Intergenic transformation of \textit{AtMYB44} confers drought stress tolerance in rice seedlings

Joungsu Joo$^1$ · Nam-Iee Oh$^1$ · Nguyen Hoai Nguyen$^1$ · Youn Hab Lee$^2$ · Yeon-Ki Kim$^2$ · Sang Ik Song$^2$ · Jong-Joo Cheong$^1$

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Abstract We transformed rice (\textit{Oryza sativa L. Japonica} cv. Ilmi) calli with the \textit{Arabidopsis} transcription factor gene \textit{AtMYB44} using \textit{Agrobacterium}-mediated transformation. The T-DNA construct to be transformed contained \textit{tflA} cDNA (encoding a toxoflavin lyase) as a selectable marker. Since toxoflavin is a photosensitizing phytotoxin, transgenic plantlets were selected based on their capacity for root development on medium containing this toxin in the light. Homozygous lines were selected by determining the segregation patterns, expression levels, and copy numbers of \textit{AtMYB44}. Intergenic genomic locations of the inserted T-DNA in the three transgenic lines were confirmed by adaptor-ligation polymerase chain reaction and analysis using FSTVAL (http://bioinfo.mju.ac.kr/fstval/), an open-access web tool used to localize the flanking sequences of the transgene. Drought tolerance of young seedlings of the transgenic lines was determined based on the recovery of wilted leaves by re-watering after 3 days of water deprivation in a 105-well (35W × 35L × 45D mm/per well) plate. The three transgenic lines showed average survival rates of 80.4, 93.5, and 72.6%, respectively, whereas wild-type plants failed to recover after re-watering. Thus, the transgenic rice plants exhibited significantly enhanced tolerance to drought stress, as was shown previously in \textit{AtMYB44}-overexpressing transgenic \textit{Arabidopsis} and soybean. These results suggest that \textit{AtMYB44} activates a drought tolerance mechanism that is conserved in both monocotyledonous and dicotyledonous plants.

Keywords \textit{AtMYB44} · Drought tolerance · Intergenic transformation · Rice · Toxoflavin lyase selection · Transcription factor

Introduction

Climate change caused by global warming is expected to cause severe damage to agricultural productivity in the near future [1]. In particular, young rice seedlings are extremely susceptible to drought stress, primarily due to their high water requirement. Developing new rice varieties with enhanced drought tolerance represents an effective way to cope with the upcoming environmental disaster. Transforming rice plants with genes that control the cellular processes underlying drought tolerance is an attractive strategy for improving rice production [2]. Candidate genes for transformation include genes for transcription factors that regulate the expression of drought tolerance genes. For instance, MYB transcription factor genes from various sources have been reported to enhance drought tolerance in transgenic rice [3–5].

The \textit{Arabidopsis} transcription factor AtMYB44 plays an important role in abscisic acid (ABA)-mediated signaling, which enhances stomatal closure to reduce water loss from the leaves [6, 7]. Transgenic \textit{Arabidopsis} overexpressing \textit{AtMYB44} exhibits enhanced drought/salt-stress tolerance,
likely due to the suppression of genes encoding a group of Ser/Thr protein phosphatase 2Cs (PP2Cs) that negatively regulate ABA signaling [6]. In addition, soybean plants transformed with AtMYB44 also exhibit enhanced drought/salt-stress tolerance [8]. Recently, AtMYB44 was found to physically interact with the ABA receptor proteins PYLs, which bind to PP2Cs, suggesting that AtMYB44 is directly involved in early ABA signaling [9–11].

We transformed Ilmi, one of the most popular commercial varieties of rice in Korea, for direct commercial use, with the AtMYB44. In addition, we employed a novel light-dependent selection marker system in the transformation procedure, which provides a robust non-antibiotic and non-herbicidal selection marker system. We obtained three homozygous lines of transgenic rice plants exhibiting high levels of expression and the appropriate intergenic location of AtMYB44. The transgenic rice plants exhibited significantly enhanced tolerance to drought stress compared with wild-type plants, as seen previously in transgenic Arabidopsis and soybean overexpressing the AtMYB44 gene. These results suggest that AtMYB44 interacts with target gene promoters, activating a tolerance mechanism that is conserved in both monocotyledonous and dicotyledonous plants. The genetically modified rice lines developed in this study should be useful for confronting the forthcoming disaster in agriculture caused by global warming and climate change.

