Doctor Thesis

Production of A Novel Transgenic Rice Using Bioactive Beads-Mediated Transformation with Large DNA Fragments

(バイオアクティブビーズ法を用いた巨大 **DNA** 導入による

新規形質転換イネの作出**)**

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Contents

Chapter 1

General introduction

1-1 Development of a novel plant transformation method using bioactive beads

Genetic engineering of plants is regarded as one of the promising approaches to solve environmental and food problems. The improvement of crop productivity is important to avoid food problems. For example, the first genetically engineered food to be commercialized is FLAVR SAVR^{TM}, which can maintain the quality and taste of tomato for long time (Kramer and Redenbaugh 1994). A variety of plant diseases give a serious deffect on the crop productivity. In order to achieve the resistance to these diseases, disease-resistant plants has been produced in many plant species, such as maize, papaya, potato, tomato, rice so far. (Gonsalves 1998; Tai et al. 1999; Song et al; 2003b; Wang et al. 2005; Khan et al. 2009) Abiotic stresses including drought, temperature extremes, and saline soils are also factors which affect the crop productivity. Genetic engineering has succeeded to enhance the abiotic stress tolerance in plants, which will lead to increase the crop productivity (Yamaguchi and Blumwald 2005; Umezawa et al. 2006; Bhatnagar-Mathur et al. 2008; Khan et al. 2009). As one of the other applications of transgenic technology, nutritionally enhanced plants have been produced. The representative example is “Golden rice”, in which β-carotene was accumulated in rice seeds (Ye et al. 2000). This approach will contribute to us by making the plants which promote our health. The plant transformation technology will facilitate the production of many kinds of useful plants. Several methods are now available for delivering exogenous DNAs into cells, for example *Agrobacterium*-mediated transformation, particle bombardment, and electroporation (Bhalla 2006; Rakoczy-Trojanowska 2002). *Agrobacterium*-mediated methods utilize the unique ability of this bacterium to introduce exogenous genes into plant cells. Particle bombardment involves bombarding

cells with DNA-coated gold or tungsten particles. Both of these methods have their respective merits or drawbacks. Particle bombardment is widely applicable, but DNA molecules can be fragmented during bombardment. It often results in the insertion of multiple gene copies and in complex rearrangements of transgenes (Hiei et al. 1994; Iyer et al. 2000). These events adversely affect the stability of the transgenes. In addition, the transformation efficiency of the particle bombardment is relatively low. Indirect DNA delivery via *Agrobacterium*-mediated transformation usually produces a higher frequency of a single copy of the transgene and results in higher transformation efficiencies, but plant species which are applicable to *Agrobacterium*-mediated transformation are still limited. It has also been reported that large DNA molecules (*ca.* 100 kb) integrated by the *Agrobacterium*-mediated method tend to be rearranged, with duplications, deletions and insertions not only in the transgenic plants but also in *Agrobacterium* (Nakano et al. 2005; Song et al. 2003a). Thus, the *Agrobacterium*-mediated method may not be the best method for transformation with large DNA fragments. In addition, it requires the construction of a specialized vector, i.e., binary vector. Bacterial artificial chromosome (BAC) libraries cannot directly be used for transformation by the *Agrobacterium*-mediated method, except for the cases of binary bacterial artificial chromosome (BIBAC) techniques discussed below.

Transformation with large DNA fragments, such as the insertion of BACs or yeast artificial chromosomes (YACs), would be useful for verifying the function of genes in the DNA insert (Ercolano et al. 2004; Somerville and Somerville 1999). Large DNA fragment insertion would also enable production of transgenic plants with complex phenotypes, particularly in the case of gene stacking or the engineering of metabolic pathways. A transformation technique for the introduction of large DNA

fragments is also a prerequisite in the development of plant artificial chromosomes. Introduction of long centromere repeats into plant chromosomes has contributed to the understanding of the functions of centromeres (Carlson et al. 2007; Phan et al. 2007; Ananiev et al. 2009).

