Establishment of High-amylose Type Japonica Rice by *Agrobacterium*-mediated Gene Transfer

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Summary

The *Wx* gene encodes a granule-bound starch synthase I (GBSSI) that plays a key role in the amylose synthesis. Previous study showed that introduction of W*x* transgene into null-mutant *wx* rice (*WxR/wx*) by direct gene transfer leads to both high- and low- amylose rice. In later generations of several transgenic *WxR/wx* lines, unstable and silenced endosperm phenotypes appeared. The unstable and silenced phenotypes are repeat-induced gene silencing caused by integration of multicopy transgenes (~11 copies per haploid genome). In order to solve the unstable phenotype emergence, binary vector pWAB was constructed and transferred into null-mutant *wx* by *Agrobacterium*-mediated gene transfer. Resultant transgenic rice, *WAB/wx* lines, carrying low copy number of the transgenes (~3 copies per haploid), showed high-amylose phenotype at the endosperm, and the phenotypes are stably transmitted to next generations.

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Key words : *Wx*, Granule-bound starch synthase I, Amylose, Japonica rice

Starch is a major component of cereal grains, and is mainly comprised of two types of α-glucans, amylose and amylopectin. The former is made of linear of slightly branched molecules and the latter is composed of highly branched molecules (Hizukuri *et al.* 2006). The ratio of these molecules affects the properties of processed starch in the food industry and the texture of cooked cereal grains. Therefore, control of the amylose content of starch is a major objective in cereal breeding.

In rice, *Wx* gene encoding a Granule-bound starch synthase I (GBSSI) is a key enzyme of amylose synthesis and wild-type allele of the rice *Wx* gene, Wx^a, also involves in synthesis of extra-long chain (ELC) of amylopectin (Hanashiro *et al.* 2008). Previous study has demonstrated that transgenic rice carrying Wx^a gene showed both high- and low-amylose rice (Itoh *et al.* 2003). In later generations of the transgenic lines, unstable and silenced endosperm phenotypes appeared. These transgenic rice carrying multiple copies of transgene (2-11 copies per haploid) resulting repeat-induced gene silencing. In order to solve the unstable silenced phenotype emergence, binary vector pWAB was constructed and transferred into null-mutant *wx* by *Agrobacterium*-mediated gene transfer. Resultant transgenic rice *WAB/wx* lines are analyzed by phenotypic assay in the pollen and the endosperm, and Southern blotting.

MATERIALS AND METHODES

Plant materials

All plants were cultivated at 28℃ day/ 26℃ night under photoperiodic condition 14.5 day/ 9.5 night in closed green house. *Oryza* sativa L. Japonica cv. Musashimochi(wx^{nmd}) was transformation. *O. sativa* L. Indica cv. Labelle, and Japonica cv. Nipponbare were used as controls.

Plasmid construction

A 6.1 kbp DNA fragment of partial digestion product of pWxR (Itoh *et al.* 1997) with restriction enzymes *Kpn*I/*Hin*dIII was obtained and was ligated into multiple cloning site of *Kpn*I/*Hin*d III digested pPZP2H-lac (Fuse *et al.* 2001). Resultant pWAB contains *Wx* promoter, Wx^a 1st intron, *Wx* cDNA and bacterial gene *nos* terminator. Predicted transcript and protein of *WAB* gene is identical to that of *WxR* and Wx^a (Itoh *et al.* 2003).

Rice transformation

Rice calli were initiated from mature dry seeds of cv. Musashimochi(wx^{nmd}) and were transformed by *Agrobacterium*-mediated gene transfer (Holster *et al.* 1978, Hiei *et al.* 1994, Terada *et al.* 2002). The copy numbers of the transgene per diploid of all transgenic lines were determined by Southern blot analysis and PCR analysis of the transgenes.

