety (17, 18). Rice is one of genetically modified crops at the earliest stage, however, most of the existing transgenic rice lines generated with improved nutritive carry the SMGs, especially antibiotic resistance genes (8-12), that would limit the commercialization and dissemination of transgenic rice for agricultural production. Several approaches have been developed to remove SMGs from transgenic plants (19, 20). Among these, the co-transformation system appears to be the simplest and could be suitable for most important crops (19).

2. Objectives

The aim of the present work was to examine whether AmA1 can be expressed specifically in the seeds of rice, and generate SMG-free transgenic rice with improved nutritive value using *Agrobacterium*-mediated co-transformation system with a twin T-DNA binary vector.

3. Materials and Methods

3.1. Plasmid Construct

The twin T-DNA binary plasmid vector pCDMAR-*AmA1-hpt* (Fig. 1), was previously constructed in our laboratory (21), which was composed of two transferring DNA region. One of T-DNA region contains the chimeric gene pGt1-AmA1-nos, carrying the 915 bp desired *AmA1* coding region (13), 1314 bp seed-specific Gt1 promoter (22) and nopaline synthase (nos) terminator. The chimeric gene is flanked by 1.0 kb tobacco Rb_7 matrix attachment regions (MARs) for high-level transgene expression in rice (23), the other T-DNA region carrying a hygromycin resistance gene (*hpt*) driven by *Cauliflower mosaic virus* (CaMV 35S) promoter. The pCDMAR-*AmA1-hpt* was transferred into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method (24), for use in rice transformation as follows.

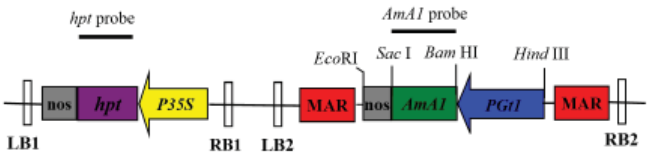

Figure 1. The T-DNA structure of binary plasmid vectors pCDMAR *AmA1-hpt*. *hpt*= hygromycin phosphotransferase; *P35S*= promoter of cauliflower mosaic virus; *nos*= 3'signal of nopaline synthase; *MAR*= tobacco Rb_7 matrix attachment regions; *AmA1= Amaranth* albumin gene; *pGt1*= rice glutelin *Gt1* promoter; *LB1, RB1*= left and right borders of the first T-DNA region; *LB2, RB2*= left and right borders of the second T-DNA region. As indicated in the figure, a full length fragment of *hpt* or *AmA1* coding region was used as a probe.

3.2. Rice Transformation and Regeneration

An elite *indica* rice variety 'MH86' (obtained from Rice Research Institute, Fujian Academy of Agricultural Sciences), was selected as the target plant in this study. *Agrobacterium*-mediated rice transformation, selection, and plant generation were performed following the procedure described by Su *et al*. (25). Briefly, embryogenic calli were induced from sterilized immature embryos (12-15 d after pollination) on NB media containing 2.0 $mg.L^{-1}$ 2,4-D. After a 4-d pre-incubation on fresh NB media, rice calli were soaked in the suspension culture of *A. tumefaciens* carrying pCDMAR-AmA1-hpt for 30 min, followed by the removal of excess broth by the sterile filter paper, transferred to the Co-medium plus 100 µM acetosyringone, and incubated in dark at 25 °C for 3 d. Co-cultivated rice calli were washed with sterile water supplemented with 250 $mg.L^{-1}$ carbenicillin and placed on selection medium containing 30 $mg.L^{-1}$ hygromycin and 250 $mg.L^{-1}$ carbenicillin. After two-month selection, resistant calli were regenerated and transferred to regeneration medium containing 2 $mg.L^{-1}$ 6-BA and 0.5 $mg.L^{-1}$ NAA). Shooting buds of about 3 cm were cut and further cultured on 1/2 MS medium containing 30 $mg.L^{-1}$ hygromycin for root development. After hygromycin-resistant T0 plantlets were well-developed, they were transferred to pots with soil for further growth and maturity.

