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Original Paper

Transgenic rice for animal feed with high tryptophan content generated by a selectable marker- and vector backbone-free technology

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Abstract We reported previously that the genetically modified rice lines expressing *OASA1D*, a feedback-insensitive anthranilate synthase (AS) α-subunit gene of rice, showed remarkable increase of free tryptophan (Trp) in calli. In the present study, we investigated transformation of "Kusahonami" (*Oryza sativa* L.), a rice variety for animal feed with this gene. In order to avoid the co-integration of vector backbone sequences and taking into account the fact that *OASA1D* gene exert resistance to 5-methyltryptophan (5MT), gene cassette vector consisting of a promoter, *OASA1D* cDNA and terminator was constructed and introduced directly to rice calli by use of potassium titanate whisker-supersonic method. Transgenicity of regenerated plants and the copy number of the transgene in the genome were analyzed by Southern blot. Fertile transgenic plants carrying low copy number of transgene and producing high level of free Trp in leaves and seeds were obtained. Genetic stability of the transgene has been demonstrated. This cultivar is a common variety for animal feed, these transgenic plants may be useful to establish an actual variety that is nutritionally improved.

Kew words: Anthranilate synthase, 5-methyltryptophan (5MT), transformation, tryptophan, whisker.

We reported previously on generation of a feedback-insensitive anthranilate synthase (AS) α-subunit gene of rice (*OASA1*), *OASA1D*. The genetically modified rice lines expressing *OASA1D* were generated by *Agrobacterium*-mediated transformation system and shown to accumulate free tryptophan (Trp) in the calli and leaves (Tozawa et al. 2001). This gene, *OASA1D*, that encodes a protein in which aspartate-323 is replaced with asparagine manifested up to about 150 fold increase in free Trp in rice calli. In addition, we confirmed that the marked overproduction of Trp was stable in the seeds of two lines, HW-1 and HW-5, under the condition of a greenhouse or a field (Wakasa et al. submitted). As a next step, we tried to make a practical transgenic line with high content of Trp for the purpose of establishing a rice variety of animal feed, because Trp is an essential dietary amino acid for animal growth. Amino acid-fortified low-protein diets to decrease nitrogen excretion in pigs and broiler chicks often require supplement of Trp (Sharda et al. 1976; Russell et al. 1983; Hahn et al. 1995).

To make transgenic rice for practical purpose such as animal feed, the following points were must be taken into account: 1) Use of an antibiotic selection system must be avoided. 2) The introduction of vector backbone sequence in the rice genome is evaded. 3) A practical variety must be used. To solve these issues, we achieved the direct transformation system with linear gene cassette lacking vector backbone sequence and selectable maker gene using a rice variety for animal feed in this study.

Rice cells expressing a mutated rice AS α-subunit gene, *OASA1D*, showed resistance to 300 μM 5-methyltryptophan (5MT) (Tozawa et al. 2001) and it has been shown that the selection system using *OASA1D* gene and 5MT for a selection agent was effective in potato, rice and *Arabidopsis* transformation (Yamada et al. 2004; Kawagishi et al. 2005). Because *OASA1D* gene acts as a selectable marker and target gene for Trp accumulation, we can eliminate the use of any selectable maker genes.

In the routine transformation protocol, the integration of "unnecessary" foreign DNA such as vector backbone sequences, selectable marker genes and *vir* genes linked to the T-DNA into the genome is unavoidable because the cassette of a transgene is connected in the

Abbreviations: AS, anthranilate synthase; IAA, indole acetic acid; 5MT, 5-methyltryptophan.
This article can be found at http://www.jspcmb.jp/

plasmid vector. Integrated vector DNA has reportedly been detected in transgenic plants generated by *Agrobacterium*-mediated transformation (Ramanathan and Veluthambi 1996; Cluster et al. 1994; Kononov et al. 1997; Tingay et al. 1997) and direct delivery procedures such as particle bombardment (Kohli et al. 1999). Vector backbone sequences may exert undesirable negative effect in *cis* regions (Artelt et al. 1991). Therefore, in this study we used an expression cassette fragment that contains only promoter, transgene and terminator, in stead of whole plasmid, for the transformation of rice.