Materials and methods

Plant materials

Oryza sativa L. japonica cv. Ilmi was used throughout this study as the wild-type rice plant. Seeds were germinated in Murashige and Skoog (MS) agar medium containing vitamins (Duchefa, Haarlem, the Netherlands) and 3% sucrose at 28 °C for 3 days without light. Germinated plantlets were grown further in a growth chamber maintained at 28 °C for 2 days under long-day lighting conditions (16 h light/8 h dark). To grow to mature plants, 5-day-old rice plantlets were transplanted and grown in a greenhouse under natural sunlight. For seed propagation to the next generation, germinated rice seedlings were transplanted and grown in a field approved for genetically modified (GM) plants at the Kyungpook National University Farm (Gunwi, Korea).

T-DNA construction

Full-length AtMYB44 (At5g67300) cDNA (EST 119B8) was obtained from the Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org). The cDNA was amplified by polymerase chain reaction (PCR) and inserted between the XbaI and KpnI sites on the multiple cloning site (MCS) of a modified pCAMLA vector, a kind gift from Dr. Ingyu Hwang of Seoul National University, Korea (Fig. 1A). pCAMLA vector was modified from the pCAMBIA1300 vector (Cambia, Canberra, Australia) by inserting the 35S-MCS(pUC19)-nos cassette of pBI121 (Takara Korea, Seoul, Korea). The tflA gene [12] was then replaced with the hpt gene to use as a marker for selecting toxoflavin-resistant plant transformants. Nucleotide sequence between the right border (RB) and left border (LB) was determined (Supplementary Figure 1) using the primer sets listed in Supplementary Table 1. The plasmid containing the T-DNA construct was introduced into Agrobacterium tumefaciens LBA4404.

Fig. 1 Transgenic rice plant. (A) DNA construct for rice transformation. The full-length AtMYB44 (At5g67300) cDNA (EST 119B8) was inserted between XbaI and KpnI sites in the multiple cloning site (MCS) of the pCAMLA vector. Nucleotide sequence between the right border (RB) and left border (LB) was determined (Supplementary Figure 1) using the primer sets listed in Supplementary Table 1. (B) Real-time PCR for expression of AtMYB44 in transgenic rice plants. The rice ubiquitin gene OsUBI1 was used as a control to normalize the expression data. Y-axis represents relative values compared to the expression level in 13-1 line plants (13-1; =1). (C) Southern blot analysis of transgenic rice plants. Genomic DNA isolated from T3 plants was digested with BamHI, separated on a 0.8% agarose gel, and transferred to a nylon membrane. The blots were probed with C-terminal side-specific primer AtMYB44 cDNA [6] integrating digoxigenin (DIG)-labeled nucleic acids.
Rice transformation

Embryogenic calli were generated from the scutellum of mature rice seeds of Ilmi as described previously [13]. Briefly, 200 hulled rice seeds were sterilized with 100 mL of 70% ethanol, shaken vigorously for 5 min, and 100 mL of 2% NaOCl solution was added for 15 min. The seeds were washed with sterile water five times, transferred to 2N6 callus-induction medium [13], and incubated at 28 °C for 4 weeks in the dark. We modified the composition of the 2N6 medium slightly by dissolving 4 g Chu (N6) basal salt mixture (Sigma-Aldrich, St. Louis, USA), 0.3 g casamino acid, 30 g sucrose, 2 mg 2,4-D, 0.5 g L-proline, 0.5 g L-glutamine, and 2.5 g Phytagel in 1 L of solution (pH 5.8). Pawar et al. [14] reported that proline and glutamine improve callus induction and subsequent shooting in rice.