3.3. Nucleic Acid Analysis

Total genomic DNA was isolated from the leaves of transgenic rice with plant mini-genomic DNA isolation Kit (Dingguo, China) for polymerase chain reaction (PCR) analysis. Two sets of primer pairs, including A1 (5′-ATGGCGGGATTACCAGTG-3′) and A2 (5′-TTAGTTGTTGGATCCCAATTC-3′) for *AmA1*, and H1 (5′-CTATTTCTTTGCCCTCGGAC-3′) and H2 (5′-AAGCCTGAACTCACCGCGAC-3′) for *hpt* were designed for PCR analyses using the Primer Premier 5.0 software. The PCR conditions of *AmA1* amplification were: 94 ºC for 5 min, followed by 30 cycles of 94 ºC (40 s), 56 ºC (40 s), and 72 ºC (1 min). The conditions of *hpt* gene amplification were: 94 ºC for 5 min, followed by 30 cycles of 94 ºC (40 s), 58 ºC (40 s), and 72 ºC (1 min). The PCR products were analyzed by electrophoresis on 1% agarose gel and imaged with a gel imaging system (Bio-RAD, U.S).

In Southern blot, genomic DNA was extracted from 5 g leaf tissues using the CTAB method (26). A 10 μg aliquot of genomic DNA was digested with suitable restriction endonuclease and separated on a 0.8% agarose gel. Following electrophoresis, the DNA fragments were transferred onto a nylon membrane (Amersham, UK) with the aid of an alkaline solution (0.4 M NaOH, 1.5 M NaCl). The *hpt* and *AmA1* probes were synthesized using the PCR DIG Probe Synthesis Kit (Roche, Swiss) and hybridization assay was performed in accordance with the protocol from DIG High Prime of DNA Labeling and Detection Starter Kit I (Roche, Swiss).

Total RNA samples were isolated from each developed rice seed 12-15 days after flowering using EASY spin plant RNA kit (Aidlab, China) and reverse-transcribed into cDNA as a template for real-time quantitative reverse transcription PCR (qRT-PCR). The gene-specific primer sets for *AmA1* were: 5′-CGAACCTTCCAAGACTTATGATG-3′ and 5′-TGGCTGATGCTGTAATCCA-3′ and rice *actin1* was used as the reference gene to normalize targeted gene expression. The qRT-PCR was performed in a 20 μL reaction volume with 0.5 μM each primer and 2× SYBR Green real-time PCR master mix (Takara, Japan). The reactions were run on ABI 7500 Real-Time PCR System using the cycling conditions described by Huang *et al*. (27). The relative expression of the *AmA1* was RQ = $2^{-\Delta\Delta Ct}$. Each sample was amplified in triplicate.

3.4. Antibody Preparation

The prokaryotic expression vector pET28-*AmA1* previously constructed (21) and the molecular chaperone vector of *E. coli*: pBB540, pBB542 (containing *groES* and the *groEL*, was a gift from Dr. Ario de Macro of EMBL) were co-transformed into BL21 (DE3) competent cells to obtain the recombinant strain BL21 (DE3)/pET28a-AmA1-C4. It was then cultured on LB medium containing 30 $\mu g.mL^{-1}$ kanamycin, 34 $\mu g.mL^{-1}$ chloramphenicol and 50 $\mu g.mL^{-1}$ spectinomycin and grown to the OD_{600} 0.6. IPTG was added at a final concentration of 0.4 mM, and the culture was shaken at 20 ºC for 10 h. Bacterial cells were collected and broke open to use the cell lysate to pass through Ni-NTA His-tag purification resin (Biomiga, U.S) in order to purify the AmA1 recombinant fusion protein. The protein samples were collected by ultrafiltration (Millipore, U.S) and sent to the Animal Center of the Institute of Zoology, Chinese Academy of Sciences, to make the rat immune polyclonal antibody.