Silicon carbide whiskers are micro-fibers 10–80 μm long and 0.6 μm in diameter. Whisker-mediated gene transfer, by virtue of its simplicity and potential for scaling up, is an attractive means of delivering DNA to plant cells. This method has been used in transformation of *Chlamydomonas reinhardtii* (Dunahay 1993), maize (Kaeppler et al. 1992), tobacco (Kaeppler et al. 1990) and *Agrostis alba* (Asano et al. 1991). More recently, Terakawa et al. (2005) reported that a whisker method by the potassium titanate combined with supersonic treatment for transformation of rice.

Given that cereal crops such as rice, maize, wheat, and oats exhibit a relatively low content of Trp in seed storage proteins, an increase in the abundance of this amino acid should improve their nutritional value for humans and animals. The use of rice for animal feed has been developed (Sakai et al. 2003). Therefore, we here investigated transformation of Kusahonami, a rice cultivar for animal feed, by use of the whisker-mediated transfer of a minimal transgene cassette containing promoter, transgene (*OASA1D*) and terminator to establish a variety with high Trp content for animal feed.

Materials and methods

Plant material and transformation

Calli were induced from mature seed of rice (*Oryza sativa* L. cv. Kusahonami), and suspension culture was prepared as reported by Urushibara et al. (2001). Ten microliter plasmid or PCR amplified DNA solution (1mg ml^{-1}) was mixed with 107.5 μl liquid medium containing 1/3 Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962) and 30 g l^{-1} sucrose. Then, 2.5 μl of POH solution containing 1 mg ml^{-1} poly-L-ornithine hydrochloride (MW 30,000) solution, after being sterilized by passage through a 0.45 μm filter (Advantec, Tokyo, Japan) was added to the plasmid or PCR amplified DNA solution and quickly mixed to allow formation of DNA/POH complex.

Preparation of whisker fibers and transformation using them were performed as described elsewhere (Terakawa et al. 2005). Briefly, 250 μl packed cell volume (PCV) of suspension cultured rice calli was dispensed into a tube. The 500 μl whisker suspension (1%, w/v)) was added to this tube. The 120 μl mixture of DNA and POH complexes was added to the tube. The suspension was centrifuged for 5 min at 20,000 *g* at 4°C. This was repeated 3 times. After being kept on ice for 10 min, the mixture of rice calli, whisker and DNA were subjected to a supersonic treatment by use of a disrupter (Bioruptor UCD-200, Cosmo bio, Tokyo, Japan) for 1 min at room temperature. The calli thus treated were then transferred into a 35×10 mm plastic petri dish, and 3 ml liquid R2 medium (Ohira et al. 1973) consisted of R2 basal salts, 2 mg l^{-1} 2, 4-dichlorophenoxy acetic acid (2,4-D) and 30 g l^{-1} sucrose. Calli were cultured on a rotary shaker (120 rpm) in the dark at 28°C.

Plasmid vector DNA and gene cassette DNA

A *Xho*I-*Sac*I DNA fragment including the maize ubiquitin gene promoter and *OASA1D* gene from a binary vector, pUASA1D (Tozawa et al. 2001), was subcloned into same restriction sites on pUBA (Toki et al. 1992; Cornejo et al. 1993) to generate pUBdD (Figure 1A). The pUBdD plasmid DNA was purified by a QIAGEN plasmid Maxi Kit (Qiagen, Tokyo, Japan) prior to use. Using a primer pair 5′CCT CTC TAG AGA TAA TGA GCA TTG CAT GTC and 5′CCC GAT CTA GTA ACA TAG ATG ACA CC, pUBdD as the template and KOD-Plus polymerase (Toyobo, Tokyo, Japan) according to manufacture's instruction, PCR amplification was carried out to produce a DNA fragment that contained the *OASA1D* gene cassette (4.4 kb) between maize ubiquitin 1 promoter and 3′ terminator signal of nopaline synthase gene (Figure 1B). The amplified DNA was purified by a PCR clean Spin Columns (BIO-RAD Tokyo, Japan).