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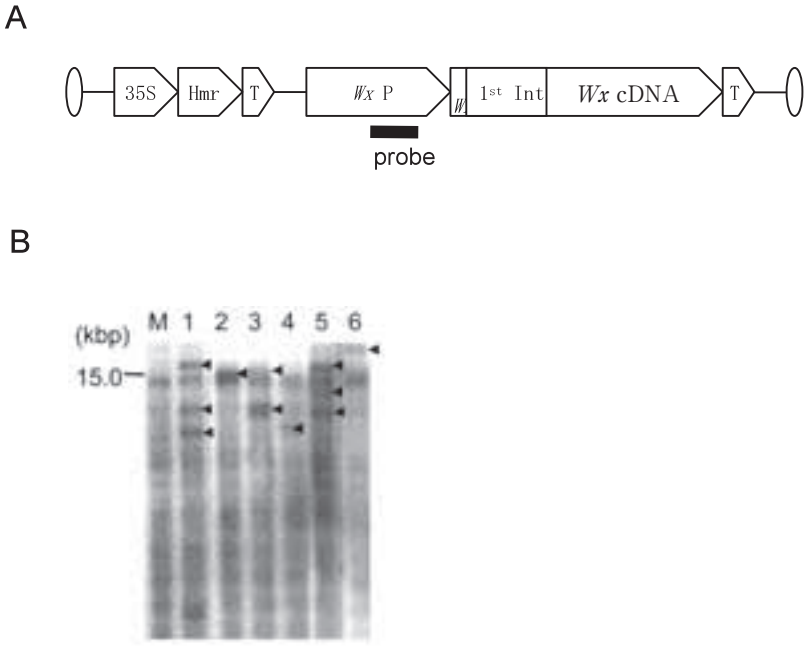

Fig. 1. Structure and integration of *WAB* gene in rice. **A**, Structure of T-DNA region of pWAB. 35S, Cauliflower Mosaic Virus 35S promoter; *Hm*^{r}, hygromycine phosphotransferase gene; T, terminator of nopaline synthase gene; *Wx*P, *Wx* promoter; 1st int, 1st intron of *Wx*^{a} gene. *Open ovals* show borders of the T-DNA. **B**, M, cv. Musashimochi; lane 1, line WAB1-2; lane 2, line WAB2-2; lane 3, line WAB2-3; lane 4, line WAB2-4; lane 5, line WAB3-3; lane 6, line WAB3-5. *Black arrowheads* indicate signals of *WAB* transgenes.

Phenotypic assay

To examine the *WAB* expression in *WAB/wx* transgenic plants, mature R0 pollen were collected and fixed by 70 % ethanol solution and then stained by potassium iodide, and R1 seeds were collected and then stained by potassium iodide.

Southern blot analysis

Genomic DNA was isolated from young leaves of transgenic plants (Walbot and Warren 1988) and 5 mg of DNA was degested with *Hin*dIII. The DNA was electrophoresed in a 0.7 % agarose gel, and then transferred onto a positively charged nylon membrane (Roche diagnostics K.K.). A 0.5 kbp fragment of *Wx* promoter region (Fig. 1A) was amplified by PCR, purified by Microcon YM-100 (Millipore Corp.), labeled with [α-^{32}P] dCTP using the *Redi*-prime labeling system (GE healthcare), and subsequently used as hybridization probe. Hybridization and washing procedures were performed according to the manufacturer's protocol of the membrane (Roche diagnostic K. K.).

Signal detection

The hybridization signals were detected by contacting with the Imaging Plate (Fuji Film Corp.) and then the signal intensity was measured by BAS-5000 system (Fuji Film Corp.).

Quantitative analysis of amylose contents

For quantitative analysis of amylose content, the amylose/ starch ratio was determined as described in Itoh et al. (2003). The total sugar concentration of the starch solution was measured using a phenol-sulfate method (Dubois *et al.* 1956). Standard curves were obtained from starch solution with various amylose/amylopectin composition ratios using by purified rice amylose and amylopectin (Takeda *et al.* 1986).

