Isolation and Characterization of *GAMYB* Mutant Rice

Yoshito TANAKA[1], Kouichi NAKANO[1], Toshiaki MITSUI[2,3], Hidetaka HORI[1] and Kimiko ITOH[1,3*]

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Summary

A R2R3-type MYB transcription factor, GAMYB, activates gibberellin inducible α-amylase gene transcription during cereal seed germination. Recent studies in several plant species suggested that the GAMYB also functions in other organs includes floral meristems, leaf, stem, flower, anther development, and floral transition. We isolated a *GAMYB* mutant in rice, designated *GAMYB*^{m}. *GAMYB*^{m} showed delay of germination, charasteristic dwarfism, partial defects of inflorescence formation and seed fertility and the *OsGAMYB* expression was decreased in GA-treated embryoless half seeds. Genomic and cDNA structure of the *GAMYB*^{m} gene showed that multiple point mutations existed along genomic region of the *OsGAMYB*.

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Gibberellin (GA) promotes *de novo* synthesis of hydrolytic enzymes at scutellar epithelial cells and aleurone layer during cereal seed germination. An α-amylase is one of major hydrolytic enzymes for starch degradation, and the expression is activated by GA at both transcriptional and translational levels. An R2R3-type MYB transcription factor, GAMYB, was identified in barley as a transcription factor that activates GA regulated α-amylase transcription (Gubler *et al*., 1995). Thereafter, the genes of GAMYB were also isolated in other plant species, rice, *Lolium temulentum*, and *Arabidopsis* (Gubler *et al*., 1995; Gubler *et al*., 1997; Gocal *et al*., 1999; Gocal *et al*., 2001).

Recent studies showed that the GAMYB also expressed various organs (Gocal *et al*., 1999; Kaneko *et al*., 2003; Gocal *et al*., 2001) and implicated multiple roles for plant development (Murray *et al*., 2003; Palatnik *et al*., 2003; Kaneko *et al*., 2004; Achard *et al*., 2004; Millar and Gubler 2005). In barley, transgenic plant carrying GAMYB:GFP fusion gene showed GFP expression in developing anther and the anther showed abnormal phenotype (Murray *et al*., 2003). In *Arabidopsis, GAMYB-like* genes, *MYB33*, *65,* and *101* were analyzed for the temporal and spatial expression and both *MYB33* and *MYB65* were predominantly expressed in inflorescence and floret (Gocal *et al*., 2001). The *MYB33* and *MYB65* were functionally redundant and the double knockout mutant showed conditional defect of anther development in *Arabidopsis* (Millar and Gubler, 2005). The *MYB33* is also involved in leaf morphogenesis through microRNA, miR159, regulated gene expression, and overexpression of *MYB33* carrying substitution mutation at their miR159-guided cleavage site led to abnormal leaf phenotype in *Arabidopsis* (Palatnik *et al*., 2003). Moreover, constitutive expression of miR159 suppressed floral transition under long-day condition in 35S::miR159 transgenic *Arabidopsis* (Achard *et al*., 2004). In *Lolium*, spatial and temporal regulated *LtGAMYB* expression was correspondingly induced with long-day induced the floral organ differentiation (Gocal *et al*., 1999).

In rice, null mutants of *GAMYB, gamyb-1, 2*, and *3*, by *Tos17* insertion were analyzed and their loss-of-function mutation caused defects of a GA inducible α-amylase gene expression, 1st internode elongation, floret and anther development. There are no changes of floweing date, leaf, root, and number of primary rachis branch in the null mutants, although the *GAMYB:GUS* fusion gene also expressed in other organs, root, and primodial of primary rachis branch (Kaneko *et al*., 2004).

We isolated mutant showed altered *OsGAMYB* expression in rice, *GAMYB*^{m}, and the mutant showed characteristic dwarfism, and short inflorescence. We analyzed the phenotype, and the genomic, and cDNA structures of *OsGAMYB* in the mutant.