Selection and regeneration

After 3–5 days, the whisker-treated calli were transferred onto the selection medium which contained N6 basal salts, 2 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose, 150 μM D, L-5MT

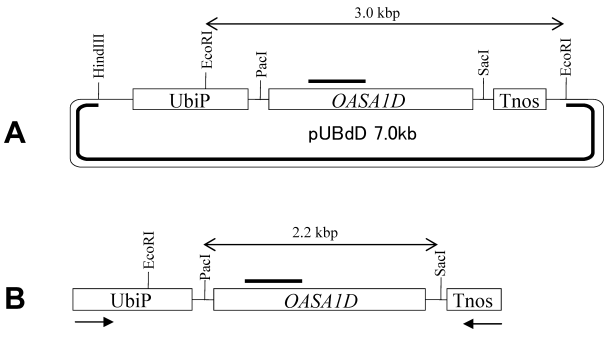

Figure 1. Schematic structure of plasmid pUBdD (A) and *OASA1D* gene cassette (B). UbiP: promoter of maize ubiquitin gene, Tnos: terminator of nopaline synthase gene. Solid lines on the *OASA1D* gene in (A) and (B) and vector backbone in (A) indicate the positions of the probes used for Southern blot analyses (see below). Two small arrows in B indicate the PCR primers used for cassette fragment amplification as well as for transformation.

and 0.3% Gelrite (Wako Chemicals, Osaka, Japan). During selection calli were kept in dark at 28°C. After a 30-day culture, the growing calli were transferred to regeneration medium (Rashid et al. 1996) and cultured at 28°C with 16-h light per day. The regeneration medium was consisted of MS basal salts, 1 mg l^{-1} NAA, 2 mg l^{-1} BAP, 20 g l^{-1} sucrose, 30 g l^{-1} sorbitol, 150 μM 5MT and 4 g/l^{-1} Gelrite. After culture for about one month, regenerated plants (approximately 1–3 cm in length) were transferred to the plastic box with 150×150 mm containing 100 ml MS medium supplemented with 30 g l^{-1} sucrose, 150 μM 5MT and 3 g l^{-1} Gelrite to allow root formation. Rooted plants were transferred to pots and grown in a greenhouse at 26 to 28°C under natural light conditions.

Southern blot analysis

Genomic DNA was isolated from fresh leaves by the CTAB method (Murray and Thompson 1980) with automatic DNA isolation system (PI-5α Kurabo, Osaka, Japan). DNA (5 μg) was digested with *Sac*I, *Eco*RI or double-digested with *Pac*I and *Sac*I. The resulting DNA fragments were subjected to electrophoresis in a 0.8% agarose gel, transferred to nylon membrane, and subjected to hybridization using *OASA1D* gene or vector backbone sequence as gene probes. The sequence corresponding to 242nd to 693rd bases of *OASA1* cDNA (accession no. AB022602) and vector backbone sequence cut from plasmid pUBdD with *Eco*RI and *Hind*III were used as the gene probes (see Figure 1). Probe labeling, hybridization, and detection were performed with the use of an ECL-based kit (Amersham Bioscience, Tokyo, Japan).

Northern and Western blot analysis

Total RNA was isolated from the leaves of transgenic plant by RNeasy Mini kit (Qiagen, Tokyo, Japan) and 10 μg RNA was subjected to electrophoresis through a 1.2% agarose-formaldehyde gel. The separated RNA was transferred to a nylon membrane and subjected to hybridization with an *OASA1D* riboprobe. Probe labeling with digoxigenin-dUTP, hybridization and immunological detection were performed with a Dig-based kit (Roche Diagnostics, Tokyo, Japan).

To detect OASA1 protein, antiserum to OASA1 was generated by injection of rabbits with the synthetic peptides CMDHKMKSRREQFAPD. The antiserum was subjected to chromatography on protein A-Sepharose to obtain a fraction enriched in IgG. The obtained antibody was cross-reacted with a single band at around 60 kDa. Total protein was extracted from the leaves of transgenic plant and 10 μl was subjected to SDS-PAGE on a gel containing 7.5% polyacrylamid for separation (Laemmli 1970), and then electro-transferred onto PVDF membranes (Hybond-P, Amersham Bioscience, Tokyo, Japan), which was then incubated either with rabbit antiserum to OASA1. The OASA1 oligopeptide antibodies were used at dilution 5000 : 1, and immunodetection was performed using ECL-Plus Western blotting kit according to manufacturer's instruction (Amersham Bioscience, Tokyo, Japan).