3.5. Protein Extraction and Western Blotting

Albumin protein was extracted from the transgenic rice seeds according to Schaeffer and Sharpe (28). The appropriate protein samples with 2× buffer solution (100 mM Tris-HCl with pH 6.8, 200 mM DDT, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue R) were mixed and denatured at 99 ºC for 10 min. Electrophoretic separation was performed on a polyacrylamide gel containing 12% separating gel and 5% stacking gel, and the protein bands were stained with Coomassie Brilliant Blue R-250. Trans-blot SD semi-dry electrophoretic transfer cell device (Bio-RAD, U.S) was used to transfer proteins to PVDF membranes. Rat anti-AmA1 antiserum was used as the first antibody, and goat anti-rat IgG-HRP conjugate was used as the secondary antibody (CWBIO, China). Protein bands were visualized in 3,3'-diaminobenzidine (DAB) substrate solution (CWBIO, China).

3.6. Protein, Amino acid, and Thousand-grain Weight (TGW) Analysis

Total protein content and amino acid composition of mature seeds from T2 homozygous transgenic lines and wild-type plant were analyzed in the Central Laboratory of the Fujian Academy of Agricultural Sciences, China. Protein content was measured by the Kjeldahl method, using a conversion factor of 5.95. Amino acid content was measured by high-speed amino acid analyzer (Hitachi L-8800, Japan). TGW was determined by counting 500 grains with the automatic seed counter (SLY-A,China), weighing the sample and evaluating the respective TGW.

3.7. Statistical Analysis

Measurements of protein content, amino acid content, and TGW were carried out in triplicate. Comparison of mean values from different data sets was analyzed for statistical significance with the Student's ***t***-test.

4. Results

4.1. Generation of Co-transformed Rice Containing Both hpt and AmA1

A total of 19 independent promising T0 transgenic rice plantlets (denoted from GA1 to GA19) were regenerated from 24 hygromycin-resistance calli within approximately 6-month of beginning of the culture.

PCR analysis by a pair of specific primers, H1 and H2, on the *hpt* gene in the T0 generation indicated that products of about 1018 bp were amplified from the genomic DNA of all of the transgenic plants and the positive control (plasmid as a template), whereas no band was detected in wild-type plant (Fig. 2A). The second pair of primers, A1 and A2 was used to identify whether the lines carried the *AmA1* (915 bp) The PCR revealed that 6 out of 19 plants were transgenic (Fig. 2B; co-transformation frequency = 31.5%).

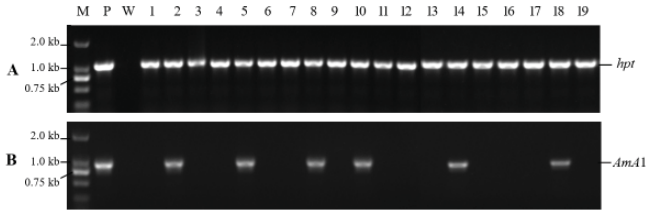

Figure 2. PCR analysis of T0 transfromants for *hpt* and *AmA1* genes. **A.** PCR amplification of the *hpt* gene; **B.** PCR amplification of the *AmA1* gene; M= molecular marker DL2000; P= plasmid pCDMAR-AmA1-hpt; W= wild type plant; 1-19= different transgenic plants.

The six co-transformed T0 plants were further identified by Southern blot, wherein DIG-labeled *AmA1* was used as the probe. The Southern result confirmed the PCR results (Fig. 3). According to the hybridization signals shown in Figure 3, the size and number of hybridization bands both varied differently among the transgenic plants, indicating that the target gene was integrated in different genomics sites of the rice transgenic lines.