MATERIALS AND METHODS

Plant materials

Oryza sativa L. cv. Toride-1 was used as control. *ΔGAMYB* was isolated from *Ac* tagged rice stocks. Screening of pooled DNA from 60, 000 independent *Ac* tagged rice were

[1]Graduate School of Science and Technology, Niigata University

[2]Faculty of Agriculture, Niigata University

[3]Center for Transdiciplinary Research, Niigata University

*Corresponding author: kimi@agr.niigata-u.ac.jp

performed by TAIL-PCR with the pairs of the *OsGAMYB* specific primers and the *Ac* specific primers. 18 plants were obtained from approximately 30,000 *Ac* tagged plants by first screening, these plants had *Ac* insertion into *OsGAMYB* locus. Then 72 self-pollinated seeds were obtained from the 18 lines (8 seeds/1 line) and used for second screening. Inheritance of *Ac* insertion at *OsGAMYB* locus was observed in 4 plants from 1 line. 4 plants had same *Ac* insertion at 5' UTR *OsGAMYB* (-161bp from translation initiation site) and designated *Δ GAMYB*. The four *Δ GAMYB* plants showed severe dwarfism and abortion. 8 BC1 plants were obtained from backcrossing between three *Δ GAMYB* lines and cv.Toride-1 and then the self-pollinated seeds (BC1S1) were used for screening of mutant plants showed moderate phenotype and restoration of fertility. Two BC_1S_1 plants, 9-8 and 9-9 were obtained from line 9 BC_1 plant and the 9-8 plant showed *dwarf* phenotype and the 9-9 plant showed *semi-dwarf* phenotype. Both 9-8 and 9-9 plants showed partial restoration of fertility and no *Ac* insertion at *OsGAMYB* locus. These mutant plants were named *GAMYB*^{m}.

Analysis of OsGAMYB expression

Embryoless half seeds of WT and *GAMYB*^{m} homozygote (BC1S5) were incubated in 10 mM acetate buffer (pH 5.2) with or without 10^{-7} M GA_3 for 24 hrs under 30°C. Total RNA was isolated from 60 embryoless half seeds (Gonzalez et al., 1980) and 4 ug of total RNA was used for northern hybridization. To detect *OsGAMYB* mRNA, 3' region of *OsGAMYB* cDNA was amplified by PCR with 5' -ATTATTGCACCTTTCGGGGG-3' and 5'-CGGCTT ATCTCCATGCACTACTTT-3' primers and used for template of the probe. To detect *RAmy1A* mRNA, 3'UTR region of *RAmy1A* cDNA was amplified by PCR with 5' -TGAGCGCACGATGACGAGACTCTCA-3' and 5' -AATTGCATCCGTAATTCGGA-3' primers and used for template of the probe. Northern hybridization and detection of the signal intensity were performed according to Tanno et al. (2005). Ribosomal DNA probe was used as control experiment. After deduction of background value, signal intensity was corrected on *18S rRNA* signal. Then the corrected signal intensities of three independent experiment was calculated and the average of the signal intensities was shown in **Fig. 2B**.

Structural analysis of genomic and cDNA of OsGAMYB

Genomic DNA was isolated from seedling of cv. Toride-1 and *GAMYB*^{m} and used for structural analysis. PCR amplified fragments of *OsGAMYB* genomic region were cloned into pGEM-T easy by TA cloning (Promega Co.) and sequenced. To analyze the structure of cDNA, total RNA was isolated from embryo at 3 days after germination according to Chirgwin et al. (1979). The region of *OsGAMYB* mRNA from 1 to 2294 nt was amplified by RT-PCR, and the fragment of the cDNA was cloned by TA cloning and sequenced.

RESULTS AND DISCUSSION

Isolation and genetic analysis of OsGAMYB mutant

We isolated a mutant, *Δ GAMYB*, carrying the maize transposable element *Ac* at the 5' UTR of *GAMYB* in rice. *Δ GAMYB* showed severe dwarfism, severe defects of inflorescence formation and abortion. Because of severe abortion of *Δ GAMYB*, we isolated fertile derivatives from *Δ GAMYB* /cv.Toride-1 BC1S1 population. Two BC1S1 plants, 9-9 and 9-8 were obtained and the 9-8 plant showed characteristic dwarfism and 9-9 plant showed semi-dwarfism, and both plants showed partial restoration of fertility **(Fig. 1A)**.