Analysis of free Trp content

A total of 100 mg of fresh leaves were ground in liquid nitrogen, extracted with 1.0 ml of chloroform : methanol : water (5 : 12 : 3, v/v/v), and centrifuged at 20,000 *g* for 10 min, and then the supernatants were pooled. The combined supernatant was mixed with 750 μl distilled water and 500 μl chloroform, vortexed vigorously for 30 s, centrifuged at 20,000 *g* for 10 min, and the aqueous phase was collected. Ten microliter of aqueous solutions was directly injected to Waters Alliance HPLC FLD System 2695 (Waters, Tokyo, Japan) on an Xterra RP18 column (4.6×150 mm). Trp was determined by fluorescence with excitation at 278 nm and emission at 348 nm.

Results and Discussion

We transformed a japonica animal feed cultivar, Kusahonami of a heavy panicle type and shows superior dry matter productivity in panicle; which is about 26% higher than that of "Nipponbare". The *OASA1D* gene cassette fragment was directly transferred into suspension cultured calli of rice using the potassium titanate whisker-supersonic transformation method. In this gene cassette, the *OASA1D* gene is under the control of the constitutive maize ubiquitin gene promoter to ensure a high-level expression in rice. Transformants were selected for 5MT resistance exerted by the expression of the *OASA1D* gene. Transformants with whole pUBdD plasmid were also produced to compare with those that are free from unnecessary DNA fragments such as vector backbone sequences.

Generation of transgenic rice

A total of 18 g rice calli transformed with the *OASA1D* gene cassette fragment were selected for resistance to 150 μM 5MT. A total of 37 resistant calli were obtained, and plants were regenerated from each of them (designated "gene-cassette transformed plants"). On the other hand, a total of 15 g calli transformed with whole pUBdD plasmid DNA produced 36 calli resistant to 150 μM 5MT. Plants regenerated were designated "whole plasmid-transformed plants". In this study, plants regenerated from the same calli were considered to belong to the same line. All of the putative transgenic plant lines were subjected to Southern blot analysis.

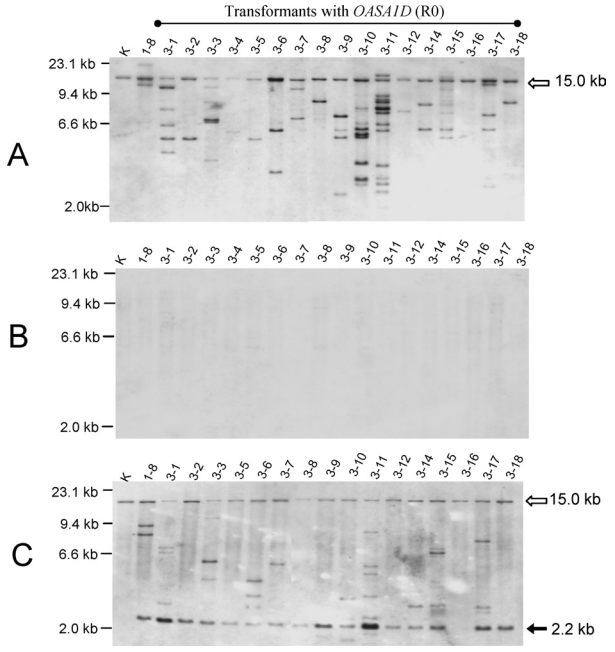

Figure 2. Southern blot analysis of R0 generation of transgenic rice transformed with the *OASA1D* gene cassette. K; Kusahonami, lines 1-8, and 3-1 to 3-18; independent transgenic lines. Probes used were a fragment of *OASA1D* DNA fragment (A and C) or the vector backbone cut by the digestion of plasmid pUBdD with *Eco*RI and *Hind*III (B) as depicted in Figure 1. Genomic DNA was digested with *Sac*I (A and B) which has a unique site in the *OASA1D* gene cassette, and the DNA was digested with *Pac*I and *Sac*I (C), which release a 2.2 kb fragment (see Figure 1). The 15.0 kb band correspond to the endogenous *OASA1* gene. See text for details.