Rice calli 1–3 mm in size were selected and cultured on fresh 2N6 solid medium for 4 days. The calli were infected with Agrobacterium harboring the DNA construct for 30 min. The infected calli were transferred to 2N6 solid medium supplemented with 250 mg/L cefotaxime (2N6-C), and grown at 28 °C for 2 weeks. Well-grown calli with no brown color were selected and transferred onto 2N6 solid medium supplemented with 250 mg/L cefotaxime and 19 mg/L toxoflavin (2N6-CT) and grown at 28 °C for 2 weeks under continuous illumination. Toxoflavin (xanthothricin; 1,6-dimethyl-pyrimido[5,4-e]-1,2,4-triazine-5,7(1H,6H)-dione) was purchased from Sigma-Aldrich. Well-grown calli exhibiting yellowish white coloration were selected for further experiments.

Regeneration of the selected calli was proceeded on MS solid medium supplemented with 250 mg/L cefotaxime and 10 mg/L toxoflavin (MS-CT) at 28 °C for 3 weeks under light conditions. The calli were then grown on MS regeneration medium containing 10 mg/L toxoflavin (MS-T) for 9 weeks, transferring to fresh medium every 3 weeks. The plantlets regenerated from calli were transferred to MS solid medium without 1-naphthaleneacetic acid or kinetin for 10 days of adaptation. The plantlets were immersed in water for 1 day and then transplanted to soil for subsequent growth in a greenhouse.

Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR), first-strand cDNA synthesis was performed using 5 μg of total RNA as a template and oligo(dT)18 primers using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Korea, Seoul, Korea) according to the manufacturer’s instructions. A one-third dilution of the cDNA synthesis reaction mixture was used, and 1 μL of the diluted cDNA mixture was added as template for subsequent real-time PCR analyses using the 2× real-time PCR Pre-Mix with EvaGreen (SolGent, Daejeon, Korea). Thermocycling and fluorescence detection were performed using a Mx3000p Real-Time PCR machine (Agilent, Santa Clara, CA, USA). PCRs were conducted at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Rice ubiquitin gene OsUBI1 was used as a control to normalize the expression data.

Genomic localization

The genomic location of the insert DNA was determined by the adaptor-ligation-based PCR method. Briefly, 500 ng of genomic DNA isolated from plants was digested with 2U of HaeIII or MspI and ligated to 50 pmol of the blunt-end adaptor AP1 [15] using 5U of T4 DNA ligase (Takara Korea, Seoul, Korea) in a 20-μL reaction volume at 37 °C for 1 h. PCR amplification was conducted using two primer sets specific for the transformed T-DNA (Supplementary Table 1) and the adaptor Ada1 [15].

The first PCR was conducted in a 20-μL reaction volume containing 1 μL of the ligation reaction mixture and PCR pre-mixture containing 0.5 pmol of each primer (AP1 and LB1 or RB1). PCRs were performed in a MJ Research PTC-200 thermal cycler and conducted as follows: initial denaturation at 95 °C for 5 min, 20 cycles of 94 °C for 30 s, and 1 min at 72 °C, followed by a final elongation step at 72 °C for 10 min. The second PCR was conducted using a different set of primers (AP2 and LB2 or RB2) with 5 μL of the first PCR product under the following conditions: initial denaturation at 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 30 s at 60 °C, and 1 min at 72 °C, followed by a final elongation step at 72 °C for 10 min.

Amplified PCR products with a minimum length of 30 bp, including the border, vector, and adaptor sequences, were isolated from a 1% agarose gel using a HiYield Gel/PCR DNA Extraction Kit (Real Biotech Corp., Banqiao City, Taiwan) and sequenced with the ABI3730XL analyzer (Applied Biosystems Inc., Foster City, CA, USA) using LB2 or RB2 primers. Genomic locations of the flanking sequences were predicted using the Flanking Sequence Tag Validator (FSTVAL, http://bioinfo.mju.ac.kr/fstval/), an open-access web tool for validating transgene flanking sequences [16].

Southern blot analysis

For genomic Southern blot analysis, approximately 10 μg of genomic DNA was digested with BamHI, separated on
0.8% agarose gels, and transferred to nylon membranes. The blots were probed with the C-terminal side-specific primer AtMYB44 cDNA [6], integrating digoxigenin (DIG)-labeled nucleic acids (Roche Applied Science, Mannheim, Germany).