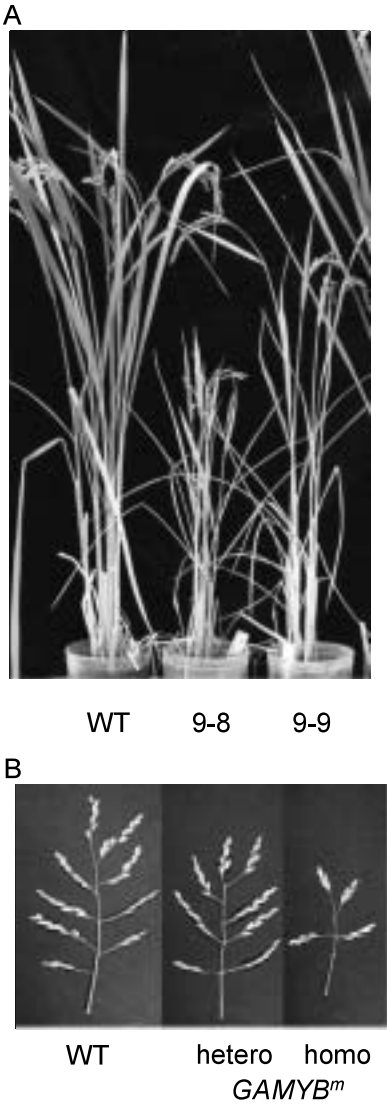

Fig. 1. Phenotype of *GAMYB*^{m} rice. (A) *GAMYB*^{m} shows dwarfism. WT, *O. sativa* L. cv. Toride-1; 9-8 and 9-9, *GAMYB*^{m} rice. (B) *GAMYB*^{m} showed defect of primary branch formation. Hetero, inflorescence of heterozygote of *GAMYB*^{m} (BC1S3); Homo, inflorescence of homozygote of *GAMYB*^{m} (BC1S3).

To test whether the mutant phenotype is stably inherited or not, offspring of line 9-8 and 9-9 were used for further analysis. The 20 and 24 seeds were obtained from 9-8 and 9-9 plants and grown for 150 days under natural light condition. A 3 to 1 segregation of *non-dwarf* (including *semi-dwarf* phenotype) and *dwarf* phenotype occurred in the BC1S2 plants from line 9-9. In contrast, no segregation occurred in the BC1S2 plants from line 9-8, and the offspring were uniformly *dwarf* (**Table 1**). These results indicated that the mutation of *OsGAMYB* stably inherited, and the 9-8 BC1S1 plant was homozygote and the 9-9 BC1S1 plant was heterozygote of the mutation. Therefore, the mutant gene may behave as a semi-dominant factor, and the *dwarf* plants were named *GAMYB*^{m}.

GAMYB involves in inflorescence formation in rice

One of the most characteristic feature of the *GAMYB*^{m} phenotype is partial defect of inflorescence formation (**Fig. 1B**). The result indicated that the GAMYB involves in inflorescence formation in rice. The mutant inflorescence showed short panicle and less primary branches. It suggested that early degeneration of the inflorescence meristem occurred in early stage of inflorescence formation, and then early transition from inflorescence meristem to floral meristem led to less branch formation in the mutant.

GAMYB^{m} *showed delay of germination*

OsGAMYB activates transcription of GA-inducible α-amylase gene, *RAmy1A*. Therefore, defect of *OsGAMYB* expression leads to delay of seed germination. To test growth rate of *GAMYB*^{m}, dry seeds were imbibed in sterile water and germinated for 6 days under 28°C, 14 hrs light and 10 hrs dark condition. Result showed that the shoot growth of *GAMYB*^{m} was slower than that of wild-type (**Fig. 2A**). Partial delay of germination may be caused by partial lack of GA responsiveness involving defect of *OsGAMYB* expression in *GAMYB*^{m}.