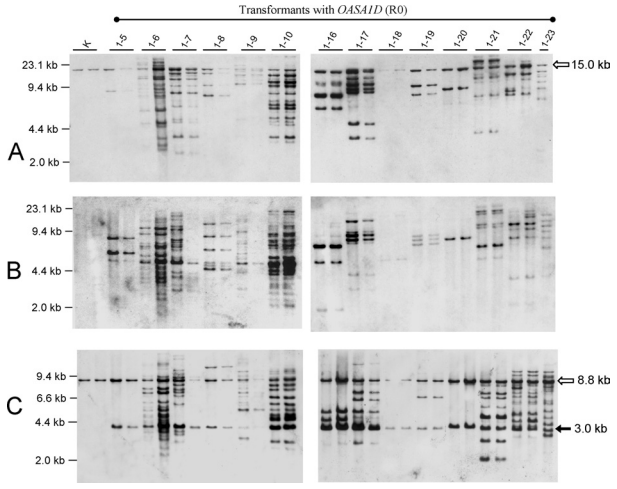

Figure 3. Southern blot analysis of R0 generation of transgenic rice transformed with the whole plasmid pUBdD. K; non-transformed control (data from two individuals are shown). Each lane represents data of from two individuals of a transgenic plant line regenerated from the same callus (except for line 1-23). Probes used were a fragment of *OASA1D* DNA fragment (A and C) or the vector backbone cut by the digestion with *Eco*RI and *Hind*III (B) as depicted in Figure 1. Genomic DNA was digested with *Sac*I (A and B) which has a unique site in both *OASA1D* gene cassette and plasmid pUBdD, and the DNA was digested with *Eco*RI (C), which release a 3.0 kb fragment. The 15.0 and 8.8 kb bands correspond to the endogenous *OASA1* gene. See text for details

Southern blot analysis to confirm transgenicity and to estimate copy number

Figures 2 and 3 show the typical Southern blot analysis results for gene-cassette transformed plants and whole plasmid-transformed plants, respectively. Prior to hybridization with the *OASA1D* gene (Figures 2A, C, 3A, C) or with vector backbone sequence probe (Figures 2B, 3B), genomic DNA from plants was restricted with *SacI* or other enzyme(s). Clearly, most of the plants, if not all, appeared to possess the band due to the *OASA1D* gene.

Since *Sac*I acts as a single cutter in both plasmid and gene cassette (see Figure 1), resulting Southern band patterns can be used as a measure for estimation of the copy number. The 15.0 kb observed in the digest with *Sac*I (Figures 2A, 3A) was considered to correspond to the endogenous *OASA1* contained 10 introns. Thus, the contribution of this band can be ignored in the estimation of the copy number of foreign *OASA1D* gene. Based on this assumption and the results obtained after digestion with *Sac*I, the lines 3-2, 3-4, 3-5, 3-8, 3-12 and 3-18 of gene-cassette transformed plants (Figure 2A) and lines 1-5, 1-18 and 1-20 of whole plasmid-transformed plants (Figure 3A) were concluded to possess a single copy of *OASA1D* gene. Also, lines 3-7 and 3-14 (Figure 2A), and 1-19 (Figure 3A) were considered to be "two-copy group". Those transgenic plants carrying one or two copies of *OASA1D* gene were designated "low copy plant". The yield of low copy plant appeared to be greater in gene-cassette transformed plants than in whole plasmid-transformed plants (see also below).

Figure 2C and 3C respectively depict Southern data for the digest of gene-cassette transformed plants with *Sac*I/*Pac*I and those for digest of whole plasmid-transformed plants with *Eco*RI. Both were probed with *OASA1D* gene. The former and latter digestion respectively generated a 2.2 kb fragment (see Figure 1B) and a 3.0 kb fragment (see Figure 1A) of the transgene. The fragments of 15.0 kb and 8.6 kb in these digests were considered to correspond to endogenous *OASA1* gene. Most of the transgenic plant lines grouped to a single or two copy ones, if not all, showed one or two bands apart from the endogenous one. The presence of larger and smaller fragment size than expected ones in some lines suggest the rearrangement of transgene in the genome.