There have been some previous reports on plant transformation with large DNA fragments. Using particle bombardment, YACs have been introduced into tomato (Van Eck et al. 1995) and tobacco (Mullen et al. 1998). The use of specialized vectors for *Agrobacterium*, referred to as BIBACs, permitted the transformation of tobacco with a 150 kb insert (Hamilton et al. 1996). In rice, a 75 kb *Aegilops squarrosa* (the D genome donor to common wheat) genome insert containing the wheat *isoamylase 1* (*TaISA1*) gene has been inserted by the *Agrobacterium* method using a BIBAC vector (Kubo et al. 2005). The fragments of *Aegilops squarrosa* genome DNA were stably transmitted to offspring plants and the transgenes were expressed in rice. Phan et al. (2007) has succeeded in introducing a 150 kb BAC using the particle bombardment. However, the number of reports on successful transformation with large DNA fragments is quite limited. In addition, as mentioned above, the rearrangement of transgenes has often been observed.

Thus, there is a strong need to develop a novel method to introduce large DNA fragments into plant cells. A new transformation method that is simple in procedure with high transformation efficiency and physical stabilization of large sized DNAs in a solution, would be ideal to address the limitation of existing methods.

1-2 Grain hardness

Grain hardness is one of the most important determinants of cereal end-product quality. In wheat (*Triticum aestivum* L.), the hardness has been reported to correlate with many flour properties such as particle size, starch damage, water absorption (Pomeranz and Williams 1990) and its direct effects on the end-product quality. For instance, soft wheat flour is generally used for cakes and cookies while hard wheat flour is used for making breads. To evaluate and manipulate the end-product quality more effectively, the molecular mechanisms of wheat grain hardness have attracted much interest in recent years.

Friabilin was identified as the first molecular marker for grain softness. The friabilin was more abundant on the starch surface of soft textured wheat than that of the hard textured wheat (Greenwell and Schofield 1986). The amino acid sequence indicated that the friabilin was a mixture of puroindoline a (PINA) and puroindoline b (PINB) (Rahman et al. 1994). The proteins are encoded by the *puroindoline a* (*Pina*) and *puroindoline b* (*Pinb*) genes located on the *hardness locus (Ha)* on the short arm of chromosome 5D (Giroux and Morris 1998; Law et al. 1978). The two genes were shown to encode wheat endosperm-specific lipid binding proteins with a unique tryptophan-rich domain (Gautier et al. 1994). These proteins, PINA or PINB, can act independently to give the intermediated-soft textured grain or can function together to give a soft textured grain (Wanjugi et al. 2007).

Rice does not have any homolog for *Pina* and *Pinb*. Therefore, the variation of rice grain texture is so small. This is the one of the reasons why the application of rice flour to processing foods is very limited at present. The creation of such variations in

rice endosperm texture should make it possible to develop a variety of new end products and food uses.

1-3 Objectives of this study

As described above, stable transgenic plants with large DNA fragments have not been obtained so far. In this study, the large DNA fragments containing *Aegilops tauschii* hardness genes have been introduced into rice using the bioactive beads method to produce the transgenic rice which has the novel characteristics in endosperm texture. The transgenic plants obtained were analyzed in the levels of DNA, RNA, and protein in Chapter 2. In Chapter 3, the author has focused on the homozygous transgenic plant 9-1-6-3 expressing *Pinb* gene in successive generations and analyzed the influence of PINB on the ultrastructure of endosperm cells and physicochemical properties of rice flour in detail. The significance of the results obtained through the study is discussed and summarized in Chapter 4. The prospects for this study are also discussed in Chapter 4.

Chapter 2

Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes

2-1 Introduction

Transformation with large DNA fragments enables multiple genes to be introduced into plants simultaneously to produce transgenic plants with complex phenotypes. There have been some previous reports regarding transformation with large DNA fragments (Van Eck et al. 1995; Hamilton et al. 1996; Mullen et al. 1998; Phan et al. 2007). Generally speaking, however, the number of reports of successful transformation with large DNA fragments is limited and the technology has not been established well.