**Drought tolerance test**

To investigate the drought tolerance of the rice lines, transgenic T2 seeds were germinated on toxoflavin selection medium, while non-transformant seeds were germinated on MS agar medium 4 days after the transgenic lines were planted to minimize the disparity in developmental stage. Germinated plantlets were transplanted to soil and grown in a greenhouse (16-h-light/8-h-dark cycle) at 28–30°C. Twenty-eight seedlings per rice line were grown in a 105-well (35W x 35L x 45D mm/per well) plate (one plant per well) for 4 weeks before exposure to drought stress, as described previously [17]. The plants were deprived of water, and the soil was air-dried for 3 days, followed by re-watering. The number of plants that survived and continued to grow was counted after 25 days.

**Results**

**Gene transformation**

Rice calli generated from the scutellum of mature Ilmi rice seeds were co-cultivated with *Agrobacterium* containing a DNA construct harboring the AtMYB44 gene and a toxoflavin lyase (tflA) gene as a selectable marker (Fig. 1A). Out of 2800 calli, 1670 calli well grown on 2N6-C solid medium were transferred onto 2N6-CT selection medium to grow at 28 °C for 2 weeks under light conditions. Of the 1670 calli, 395 with yellowish white coloration were selected for further experiments. Seventy-three plantlets regenerated from the calli were transferred to MS solid medium and transplanted into soil in plastic pots (105W x 95L mm) for growth in a greenhouse. Among the 73 regenerated plantlets, 59 individuals yielded 50–100 T1 seeds, which were subsequently dried.

The well-dried T1 seeds were germinated on MS solid medium containing 10 mg/L toxoflavin and cultivated for 3 days in the dark and 4 days in the light. T2 seeds of each line were segregated into next generation, and stable integration of the transgene AtMYB44 was examined by PCR amplification (data not shown). All T3 plants of four T2 lines (2, 9, 13, and 14) yielded AtMYB44 PCR fragments, identifying these lines as homozygotes. Quantitative real-time PCR revealed that AtMYB44 was expressed in all the transgenic lines which survived in the toxoflavin-tflA selection system (Fig. 1B). Based on the expression level, sublines 2-1, 9-2, 13-1, and 14-2 were selected for further experiments. Southern blotting indicated that T3 plants of the sublines 2-1, 9-2, and 13-1 contained a single copy of the transgene (Fig. 1C). For 9-2 line, the upper band at 23-kb position appears to be an artifact, when considering its size and DIG intensity.

Germination of non-transformants (wild type) was unaffected upon growth on MS solid medium containing 10 mg/L toxoflavin, but root development was affected significantly (Fig. 2A). Consequently, transgenic plantlets were selected based on their tolerance to the toxoflavin-containing medium as demonstrated by root development. Eight sublines of each T2 seed were germinated on toxoflavin selection medium as described above. Sublines

![Fig. 2 Toxoflavin-tflA selection. (A) Growth of non-transformed wild-type rice plants on MS solid medium without (MS) and with (MS + Tx) 10 mg/L toxoflavin. (B) Growth of transgenic rice plants (T2 sublines on MS medium containing 10 mg/L toxoflavin. Well-dried T2 and none-transformant (NT) seeds were germinated in dark conditions for 3 days and grown in light conditions for 4 days. Toxoflavin tolerance was determined based on root development.](image-url)
from lines 2, 9, 13, and 14 showed positive rates higher than 90%, whereas those from negative line 8 showed a lower rate (Fig. 2B). The selected plantlets were transplanted and grown in a field to obtain seeds for the next generation.

**Genomic localization**

We examined whether the transformed gene was inserted in an intergenic location so as not to alter the expression of surrounding genes. The genomic location of the insert DNA was determined by flanking sequence analysis. Genomic DNA isolated from plants was digested with HaeIII or MspI and ligated to an adapter. PCR products using primer sets specific for the T-DNA and the adaptor were isolated from an agarose gel and sequenced (Fig. 3).