When probed with vector backbone sequence, no hybridization bands were detected in all gene-cassette transformed plants (Figure 2B), whereas all whole plasmid-transformed plants showed bands due to the vector backbone sequence (Figure 3B). The latter result was consistent with the report that the vector backbone sequence was detected in the genome of transgenic plants obtained by T-DNA integration through *Agrobacterium* infection (Ramanathan and Veluthambi

Table 1. Transformation frequency.

DNA	Weight of calli used for transformation (g)	Number of calli with 5MT resistant shoots	Number of transgenic plants[a]	Frequency of transgenic low copy plant (%)[b]
Cassette fragment	6.0	8	7	2 (28.6)
(Linear)	6.0	12	12	5 (41.7)
	6.0	17	16	8 (50.0)
Total	18.0	37	35	15 (42.9)
Whole pUBdD	7.5	23	21	4 (19.0)
(Circular)	7.5	13	13	2 (15.4)
Total	15.0	36	34	6 (17.6)

Number of transformants after transfer gene cassette fragment (linear) or whole pUBdD plasmid (circular) using the whisker-supersonic method followed by selection with 150 μM 5-methyltryptophan (5MT).

[a] Transgenicity of plants were confirmed by Southern blot analysis using *OASA1D* gene probe.

[b] Plants carrying one or two copies of *OASA1D* was designated low copy plant.

1996; Cluster et al. 1994; Kononov et al. 1997; Tingay et al. 1997). The present result that gene-cassette transformed plants carry no other foreign DNA but *OASA1D* gene shows the advantage of the present transformation method for production of transgenic crops for human and animal feeds.

The OASA1D gene cassette versus whole plasmid DNA transformation

Table 1 summarizes the data of gene-cassette transformed plants and whole plasmid-transformed plants. The fact that 35 and 34 transgenic plant lines were obtained by transformation with the *OASA1D* gene cassette alone and with whole pUBdD plasmid DNA, respectively, indicates that the presence of vector backbone sequence does not increase the yield of transgenic plants, and thus confirms that the present method using the *OASA1D* gene cassette alone as a vector has a great advantage (no risk of "co-integration" of vector backbone sequences into the genome) in rice transformation.

The data that total of four lines (including the line 3-16 shown in Figure 2) did not contain *OASA1D* gene in spite of their resistance to 5MT suggest that the integrated *OASA1D* gene was lost during plant regeneration process, or that 5MT resistance was produced by somaclonal variation.

The yield of low copy plant (see above) was markedly higher in gene-cassette transformed plants than in whole plasmid-transformed plants; 42.9% (15 out of 35) and 17.6% (6 out of 34), respectively, were low copy plants (Table 1). Characterization of integration site of the introduced transgene is essential in the safety assessment, and thus low copy plants are more advantageous in practical use as human and animal feeds. Reason(s) why gene-cassette transformation produces high yield of low copy plants remains unclear. However, it is possible that the vector backbone sequences play a key role in the integration process. Fu et al. (2000) proposed that backbone elements promote high-copy-number integration events by providing extensive regions of homology, and that the removal of the backbone would (quantitatively) limit the amount of homologous recombination. In addition, they suggested that the vector backbone has been shown to provide a number of recombination hotspots, and that removal of these sequences would limit the influence of recombinogenic elements in the process of integration. In each case, the minimal cassette would provide shorter regions of homology and fewer recombination hotspots than whole plasmid DNA (Fu et al. 2000).