We have developed a novel and efficient transformation method with the capacity to deliver large DNA fragments (Sone et al. 2002; Mizukami et al. 2003; Liu et al. 2004a; Liu et al. 2004b). The method employs calcium alginate microbeads (referred to as bioactive beads) to immobilize DNA molecules on the surface of the beads. Immobilized DNAs on the calcium alginate microbeads are physically stabilized and can be accumulated on a limited area of the cell surface when the beads are attached to cells. This makes it possible to efficiently introduce large DNA fragments. Polyethylene glycol (PEG) is additionally employed to introduce the DNA into cells, making the transformation efficiency 5- to 10-fold higher than that of PEG treatment only (Sone et al. 2002). The bioactive beads-mediated transformation is simple, low cost, and widely applicable. This method has been successfully applied to the transformation of yeast, tobacco BY-2, tobacco SR-1, egg plant, carrot, rice, and mammalian cells (Higashi et al. 2004; Liu et al. 2004b). Chromosomal DNA of up to 450 kb has been introduced into yeast cells by this method (Mizukami et al. 2003). In plants, 124 kb of YAC has been introduced into tobacco BY-2 suspension culture cells (Liu et al. 2004a). However, it has not been yet demonstrated that this method can produce regenerated transgenic

plants containing large DNA inserts.

In Chapter 2, I have reported the successful transformation of rice plants with large DNA fragments using the bioactive beads method. As rice is an important crop and is also a model plant among monocotyledons, the development of effective genetic engineering for this species is of considerable significance. The results presented in Chapter 2 demonstrate that the bioactive beads method can produce transgenic rice plants harboring large fragments of *Ae. tauschii* DNA.

2-2 Materials and Methods

2-2-1 Plant materials and culture methods

Embryogenic calli were induced from mature rice kernels (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) as previously described by Otani and Shimada (1992).

HY-1 cells from wheat (cv. Haruyutaka) were kindly provided by Prof. Shimada (Ishikawa Prefectural University). The calli were cultured on modified Linsmaier and Skoog (LS) medium (LS medium + 2 mg/l 2,4-D, 3% sucrose, 0.25% gellan gum, pH 5.8) at 26°C under light, and were sub-cultured once every 3 weeks.

2-2-2 BAC DNA construction

The pBI BAC 10-60 was constructed by introducing the partially-digested BAC10 clone (Turnbull et al. 2003) into the pBI101Hm vector (Clontech) with the hygromycin-resistance gene (*HPT*), and contains the *Ae. tauschii* genes for PINA, PINB, and GSP-1 (Suzuki et al., unpublished results)(Figure 2-1).