Table1. Segregation of the *dwarf* phenotype in offspring of *GAMYB*^{m}

BC1S1 line	*non-dwarf*	*dwarf*	no germination
9-8	0	13	7
9-9	17	5	2

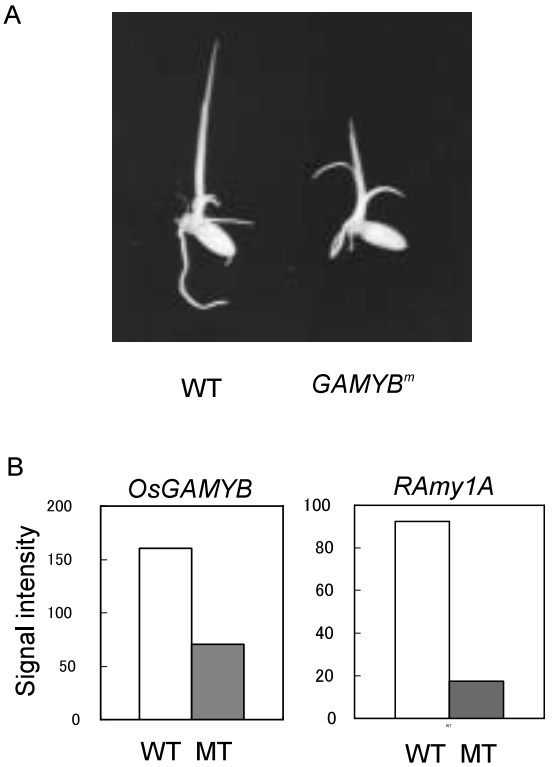

Fig. 2. Altered expression of *OsGAMYB* in *GAMYB*^{m}. (A) *GAMYB*^{m} showed delay of germination. (B) Quatntitative analysis of *OsGAMYB* and *RAmy1A* expression by northern blotting. WT, cv Toride-1; MT, homozygote of *GAMYB*^{m}.

OsGAMYB expression was altered in GA-treated embryoless half seeds of GAMYB^m

To clarify the causes of delay of germination, *OsGAMYB* expression was analyzed by northern blotting. Embryo (including scutellum tissues) provides endogenous gibberellin, and removal of the embryo allow to assess the GA-responsive *OsGAMYB* and *RAmy1A* transcription in remained embryoless half seed by exogenous GA_3 treatment. Embryoless half seeds of both mutant and wild type were treated with final concentration 10^{-7} M GA_3 in sodium acetate buffer (pH 5.2) for 24 hrs, and then total RNAs were isolated and used for the northern blotting. Quantitative data of the signal intensity was calculated and shown in **Fig. 2B**.

Results showed that the level of *RAmy1A* transcript decreased to approximately 20% in *GAMYB^m* (**Fig. 3B**, right) and the level of *OsGAMYB* transcript decreased to approximately 56% in *GAMYB^m* (**Fig. 3B**, left). These results showed that the mutant gene is a weak allele, but not the null allele, and partial defect of *GAMYB* expression led to partial defect of GA-responsiveness like as the decrease of GA-inducible *RAmy1A* expression.

Structural analysis of OsGAMYB gene

Genomic structure of *OsGAMYB* was analyzed in both WT and *GAMYB^m*. 13 point mutations were identified in the genomic region of *OsGAMYB* gene in *GAMYB^m*. Interestingly, both *Ac* insertion and target duplication were not found in the *GAMYB^m* gene and its 5' flanking region (**Fig. 3A**). All mutations did not exist in significant domain or *cis*-element, except A to G substitution mutation generated a lysine to arginin substitution at 271st amino acid (**Fig. 3B**). The the K271R substitution existed at the putative domain for negative regulation of GAMYB activity (Gubler *et al*., 1999; Washio *et al*., 2003).