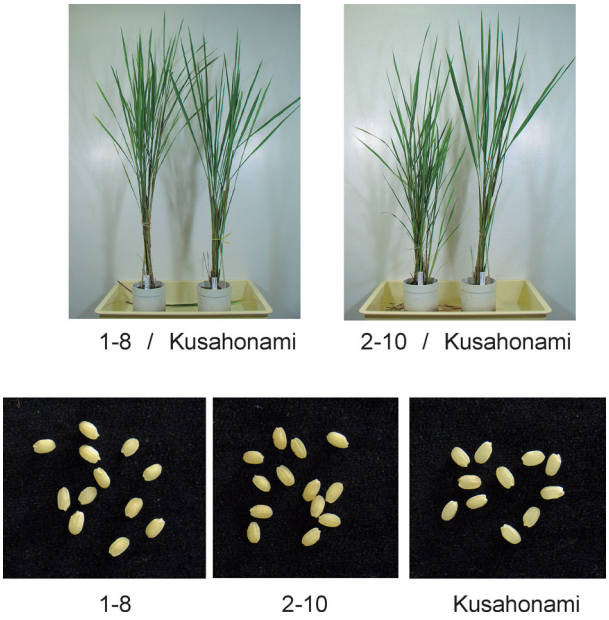

Figure 4. Morphology of transgenic rice plants (R2 generation) expressing *OASA1D* in a pot (upper panels), and the mature seeds (lower panels). Kusahonami: non-transgenic plant or seed.

Morphology and fertility

Among 35 transgenic plants obtained by the gene cassette method, four of them died before maturation. Somaclonal mutation during culture and transformation procedure might cause their weakness. Other 31 plants reached to the maturation stage normally. Representative plants and seeds are shown in Figure 4.

The transgenic rice of Nipponbare expressing *OASA1D* gene reportedly showed low spikelet fertility (Yamada et al. 2004; Wakasa et al. submitted). Transgenic plant lines obtained in this study showed rather low spikelet fertility (35 to 50%), and five plant lines (out of 31) produced no seeds, suggesting the existence of some influences by the expression of *OASA1D* gene as observed transgenic Nipponbare. However, since even non-transformed wild type plants of Kusahonami when grown in a greenhouse showed low spikelet fertility (around 50%), it was difficult to figure the influence of *OASA1D* gene to spikelet fertility in our transgenic plants obtained here. From 26 plants that formed succeeding generations (R1 and R2), several lines were chosen, and further analyses on the gene expression and Trp content were carried out.

Expression of transgene

In order to assess the stability of transgene expression following transformation with gene cassette constructs, we monitored the expression of *OASA1D* in the R2 generation. RNA and proteins were isolated from the leaves of nine low copy transgenic plants, and subjected to northern and western blot analyses, respectively. Results are shown in Figure 5. Transgenic plants exhibited an abundant RNA (at ca. 2.0 kb) hybridizable with an *OASA1D* riboprobe (Figure 5A), indicating that the *OASA1D* gene under the control of the maize ubiquitin promoter was expressed at a high level in the leaves of transgenic plants. Consistent with this northern data is the result that transgenic plants showed a 60 kDa protein band in the western blot analysis using an OASA1 antibody (Figure 5B). Wild type Kusahonami showed a very weak band with an *OASA1* riboprobe and with the antibody for OASA1, suggesting the expression of the endogenous AS α subunit gene at a low level.

Although some transgenic plant lines (2-4 and 3-18) showed high level of *OASA1D* RNA, the OASA1 protein level was low. Reason for this remains unclear. There seemed no clear correlation between the copy number of transgene and the level of expression. For example, single copy lines (2-3, 3-4 and 3-18), two copy ones (1-7, 2-4 and 2-10) and multi copy ones (1-8, 3-6 and 3-9) presented no proportional level of *OASA1D* RNA and proteins to the copy number of transgene (Figure 5). The fact that they all survived during the 5MT selection in the callus and plantlet stages indicates that the level of expression was enough to exert resistance to 5MT. It is consistent that the OASA1 protein level of many transgenic Kusahonami lines was comparable with HW-1 and HW-5, which were the elite lines developed to the field trial (Wakasa et al. submitted).

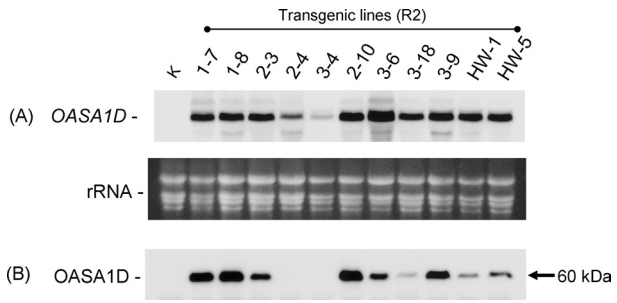

Figure 5. Expression analysis of *OASA1D* transgenes in transformants (R2 generation). (A) Northern blot analysis of total RNA in leaves from transgenic rice with an *OASA1D*-specific riboprobe (upper panel). The rRNA bands in the ethidium bromide-stained gel (lower panel). (B) Western blot analysis of protein extracts from transgenic rice leaves. Antiserum to OASA1 protein was generated by injection of rabbits with the synthetic peptide. K; non-transformed control of Kusahonami, all transgenic lines are of Kusahonami, except for HW-1 and 5 that are transgenic lines of cv. Nipponbare.