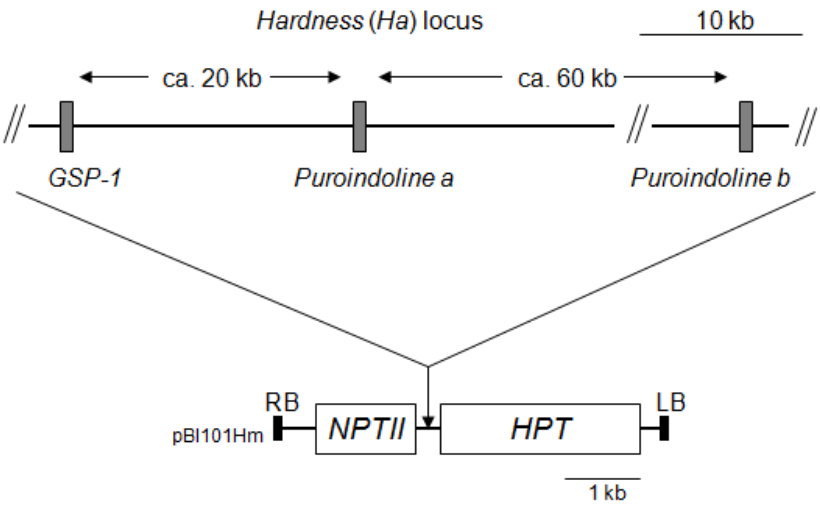


Figure 2-1 Schematic diagram of the construct, pBI BAC10-60, used in this transformation. The pBI BAC 10-60 was constructed by introducing the partially-digested BAC10 clone (Turnbull et al. 2003) into the pBI101Hm vector (Clontech) with the hygromycin-resistance gene (*HPT*), and contains the *Ae. tauschii* genes for PINA, PINB, and GSP-1 (Suzuki et al., unpublished results). The *HPT* gene was used for selection of transgenic plants.

2-2-3 Preparation of bioactive beads

The bioactive beads were prepared according to the protocol described previously by Sone et al. (2002) with several modifications. BAC DNA was extracted using a Qiagen Plasmid Midi Kit (Qiagen, Tokyo, Japan). Ten microgram of the BAC DNA was used for preparing the bioactive beads. The BAC DNA solution was mixed with 450 µl of 100 mM $CaCl_2$. Nine hundred microliters of isoamyl alcohol was added to a 1.5-ml microtube containing 100 µl of 0.5% sodium alginate solution to form a water/oil emulsion. An ultrasonic disrupter (UR-20P; Tomy Seiko, Tokyo, Japan) set to maximum power was used for emulsification. After emulsification for 10 s, 500 µl of $CaCl_2$ solution containing the BAC was added immediately. The solution was then mixed

gently. The microtube was centrifuged at 5,000 rpm for 3 min to precipitate the bioactive beads. The upper isoamyl alcohol phase was removed, without removing the bioactive beads located around the interface. After adding 100 mM $CaCl_2$, the solution was mixed gently and centrifuged at 5,000 rpm for 3 min in order to completely eliminate the isoamyl alcohol. This washing step was repeated at least twice, and the final volume was adjusted to 50 μl with 100 mM $CaCl_2$.

2-2-4 Transformation of rice protoplasts

Rice protoplasts were isolated from mature kernel-derived suspension cells according to the protocol described by Otani and Shimada (1992). The transformation was performed according to the protocol described by Liu et al. (2004b) with several modifications. A protoplast suspension (500 μl) of 2×10^6 cells/ml was transferred to a 15-ml glass centrifuge tube. The bioactive beads with BACs were gently mixed with the protoplast suspension, after which 825 μl of 40% (w/v) PEG CMS6 solution (40% PEG 6000, 0.4 M mannitol, 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$, pH 7~9; sterilized by passage through a 0.45-μm pore filter) was added, and the final concentration of PEG was adjusted to 24%. After 10 min of PEG treatment, 815 μl of a 0.2 M $CaCl_2$ solution (0.2 M $CaCl_2$, 0.4 M mannitol, pH 5.8) was added to dilute the PEG, and mixed to disperse the protoplasts. This procedure was repeated three times after which the centrifuge tube was filled with W5 solution (Menczel and Wolfe 1984). The suspension was centrifuged for 1 min at 800 rpm, and the precipitated protoplasts were washed again with W5 solution. The protoplasts were then suspended in 1 ml of R2P medium (R2 medium + 100 mg/l myo-inositol, 2 mg/l 2,4-D, 137 g/l sucrose, pH 6.0).