Using a primer designed from the LB sequence, two bands were obtained from line 2-1 plants. FSTVAL analysis [16] revealed that the two inserted sequences were located 123 bp apart from each other at 12.855 and 12.978 kb upstream of the hypothetical conserved gene Os06t0576600-00 (Table 1). Nucleotide sequences of the fragments (Supplementary Figure 2) suggested that the LB sides of the two fragments are contiguous with one another in an opposite direction. No PCR product was obtained when the primer designed from the RB-side sequence was used.

In case of plants from line 9-2, a single band was obtained when amplifying with a primer designed from the LB-side sequence. FSTVAL analysis suggested that the insert was located 4.818 kb upstream of the gene Os05t0515200-01, which encodes a cytochrome P450 family protein. PCR amplification revealed two distinct bands when the primer designed from the RB sequence was used, but we failed to determine their sequences, possibly due to modification of the RB-side sequence of the insert.

When primers designed from either the LB- or RB-side sequence were used with plants from line 13-1, a single PCR product was obtained. FSTVAL analysis suggested that the inserted sequence was located approximately 6.7 kb upstream of Os02t0313400-01, which encodes an apoptosis inhibitory 5 family protein.

FSTVAL analysis of plants from line 14-2 indicated that two inserted sequences were located approximately 0.062 kb upstream of Os03t0421700-00 and 0.827 kb upstream of Os06t0611900-01, respectively. Therefore, we conclude that line 14-2 contains duplicated transgenes which were inserted in promoter regions located on different chromosomes. Thus, the inserted T-DNAs have likely disturbed the promoters of the two adjacent genes.

**Phenotypes of transgenic plants**

T2 transgenic rice seeds were sown and cultivated in a field approved for GM plants. During the early stage of vegetative growth, the transgenic plants were dwarfed compared with wild-type Ilmi plants, as observed previously for AtMYB44-transformed *Arabidopsis* [6]. When fully grown, the average culm length was 59 cm for subline 2-1 plants and 65 cm for subline 9-2 plants, while that of wild-type plants was 68.8 cm. Line 13-1 plants were 74 cm in height, which is comparable to wild-type plants. The extent of growth retardation was correlated with the expression level of AtMYB44 in the transgenic plants (Table 2).

We investigated the agronomic characteristics of T2 plants when they were harvested to obtain T3 seeds. For most lines, the panicle length, number of panicles per plant, number of spikelets per panicle, and total number of spikelets were comparable to those of wild-type Ilmi plants (Table 2). For T3 seeds, the grain-filling rates (number of filled grains/total number of spikelets) of sublines 2-1, 9-1, 9-2, and 13-3 were comparable (higher than 90%) to that of wild-type plants (92.6%).

Using T2 sublines germinated on toxoflavin selection medium, drought tolerance of the transgenic rice plants was compared with that of untransformed control Ilmi plants. The untransformed control seeds were germinated on MS medium 4 d after those of the transgenic lines to minimize
Table 1 The genomic location of the inserted DNA determined by analysis of the flanking sequences

| Query name | Length (bp) | Chr. no. | Chr. start | Chr. end | Type     | Gene_ID (location) | Description (gene) |
|------------|-------------|----------|------------|----------|----------|--------------------|-------------------|
| 2-1        | 284         | Chr 06   | 23252584   | 23252867 | Intergenic | Os06t0576600-00 upstream 12.855 kb | Hypothetical conserved gene |
| H3_LA      |             |          |            |          |          |                    |                   |
| 2-1        | 141         | Chr 06   | 23252261   | 23252321 | Intergenic | Os06t0576600-00 upstream 12.978 kb | Hypothetical conserved gene |
| H3_LB      |             |          |            |          |          |                    |                   |
| 9-2        | 151         | Chr 05   | 25635469   | 25635619 | Intergenic | Os05t0515200-01 upstream 4.818 kb | Cytochrome P450 family protein |
| H3_L       |             |          |            |          |          |                    |                   |
| 13-1       | 412         | Chr 02   | 12393338   | 12392927 | Intergenic | Os02t0313400-01 upstream 6.715 kb | Apoptosis inhibitory 5 family protein |
| H3_L       |             |          |            |          |          |                    |                   |
| 13-1       | 63          | Chr 02   | 12393346   | 12393408 | Intergenic | Os02t0313400-01 upstream 6.707 kb | Apoptosis inhibitory 5 family protein |
| M_R        |             |          |            |          |          |                    |                   |
| 14-2       | 54          | Chr 03   | 18190642   | 18190695 | 5'-Upstream-1000 | Os03t0421700-00 upstream 0.062 kb | Similar to fumarylacetoacetate hydrolase domain-containing protein 1 |
| H3_LA      |             |          |            |          |          |                    |                   |
| 14-2       | 155         | Chr 06   | 25281209   | 25281055 | 5'-Upstream-1000 | Os06t0611900-01 upstream 0.827 kb | Similar to glycine decarboxylase P subunit |
| M_R        |             |          |            |          |          |                    |                   |