4.2. Selection of SMG-free, Homozygous Transgenic Lines

To obtain SMG-free plants, the six T0 co-transformants were planted in the greenhouse and allowed to self-

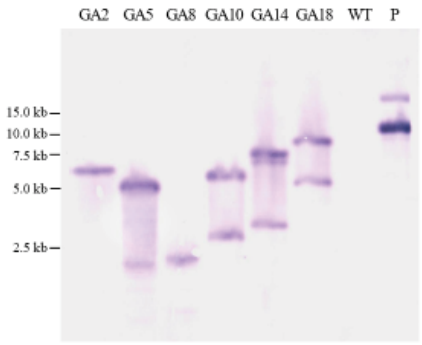

Figure 3. Southern blot analysis of T0 co-transformants. WT= wild type plant; P= plasmid pCDMAR-AmA1-hpt. Genomic DNAs were digested with *Bgl* II and hybridized with the DIG-labeled *AmA1* coding sequence.

pollinate. The total DNA was extracted from the T1 seedlings for both PCR and Southern analyses. As shown in Figure 4, the plants in lanes 4, 10 and 11 were positive for the *AmA1*, but negative for the *hpt* gene

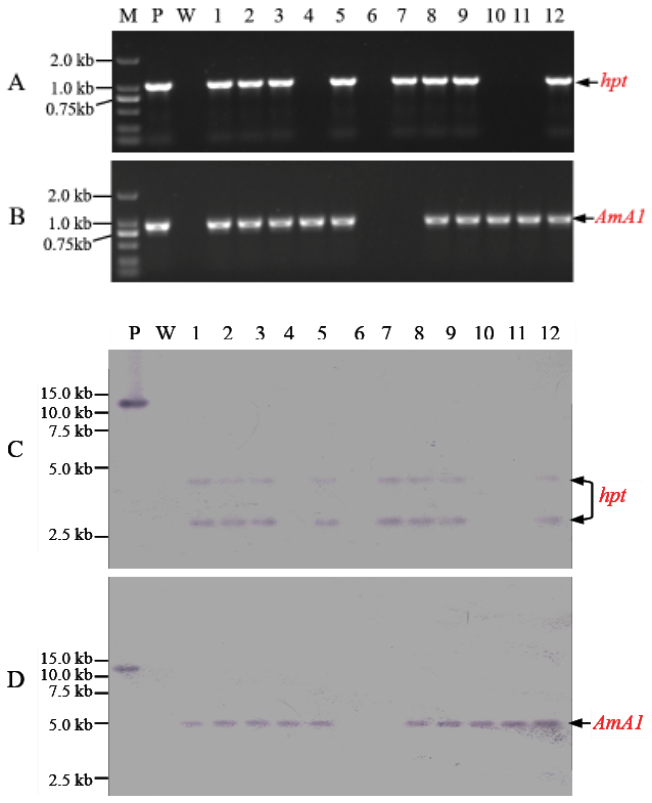

Figure 4. PCR and Southern blotting analyses of T1 progenies from T0 co-transformants GA8. **A.** PCR amplification of the *AmA1* gene; **B.** PCR amplification of the *hpt* gene; **C.** Southern blotting analysis for the *hpt* gene; **D.** Southern blotting analysis for the *AmA1* gene; M= molecular marker DL2000; P= plasmid pCDMAR-AmA1-hpt; W= wild type plant; 1-12= different transgenic individual plants of T1 progenies from T0 co-transformants GA8. Genomic DNA digested with *Hind*III was loaded in each lane.

Table 1. Segregation of *AmA1* and *hpt* in T1 plants

T0 Co-transformants	T1 progenies screened by PCR analysis					Frequency of SMG-free transgenic plant (%)
	Total number	*AmA1*$^+$*hpt*$^-$	*AmA1*$^-$*hpt*$^+$	*AmA1*$^+$*hpt*$^+$	*AmA1*$^-$*hpt*$^-$	
GA2	60	9	3	40	8	15.0
GA5	73	22	2	48	1	30.1
GA8	72	28	4	38	2	38.8
GA10	56	10	6	35	5	17.8
GA14	48	0	0	48	0	0
GA18	76	5	9	59	3	6.6
Total	385	74	24	268	19	-

$^+$ and $^-$ mean positive and negative in PCR detection, respectively.