2-2-5 Regeneration of transgenic rice plants

Rice plants were regenerated from protoplasts according to the protocol described by Hayashimoto et al. (1990) and Otani and Shimada (1992) with several modifications. The protoplast suspension was mixed with an equal volume of prewarmed (60°C) R2P medium containing 2.5% (w/v) Seaplaque® agarose (Lonza, Switzerland). The agarose mixture was poured into a Petri dish that had been stored at –20°C. The solidified agarose block containing protoplasts was cultured in 5 ml of liquid R2P medium with wheat HY-1 nurse cells at 26°C in the dark with gentle shaking. After 14 d, the agarose blocks were transferred to liquid R2P medium containing 50 μg/ml hygromycin in order to select transformed colonies. After 14 or 28 d, the agarose blocks containing colonies of at least 1 mm in diameter were transferred to N_6AS medium (N_6 medium + 100 mg/l myo-inositol, 2 mg/l 2,4-D, 3% (w/v) sucrose, 0.3% agarose type I, pH 6.0) and maintained for 2 to 4 weeks until the colony size reached 2 mm in diameter. The hygromycin-resistant protoplast colonies were then transferred to regeneration medium [LS medium + 3% sucrose, 3% sorbitol, 2 g/l casamino acid, 1 g/l 2-(N-morpholino) ethanesulfonic acid (MES), 2 mg/l NAA, 1 mg/l kinetin, 0.4% gellan gum, pH 5.8] containing 50 μg/ml hygromycin and cultured at 26°C under a 16-h light/8-h dark photoperiod. The regenerated calli that formed shoots were transferred to LS hormone-free medium (LS medium + 3% sucrose, 0.25% gellan gum, pH 5.8) supplemented with 50 μg/ml hygromycin and maintained at 26°C under a 16-h light/8-h dark photoperiod until sufficient growth of the transgenic rice plants had been obtained. The plants were subsequently transferred to pots and grown in a greenhouse at 26°C. The fertility of the transgenic plants was assessed at the time of kernel harvest. Fertility was calculated as the proportion (%) of fertile kernels to all kernels.

2-2-6 PCR analysis

Total DNA was isolated from the leaves of transgenic plants (T_0, T_2) using DNeasy Plant Mini Kits (Qiagen, Tokyo, Japan). PCR was then performed with primers for the *HPT*, *NPTII*, *Pina*, *Pinb*, and *GSP*-1 genes. The nucleotide sequences of the primers used for the PCRs are shown in Table 2-1 (Turnbull et al. 2003; Liu et al. 2004b). The thermal cycle conditions for the PCR were as follows: 95°C for 2 min, and then 35 cycles of 95°C for 15 s, 50~55°C (depending on the genes, Table 2-1) for 30 s, and 72°C for 40 s using Go Taq Green Master Mix (Promega, WI, USA).

Table 2-1. Nucleotide sequences of primers used for PCR, RT-PCR analysis

Target gene	Primers	Expected length of PCR product (bp)	Annealing temperature (°C)
Pina	5'-ATGAAGGCCCTCTTCCTCATAGG-3' 5'-TCACCAGTAATAGGCAATAGTGCC-3'	450	50
Pinb	5'-ATGAAGACCTTATTCCTCCTA-3' 5'-TCACCAGTAATAGCCACTAGGGAA-3'	450	55
GSP-1	5'-GAATTGCGAGGAAGAGCAGC-3' 5'-GCTAGTGATGGGGATGTTGC-3'	328	55
NPT II	5'-GGCTATGACTGGGCACACCA-3' 5'-GCGATACCGTAAACCACGAG-3'	680	53
HPT	5'-GATGTAGGAGGGCGTGGATA-3' 5'-AGCAATCGCGCATATGAAAT-3'	348	55
Actin	5'-ACATCGCCCTGGACTATGAC-3' 5'-TGGAATGTGCTGAGAGATGC-3'	406	50

2-2-7 Hygromycin resistance test of transgenic kernels

Transgenic kernels (T_1, T_2) were sown on LS hormone-free medium containing 50 μg/ml hygromycin and cultured at 26°C under a 16-h light/8-h dark photoperiod. After 14 d, the number of germinated hygromycin-resistant kernels was counted.