*a* Numerals, subline number; H3, *Hae*III digest; M, *Msp*I digest; L, LB-side primer; R, RB-side primer; A, upper band; B, lower band

*b* Length of flanking sequence matched to the rice genome sequence database (Supplementary Figures 2)
the disparity in developmental stage. The 4-week-old plants wilted after 3 days of water deprivation (Fig. 4). Re-watering the wild-type plants for 25 days did not rescue the wilted plants, whereas transgenic T2 lines 2-1, 9-2, and 13-1 showed significantly enhanced drought tolerance based on the recovery of wilted leaves, with average survival rates of 93% (52 out of 56 plants tested), 100% (36 out of 36), and 92% (36 out of 39), respectively. A total of 124 out of 131 transgenic T2 plants showed significantly enhanced drought tolerance.

Discussion

We transformed the Arabidopsis transcription factor gene AtMYB44 into rice plants. The transgenic rice plants exhibited significantly enhanced tolerance to drought stress compared with wild-type plants (Fig. 4), as observed previously in AtMYB44-overexpressing transgenic Arabidopsis [6] and soybean [8]. These results suggest that AtMYB44 interacts with specific sequences in target gene promoters and/or specific proteins and activates a tolerance mechanism that is conserved in dicotyledonous (Arabidopsis, soybean) and monocotyledonous (rice) plants. The two species contain homologous molecular components that activate the same specific cellular mechanism, including transcription factor(s) with identical roles, as well as the same cis-elements on the target gene promoters. AtMYB44 contacts its specific binding site, AACnG (where n indicates A, G, C, or T), in target gene promoters to regulate their expression [18]. Phosphorylation is reportedly required for the biological function of AtMYB44, suggesting that the control of AtMYB44 is tightly linked to mitogen-activated protein kinase cascade signaling [19, 20]. AtMYB44 physically interacts with the ABA receptor PYL8 [9, 10] and PYL9 [11], suggesting that AtMYB44 is involved in early ABA signaling. PYL proteins are ABA receptors that bind to and inhibit PP2Cs in an ABA-dependent manner [21, 22]. In a recent study of the drought tolerance response in rice, interactions between OsPYL members and OsPP2Cs were also detected [23].

In typical transformation experiments, a selectable marker gene is inserted into the DNA construct used for

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Table 2 Phenotypes of the T2 transgenic plants and T3 seeds

| Subline | T2 plants | T3 seeds |
|---------|-----------|----------|
|         | CL (cm)   | PL (cm)  | NP  | NSP | TNS   | NFG | FR (%) | TGW (g) | 1000GW (g) |
| NT      | 68.8 ± 3.0 | 18.8 ± 1.1 | 11.4 ± 1.5 | 104.0 ± 13.1 | 1184.9 ± 188.4 | 1099.2 ± 187.4 | 92.6 ± 2.6 | 25.9 ± 5.6 | 23.5 ± 1.9 |
| 2-1     | 59.0 ± 1.7 | 18.3 ± 0.6 | 9.7 ± 0.6 | 119.9 ± 8.9 | 1159.3 ± 122.1 | 1046.0 ± 126.0 | 90.1 ± 1.7 | 22.6 ± 3.8 | 21.6 ± 1.6 |
| 9-2     | 65.0 ± 4.2 | 19.0 ± 1.4 | 13.0 ± 1.4 | 96.0 ± 12.0 | 1239.5 ± 20.5 | 1151.0 ± 18.4 | 92.9 ± 0.1 | 26.0 ± 2.3 | 22.5 ± 1.6 |
| 13-1    | 74.0 ± 1.0 | 18.3 ± 0.6 | 13.0 ± 1.0 | 102.1 ± 3.9 | 1328.7 ± 125.5 | 1185.3 ± 126.2 | 89.1 ± 1.2 | 25.3 ± 3.6 | 21.3 ± 0.9 |

Six individual plants were selected from each subline, and the average values and standard deviations are presented. 