(*AmA1*$^+$*hpt*$^-$), appearing as the SMG-free transgenic events. Then, 74 SMG-free plants derived from five T0 co-transformants (GA2, GA5, GA8, GA10, GA18) were segregated from 385 T1 progeny and the frequency of SMG-free plant differed among the five co-transformants, the highest reaching 38.8% in GA8 and the lowest only 6.6% in GA18 (Table 1). No SMG-free plant was found in T1 progenies of line GA14.

Further, five SMG-free plants were chosen randomly from each line of T1 generation and grown in the restricted greenhouse. Their DNA was screened to find homozygous transgenic lines via PCR. It was shown that five T2 transgenic plants, GA2-2, GA5-10, GA8-5, GA10-2, GA18-7, were identified as putative SMG-free, homozygous transgenic lines. Subsequently, T3 progenies derived from these homozygous T2 lines showed 100% PCR positive for *AmA1* (data not shown).

4.3. Expression of AmA1 in Transgenic Rice Seed

Real-time PCR analysis showed that the *AmA1* transcripts were present at variable levels in the developing seeds of T2 generation homozygous lines, GA2-2, GA5-10, GA8-5, GA10-2, GA18-7. The highest transcription level was measured in line GA8-5 and no *AmA1* transcripts were found in wild type plant (Fig. 5).

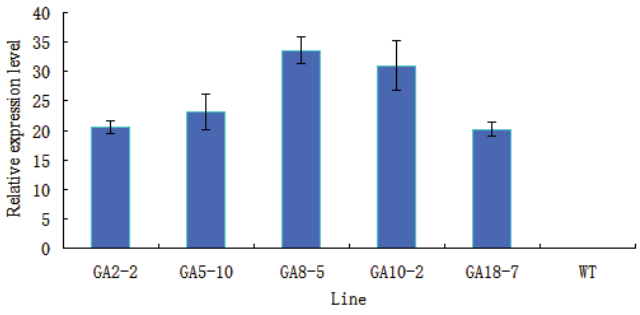

Figure 5. Real-time PCR analysis of AmA1 expression in transgenic rice seeds. WT= wild type plant.

To detect the accumulation of AmA1 in transgenic rice seeds, albumin proteins extracted from T2 generation seeds of five homozygous lines were subjected to SDS-PAGE and Western blotting. The SDS-PAGE separation showed a band of 35 kDa in all five transgenic lines, while, as expected, there was no signal detectable in wild type plant (Fig. 6A). Presence of the 35 kDa large polypeptide of AmA1 in the transgenic rice seeds was further confirmed by immuno-detection with the polyclonal anti-AmA1 antibody using a HRP conjugated secondary antibody (Fig. 6B). The results showed that AmA1 was accumulated in the T2 seeds of the five homozygous transgenic lines.

4.4. Increase of Both Protein and EAA Contents in Transgenic Rice Seed

Mature T2 seeds, which collected from the five marker-

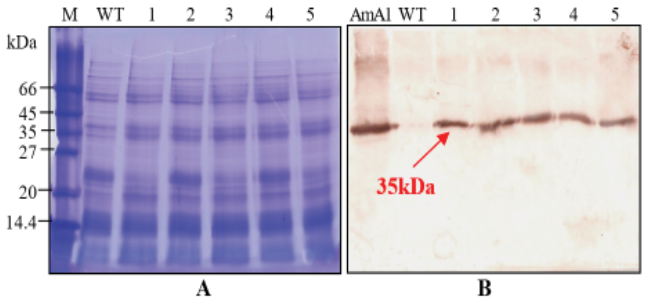

Figure 6. SDS-PAGE and western blot analysis of AmA1 in transgenic rice seed. **A.** SDS-PAGE patterns of the albumin fractions in transgenic rice seed samples. **B.** Western blotting of the protein extracted from the same seeds. 1-5= transgenic lines GA2-2, GA5-10, GA8-5, GA10-2, GA18-7; M= protein marker; WT= wild type; AmA1= albumin proteins extracted from seed of *Amaranthus hypochondriacus*.