2-2-8 Reverse transcription–PCR (RT-PCR) analysis

Mature kernels were frozen in liquid nitrogen. Total RNA was extracted from these frozen kernels using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA solution was then treated with TURBO DNA-free DNase (Ambion, Austin, TX, USA). First-strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's protocol. RT-PCR was performed with primers for the *actin*, *GSP-1*, and *Pinb* genes. The *actin* primers were used to confirm the successful cDNA preparation. The nucleotide sequences of the primers are listed in Table 2-1.

2-2-9 Southern blot analysis

Total DNA was extracted from the young leaf tissue of transgenic and control rice plants using DNeasy Mini Kit (Qiagen, Tokyo, Japan). Total DNA (approximately 5 μg) and BAC DNA were digested with *Hin*dIII, and fractionated on a 1% agarose gel. After electrophoresis, the DNA was transferred to nylon membranes (Roche Diagnostic Systems, Indianapolis, IN, USA). Hybridization was carried out in Hybri Easy Hyb (Roche Diagnostic systems) at 40°C. The membrane was washed twice in 2 × standard saline citrate (SSC), 0.1% SDS at room temperature for 5 min each, then twice in 0.5 ×

SSC, 0.1% SDS at 68°C for 15 min each. The digoxigenin-labeled probes were prepared by PCR using PCR DIG Labeling Mix (Roche Diagnostic Systems). Detection of the hybridized probe was carried out according to the instructions in the manual supplied with a DIG Luminescent Detection Kit (Roche Diagnostic Systems) using CSPD as the substrate. The band intensities were analyzed with the Image J program (http://rsb.info.nih.gov/ij/download. html) (Abramoff et al. 2004).

2-2-10 Fluorescence in situ hybridization (FISH)

The details of chromosome preparation, probe labeling, *in situ* hybridization, and signal detection are as described in Akiyama et al. (2004), Fukui et al. (1994), and Ohmido et al. (1998) with the following minor modifications. For chromosome preparation, the root tips of rice were macerated with an enzyme mixture [1.33% Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co. Ltd.,, Tokyo, Japan), 3.5% Macerozyme R-10 (Yakult Pharmaceutical Industry Co. Ltd.,, Tokyo, Japan), 0.23% Pectolyase Y-23 (Kyowa Chemical Products Co. Ltd., Osaka, Japan), 0.33 mM EDTA, pH 4.2] at 37°C for 40 min. For FISH, the slides were denatured at 85°C with 70% formamide in 2 × SSC for 90 s after pretreatment.

Following hybridization, the digoxigenin (DIG)-labeled probes were detected using the Fluorescent Antibody Enhancer set for DIG detection (Roche Diagnostic Systems, Indianapolis, IN, USA) according to the manufacturer's instructions. The slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 1.5 μg/ml DAPI and observed under an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a cooled charged-coupled device (CCD)

camera (Cool-SNAP TMHQ2; Photometrics Image Point, Tuscon, AZ, USA). Image processing was performed with the softwares: IPLab (Visitron Systems GmbH, Puchheim, Germany) and Adobe Photoshop version 7.0 (Adobe Systems Inc., CA, USA).

2-2-11 Isolation of Triton X-114-soluble proteins, SDS-PAGE

Triton-soluble proteins were isolated by phase partitioning of Triton X-114 according to the protocol described by Giroux and Morris (1998) with several modifications. Crushed whole kernels were added to 1% (v/v) Triton X-114 in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) at 4°C and were mixed for 30 min. After centrifugation at 10,000 × *g* for 5 min, the supernatant was transferred to a new tube and re-centrifuged. The supernatant was incubated at 65°C for 30 min and re-centrifuged at 25°C. The lower detergent phase was transferred to a new tube and the phase partitioning was repeated. After phase partitioning, the proteins in the detergent-rich phase were precipitated with 80% (v/v) acetone. The pellets were washed with acetone and dried followed by the addition of SDS sample buffer (without reducing agents). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard method (Laemmli method) using a 17.5% gel and visualized by silver staining.