RESULTS AND DISCUSSION

Transformation of WAB genes into Japonica wx rice

To study the effect of *WAB* gene expression on amylose synthesis in Japonica rice, binary vector pWAB was constructed and introduced into *Oryza* sativa L. Japonica cv. Musashimochi (*wx*^{nmd}). Predicted transcript of *WAB* is identical to that of *WxR* (Itoh *et al.* 1997) and also identical to that of *Wx*^{a} (Hanashiro *et al.* 2008). Six independent transgenic plants were obtained and used for subsequent analysis.

Estimation of WAB copy numbers in the transgenic rice.

To extimate copy number of integrated *WAB* transgene, Southern blot analysis was performed. Genomic DNAs were isolated from young leaves of transgenic R0 plants and then

Table 1. Summary of the *WAB/wx* lines

Line	Number of *WAB* loci	Estimated copy number of *WAB*	Pollen phenotype*	Endosperm phenotype*
WAB1-2	2	3	*Non-waxy*	*Non-waxy*
WAB2-2	1	1	*Non-waxy*	*Non-waxy*
WAB2-3	2	2	*Non-waxy*	*Non-waxy*
WAB2-4	1	1	*Non-waxy*	*Non-waxy*
WAB3-3	1	3	*Non-waxy*	*Non-waxy*
WAB3-5	1	1	*Non-waxy*	*Non-waxy*

* Homozygote of *WAB* loci.

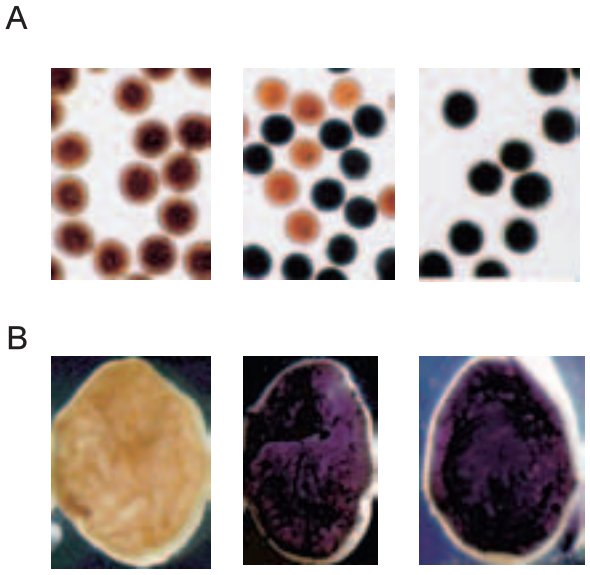

Fig. 2. Stable expression and inheritance of *WAB* in rice. **A,** Pollen grains were stained by potassium iodide and amylose accumulation was detected as dark purple colored pollen grains. Dark purple and brown colored pollen grains were segregated in 1 : 1 ratio in R0 plants of WAB3-5. Left, cv. Musashimochi, Center, R4 generation of the line WAB3-5, Right, cv. Labelle. **B**, Stable expression and inheritance of *WAB* is shown in the endosperm of WAB3-5. *Left*, cv. Musashimochi; *Center*, R4 homozygote of the line WAB3-5; *Right*, cv. Labelle.

digested with *Hin*dIII (Fig. 1A). The 0.5 kbp promoter region was used as a probe for the detection of the endogenous gene and integrated *WAB* transgenes. Since the pWAB has a single *Hin*dIII site at the end of its terminator (Fig. 1A), when the genomic DNA was digested with *Hin*dIII, each integrated *WAB* copy is detected as a distinct band size by Southern blot analysis (Fig. 1B, *arrowheads*). When the intact WAB gene was integrated in the chromosome, the size of a band should be larger than 7.8 kbp (Fig. 1A). The number of transgene loci were determined by estimation of segregation ratio of purple/brown colored pollen numbers in *WAB/wx* R0 plants.