Table 2. Protein content (%) and TGW (g) of transgenic rice with *AmA1*

Homozygous transgenic lines	Total protein content (g.100 g^{-1} DW)	Increase of total protein content (%)	TGW (g)	Decrease of TGW (%)
GA2-2	8.56±0.20^{ns}	1.06	23.78±0.30**	5.63
GA5-10	9.48±0.23**	11.92	22.95±0.20**	8.93
GA8-5	9.56±0.23**	12.87	24.33±0.31*	3.45
GA10-2	8.85±0.27^{ns}	4.49	21.68±0.48**	13.97
GA18-7	8.77±0.31^{ns}	3.54	22.22±0.47**	11.83
WT	8.47±0.09	-	25.20±0.15	-

Values are presented as the mean ± SE; * and ** mean the least significant differences at 0.05 and 0.01 probability levels, respectively, when compared with the wild types; ns: not significant; WT: the wild type.

free, homozygous transgenic lines were subjected to analyses of crude protein, TGW and EAA contents. It was shown that the total protein content varied from 8.56% to 9.56% and showed an increase by 1.06% to 12.87% in compared with wild type control (Table 2). Whereas, the TGW of these transgenic lines decreased at different levels (5.63~13.97%) and no obvious correlation was found between the protein content and the TGW (Table 2).

The increase in total protein content was accompanied by the increase of seven EAAs content compared to the wild type control (Table 3). The highly expressed line (GA8-5) showed a significant content increase of 10.8% and 9.1% for lysine and threonine, respectively. In addition, the contents of several other EAAs, including valine, isoleucine, leucine and phenylalanine, were also increased by 14.6%, 12.1%, 14.7% and 15.6%, respectively (Table 3).

5. Discussion

It has been demonstrated that the twin T-DNA vector system, involving transforming with one binary vector having two T-DNAs, one containing SMG and one gene of interest (GOI), is successful in producing

Table 3. EAA content of transgenic rice seed with *AmA1*

EAAs	EAA content (g.100 g^{-1} seed dry weight)					
	WT	Line GA2-2	Line GA5-10	Line GA8-5	Line GA10-2	Line GA18-7
Lysine	0.37±0.01	0.39±0.01^{ns} (5.4)	0.40±0.02* (8.1)	0.41±0.02* (10.8)	0.38±0.01^{ns} (2.7)	0.38±0.01^{ns} (2.7)
Threonine	0.33±0.01	0.33±0.01 (0)	0.36±0.02^{ns} (9.1)	0.36±0.01* (9.1)	0.34±0.01^{ns} (3.0)	0.34±0.01^{ns} (3.0)
Methionine	0.15±0.01	0.15±0.01 (0)	0.17±0.01^{ns} (13.3)	0.16±0.0 1^{ns} (6.7)	0.16±0.00^{ns} (6.7)	0.16±0.0 1^{ns} (6.7)
Valine	0.48±0.02	0.48±0.02 (0)	0.54±0.01** (12.5)	0.55±0.02* (14.6)	0.50±0.01^{ns} (4.2)	0.50±0.00^{ns} (4.2)
isoleucine	0.33±0.01	0.33±0.01 (0)	0.37±0.00** (12.1)	0.37±0.00** (12.1)	0.34±0.01^{ns} (3.0)	0.34±0.^{01ns} (3.0)
Leucine	0.68±0.03	0.68±0.02 (0)	0.76±0.02* (11.8)	0.78±0.03* (14.7)	0.72±0.01^{ns} (5.9)	0.71±0.02^{ns} (4.4)
Phenylalanine	0.45±0.01	0.46±0.01^{ns} (2.2)	0.51±0.02** (13.3)	0.52±0.01** (15.6)	0.48±0.01** (6.7)	0.47±0.01^{ns} (4.4)

Values are presented as the mean ± SE; amino acid increase (%) in transgenic rice in relation to wild type is presented in parentheses; * and ** mean the least significant differences at 0.05 and 0.01 probability levels, respectively, when compared with the wild types; ns: not significant; WT: the wild type.