Genomic structure of *OsGAMYB* consists of five exons and four introns (**Fig. 3A**). First reported cDNA structure consists of four exons and three introns (accession no. X98355: Gubler *et al*., 1995). Recently, characterization of the 27,000～ full length cDNA structures was accomplished by Rice Genome Project and revealed that three alternative forms of *OsGAMYB* mRNAs are expressed in callus or other organs (Kikuchi *et al*, 2003). Alternative splicing generates three splice variants. A first splice variant lacks all introns (accession no. AK119607), second splice variant lacks the 1st ～ 3rd introns and has the 4th intron (accession no. AK102841, X98355), and third splice variant lacks the 1st and 2nd introns and has 3rd and 4th introns (accession no. AK063951). These splice variants were found to be differentially distributed among organ and tissues. Therefore, these splice variants may be functionally differentiated in rice, and the several mutations in 1st and 2nd introns may affect splicing of *OsGAMYB* pre-mRNA in the mutant. Our data are not sufficient to identify the causal mutation, and further analysis by reverse genetics strategies are required for identification

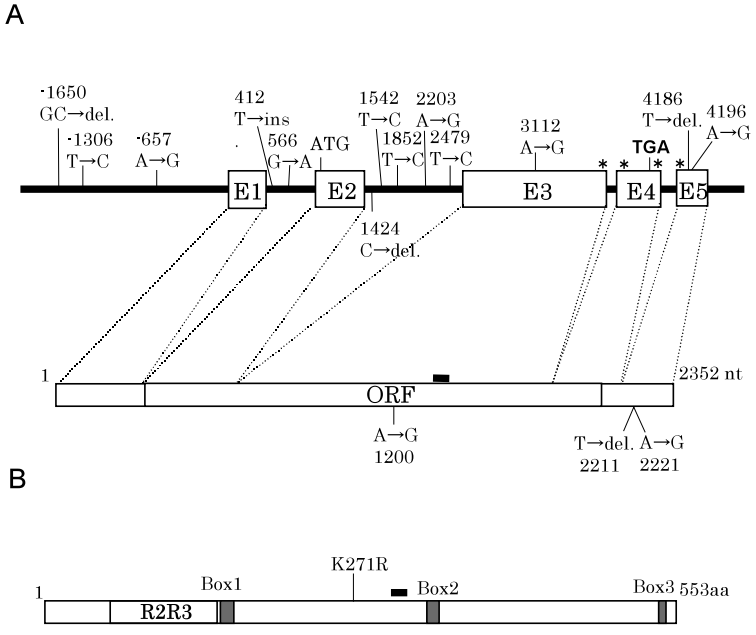

Fig. 3. Structure of *GAMYB^m* gene. (A) Map position of the point mutations in genomic and cDNA of *OsGAMYB* gene in *GAMYB^m*. Map position is based on X98355.1 (GenBank accession no.). (B) Protein structure of GAMYB in mutant. Box1～3, conserved region among HvGAMYB, AtMYB33, 65 and 101(Gocal *et al*., 2001); R2R3, DNA binding site; *black bar*, complementary sequence of *OsmiR159a* with 2 mismatches (Achard *et al*., 2004).

of causal mutation in the *GAMYB*^{m}.

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イネにおける GAMYB 突然変異体の単離と解析

田中義人[1]・中野浩一[1]・三ツ井敏明[2,3]・堀　秀隆[1]・伊藤紀美子[1,3*]

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要　約

R2R3タイプの MYB 様転写因子のひとつである GAMYB は、発芽期の単子葉穀類において、ジベレリン誘導型 α - アミラーゼの転写を活性化する。最近の他の植物における最近の研究によって、GAMYB が発芽種子以外の他の器官、花芽分裂組織、葉、茎、花、葯の発生や花芽形成に機能する事が示唆されている。私たちは、イネにおける *GAMYB* 突然変異体、*GAMYB*^{m} を単離した。*GAMYB*^{m} は、発芽の遅延、特徴的な矮性、花序形成の部分的欠損を示し、ジベレリン処理を行った無胚半切種子における *OsGAMYB* mRNA 蓄積量も低下していた。ゲノム DNA および cDNA 構造の解析によって、*GAMYB*^{m} 遺伝子に複数の点突然変異が生じている事が明らかになった。

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[1] 新潟大学大学院自然科学研究科

[2] 新潟大学農学部

[3] 新潟大学超域研究機構

* 代表著者 :kimi@agr.niigata-u.ac.jp