CL culm length (cm), PL panicle length (cm), NP number of panicles per plant, NSP number of spikelets per panicle, TNS total number of spikelets per plant, NFG number of filled grains per plant, FR filling rate (NFG/TNS, %), TGW total grain weight per plant (g), 1000GW 1000-grain weight (g)

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Fig. 4 Drought tolerance test. Seedlings from each rice line were grown in a 105-well (35W × 35L × 45D mm/each well) plate (one plant per well) for 4 weeks before exposure to drought stress. The plants were not watered for 3 days, and the number of plants that survived was counted 25 days after re-watering. Numbers in parentheses indicate the number of plants that survived/tested in two independent experiments.
transformation, leading to preferential growth of transformed cells on selection medium. In general, antibiotic- or herbicide-resistance genes are used as markers. The presence of such genes in GM crops has generated a number of environmental and consumer concerns [24–26], despite scientific evidence supporting the safety of GM crops. In this study, we employed the tflA-toxoflavin selection system [12, 27] and selected transgenic plantlets based on their root development on selection medium (Fig. 2B).

Toxoflavin is a major virulence factor in bacterial rice grain rot caused by Burkholderia glumae [28]. Toxoflavin has phytotoxic effects on many plant species by producing reactive oxygen species in the presence of light [29, 30]. The tflA gene encodes a toxoflavin-degrading enzyme in Paenibacillus polymyxa, a bacterium isolated from healthy rice seeds [12]. The use of selectable marker originated from a non-pathogenic microorganism will help alleviate public concerns over the safety of environment and food derived from transgenic crops.

The expression of transgenes in gain-of-function transgenic plants or plants produced using a reverse genetic approach should be analyzed in terms of genomic context (i.e., genic and intergenic regions). The transformed gene should be inserted in an intergenic location so as not to alter the expression of surrounding genes. To precisely predict the genomic location of the transgene, we isolated the flanking regions of the T-DNA in the AtMYB44 transgenic rice plant lines using the adaptor-ligation PCR method [15], sequenced these regions, and analyzed them using the open-access web tool, FSTVAL [16]. The results indicate that the insertion sites in lines 2-1, 9-2, and 13-1 are intergenic (Table 1). Using a primer designed based on the LB sequence, two bands were obtained from line 2-1 plants (Fig. 3). Analysis of the nucleotide sequences of the fragments (Supplementary Figure 2) suggested that the LB sides of the two fragments are contiguous with one another in an opposite direction. Southern blot analysis (Fig. 1C) revealed that plants from line 2-1 contain a single copy of the transgene. Thus, it appears that one of the two inserts was severely modified and lacks a part of the AtMYB44 sequence. For plants from lines 2-1 and 9-2, we failed to obtain PCR product when the primer designed from the RB-side sequence was used, and thus, this sequence may have been modified during the insertion process. In the current study, PCR amplification was more efficient using primers from the LB-side sequences than from the RB side, which is consistent with a previous report [15]. Border (LB or RB) sequences are thought to be extensively modified when inserted into the genome during transformation [31]. It appears that line 14-2 contains duplicated transgenes, which were inserted in promoter regions located on different chromosomes, and thus, the T-DNA insertions have likely disturbed the promoters of the two adjacent genes, respectively.

We transformed Ilmi, one of the most popular commercial varieties of rice certified by the Korean government. The Korean word ilmi means ‘best taste.’ Thus, it would be possible to commercialized the transgenic rice breed developed in this study without (or with) further backcrossing with other popular varieties to improve the agricultural traits of the new variety. Before then, however, further studies are needed to determine the detailed agronomic characteristics of the GM rice plants and to assess their safety for commercial use.

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