The estimated copy numbers and loci of the *WAB* transgenes were summarized in Table 1. All *WAB/wx* transgenic lines carrying 1 to 3 copies of *WAB*, and the integration sites of the genomic DNA were 1 to 2 loci. Average number of the integrated *WAB* transgene was ca. 1.8 per haploid genome for the *WAB/wx* lines. In contrast, previous studies showed that the average number of *WxR* transgene was ca. 5.4 per haploid genome (Itoh *et al.* 2003).

Stable inheritance of WAB expression in later generation.

All *WAB* transgene in *WAB/wx* lines were inherited in a Mendelian manner, and the expression of the *WAB* transgene stably transmitted over several generations. Inheritance of the *WAB* in line WAB3-5 is shown as a typical example in Fig. 2. The results indicated that less or equal to 3 copies of the integrated transgenes led to the stable expression and inheritance of the *WAB*.

Quantitative analysis of apparent amylose content of the WAB/wx seeds.

To examine the effect of the *WAB* expression on amylose accumulation in the *WAB/wx* endosperms, amylose contents of the seeds were quantitatively assayed. Amylose content of the WAB3-5 starch is two times higher than that of Nipponbare starch (Fig. 3). The results indicated that the Wx^a expression in the Japonica rice generates to high amylose starch and supported previous study conclusion (Itoh *et al.* 2003).

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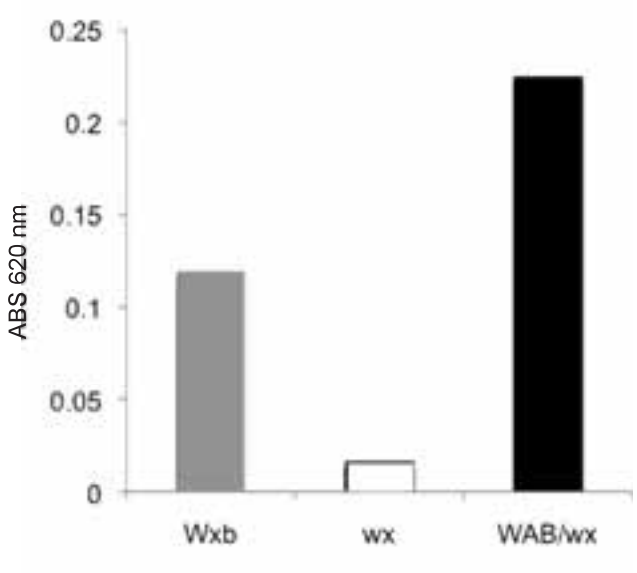

Fig. 3. Quantitative analysis of amylose content of R4 transgenic rice. *WAB* homozygote of the line WAB3-5 was used for quantitative analysis of apparent amylose content by iodine affinity assay. Wx^b, Nipponbare; *wx*, cv. Musashimochi, *WAB/wx*, R5 seeds of line WAB3-5.

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形質転換法による高アミロース日本稲の確立

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要　約

デンプン顆粒結合型デンプン合成酵素をコードする *Wx* 遺伝子はアミロース合成を司る鍵酵素である。これまでの研究から、直接導入法により *wx* 欠損変異体に外来 *Wx* 遺伝子を導入したイネ（*WxR/wx*）では、高アミロース型と低アミロース型双方の表現型を示した。そのうち複数の系統の後代において、不安定かつ不活性化された表現型が現れた。この原因は、外来遺伝子が多コピー導入されたことにより、反復配列誘導性ジーンサイレンシングが起きたためである。この不安定な表現型の出現を解決するために、バイナリーベクター pWAB を構築し、アグロバクテリウム法により *wx* 欠損変異体に導入した。得られた形質転換イネ、*WAB/wx* 系統では低コピー数の外来遺伝子が確認され、胚乳において高アミロース型であり、その胚乳形質は安定に次世代に遺伝した。

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キーワード：*Wx*、デンプン結合型デンプン合成酵素Ⅰ、アミロース、日本稲

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