SMG-free transformants at reasonable frequencies (19). Yu *et al*. (29) and Jiang *et al*. (30) achieved the co-transformation frequency of 29.87% and 43.8%, respectively, when rice was transformed with the twin T-DNA vector system. Sun *et al*. (31), working on chrysanthemum, also employed twin T-DNA vector system and reported a co-transformation frequency of 38.4%. Our co-transformation frequency (31.5%) was comparable to those obtained by other groups (29-31).

In a co-transformation experiment, when the two separate T-DNAs integrate at unlinked sites in the host genome, the gene of interest can segregate away from SMG in successive generations and SMG-free plants were subsequently obtained (19, 29). In this study, from 6 lines transformed with the *AmA1* and *hpt*, segregation occurred in 5 lines only contained *AmA1* gene, indicating an isolation frequency of SMG-free lines of about 83.3% among co-transformants. This is much higher than the previous studies (1, 29, 30, 32), this is perhaps due to factors such as different receptor and integrated position of targeted gene in the receptor genome. Similar results were observed in co-transformation experiment conducted by Hou *et al*. (33), which showed 12 out of 13 *indica* rice co-transformants displayed a segregation of the SMG and target gene in T1 generation, using nearly the same twin T-DNA binary vector system as in the current study. No SMG-free plant was found in T1 progenies of line GA14, this might be caused by the integration of both the *AmA1* and *hpt* genes at linked loci.

To date, genetic engineering technology has been used to modify the amino acid composition of rice proteins and several transgenic strategies have been developed (4, 6), among which, the most common strategy is to transfer and over-express the gene encoding a heterologous or homologous protein being rich in a desirable EAA into rice (1, 4, 6, 8, 11, 34). The key factor contributing to the success of this approach is the availability of candidate EAA-rich protein genes and appropriate regulatory elements such as a seed-specific promoter and a signal peptide sequence.

The AmA1 from *A. hypochondriacus* is a well-balanced protein in terms of amino acid composition and even better than the values recommended by the World Health Organization for a nutritionally rich protein (13). More importantly, AmA1 is a non-allergenic protein in its purified form (15). Previous studies on AmA1 have shown that its cDNA can be expressed successfully in wheat grains (16) and potato tubers (14, 15), exhibiting a remarkable increases in most EAAs of transgenic lines. These findings demonstrate the feasibility of using the *AmA1* gene in genetic engineering to improve the nutritive value of crops.

The Gt1 promoter used in this study is endosperm-specific and strongly expressed in the outer endosperm, which had been demonstrated with the GUS reporter gene in transgenic rice (35). Many proteins have been successfully expressed in rice seeds using Gt1 promoter such as bean seed storage protein β-phaseolin for improving the nutritional quality of rice grains (36), human lactoferrin for use in infant formula (37), and CALB as a biocatalyst for biodiesel production (38). As expected in this study, AmA1, an EAA-rich seed storage protein, was successfully expressed and accumulated in transgenic rice grains under the control of rice Gt1 promoter, with a significant increasing in both protein content and most EAAs contents. It might be that the decrease of total protein affected the starch accumulation during rice grain development, and consequently the TGWs of most transgenic lines dropped considerably. As an important factor related to rice grain yield potential (39), TGW decrease will have negative effects on the total protein biomass in large scale production (38). Nevertheless, a transgenic line (GA8-2) was obtained, which had highest increase in both protein content and most EAAs content, but only slight decrease in thousand-grain weight (3.45%). The next step of the research will focus on assessment of the major agronomic characters of line GA8-2 in field and digestibility of AmA1 in the transgenic rice seed.

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