Table 2. Free tryptophan (Trp) content in leaves and seeds of transgenic plants (R2 generation) grown in a greenhouse.

Progeny	Free Trp in leaves (nmol g^{-1} fresh leaves)		Free Trp in seeds (nmol g^{-1} dry seeds)	
Kusahonami	66.5±5.7		67.9±24	
1-7	15638.3±480	(235.2)[a]	13391.2±438	(197.2)[a]
1-8	28709.4±1037	(431.7)	21438.1±1232	(315.7)
2-3	20949.2±1097	(315.0)	2319.3±227	(34.2)
2-4	3125.8±62	(47.0)	918.2±86	(13.5)
2-10	7460.1±743	(112.2)	1167.9±171	(17.2)
3-4	4734.3±305	(71.2)	8852.0±1544	(130.4)
3-6	2872.3±61	(43.2)	405.0±154	(6.0)
3-9	3915.2±137	(58.8)	1347.3±242	(19.8)
3-18	90.8±11	(1.4)	403.5±248	(5.9)
HW-1	376.8±72	(5.7)	3079.5±553	(45.4)
HW-5	329.3±70	(5.0)	2410.1±391	(35.5)

Values represent free Trp content in leaves (nmol g^{-1} fresh leaves) and seeds (nmol g^{-1} dry seeds) as means±SD for three leaves or seeds. Kusahonami; non-transgenic Kusahonami, HW-1 and 5; transgenic Nipponbare.

[a] Numbers in parentheses represent values relative to wild type Kusahonami.

Free Trp content in leaves and seeds of transgenic lines

Table 2 summarizes the free Trp content in leaves and seeds of transgenic plants of Kusahonami (R2 generation) grown in a greenhouse. For comparison the data of two transgenic Nipponbare plant lines (Wakasa et al. submitted) also are shown. Free Trp content in leaves of transgenic Kusahonami varied from 90.8±11 to 28709.4±1037 (nmol g^{-1} fresh leaves), which was more than 400 times greater than that of wild type (66.5±5.7 nmol g^{-1} fresh leaves). Free Trp content in seeds varied from 403.5±248 to 21438.1±1232 (nmol g^{-1} dry seeds), which was at most more than 300 times greater than that of wild type (67.9±24 nmol g^{-1} dry seeds). The content of free Trp through R1 seeds to R2 seeds remained stable. Free Trp contents of transgenic Nipponbare plant

lines shown here was at most 376.8 ± 72 nmol g^{-1} fresh leaves and 3079.5 ± 553 nmol g^{-1} dry seeds that were comparable to transgenic Kusahonami. More recently, we found that transgenic Nipponbare plants expressing *OASA1D* gene accumulate up to 23705 ± 11681.2 nmol g^{-1} dry seed (Wakasa et al. submitted). These results are consistent with the report that high level accumulation of free Trp by transgenic rice and potato plants expressing *OASA1D* gene has been reported (Tozawa et al. 2001; Yamada et al. 2004).

Conclusions

The present study has revealed that transformation of Kusahonami, a cultivar for animal feeds, using the whisker-supersonic mediated gene transfer of PCR-amplified gene cassette of the *OASA1D*, a feedback-insensitive anthranilate synthase (AS) α-subunit gene of rice has the following advantages.

(i) No need for selection reagent but 5MT.

(ii) High transformation frequency comparable to whole plasmid transformation.

(iii) More than two times higher yield of low copy transgenic plants as compared with whole transformation.

(iv) No vector backbone and selectable marker gene are present in the genome of transgenic plants.

(v) High level of free Trp in leaves and seeds of transgenic plants; >400 and >300 times greater respectively as compared with the wild type.

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