2-2-12 In-gel digestion

Pieces of gel containing the proteins of interest were excised for in-gel digestion with trypsin according to the protocol as described previously by Gharahdaghi et al. (1999)

and Wilm et al. (1996). Briefly, gel pieces obtained from gel electrophoresis were destained by incubation in 1 ml of 25 mM ammonium bicarbonate in 30% acetonitrile. Gel pieces were then dehydrated in 100% acetonitrile and dried in a SpeedVac (Thermo Electron Corp., Waltham, MA, USA). For reductive alkylation, the gels were incubated for 1 h at 56°C in 25 µl of 10 mM dithiothreitol in 25 mM ammonium bicarbonate, then washed with 25 mM ammonium bicarbonate followed by replacement of the solution with 25 µl of 55 mM iodoacetamide in 25 mM ammonium bicarbonate and incubation for 45 min at 37°C in the dark. After washing with 25 mM ammonium bicarbonate, gel pieces were dehydrated in 100% acetonitrile and dried for 30 min in a SpeedVac. The dried gel pieces were then rehydrated for 45 min at 4°C in 5-30 µl of a solution of 50 mM ammonium bicarbonate containing 2-3 pmol of trypsin. After incubation for 16 h at 37°C, peptides were eluted with 50 µl of 5% trifluoroacetic acid in 50% acetonitrile for 10 min at room temperature. The second elutions of the peptides were performed with 25 µl of 5% trifluoroacetic acid in 50% acetonitrile for 10 min at room temperature. The first and second eluates were pooled.

2-2-13 Protein identification by mass spectrometry

Protein identification was performed by MS/MS ion searching using micrOTOF-Q. A mass spectrometer (Bruker Daltonics GmbH) equipped with UltiMate liquid chromatography (Dionex Corp., Sunnyvale, CA, USA) was also employed for the protein identification. Digested peptide solutions were concentrated on a (0.5 cm × 300 µm i.d.) trapping column packed with C18 PepMap 100 (LC Packings, DIONEX) using buffer A (acetonitrile/water (5:95, v/v) with 0.1% formic acid) delivered at 25 µl/min. The trapping column was switched on-line with the analytical column after 5 min

loading time. Chromatographic separation of peptides was performed using a C18 PepMap 100 column (15 cm × 75 μm i.d., LC Packings, DIONEX) and using a linear gradient of buffer B (a mixture of acetonitrile/water (95:5) with 0.1% formic acid) in buffer A as follows: from 10% to 45% of buffer B in 20 min, switched to 100% buffer B for 10 min, followed by 5 min re-equilibration with buffer A at a constant flow rate of 0.3 μl/min.MS/MS spectra were analyzed using the MASCOT software employing the NCBI nr Database. Masses were compared with plant protein database at 0.4 Da mass tolerance. Identification was considered positive when high scores and three peptide sequences were obtained.

2-3 Results

2-3-1 Transformation of rice with BACs using the bioactive bead method

The rice plants were transformed with pBI BAC 10-60 using the bioactive beads method. The pBI BAC 10-60 contains a set of hardness genes, the *NPTII* gene and the *HPT* gene. In nearly all the *Triticum* and *Ae. tauschii* diploids studied to date, the hardness is controlled by a single locus comprised of the *Pina*, *Pinb* and *GSP-1* genes (Bhave and Morris 2008). These three genes are called hardness genes. The pBI BAC 10-60 covers the genomic region containing these three genes (Figure 2-1). Protoplasts were prepared from rice calli and mixed with bioactive beads containing the BAC DNA. A PEG solution was then added to the mixed solution in order to introduce the BAC DNA into the protoplasts. The regeneration of rice plants was performed according to the protocol described by Hayashimoto et al. (1990) and Otani and Shimada (1992) with several modifications. Transgenic plants were selected by hygromycin resistance (Figure 2-2A). Nine plants transformed with pBI BAC 10-60 were obtained. The transgenic rice plants exhibited no aberrant phenotypes and were indistinguishable from non-transgenic rice plants during plant growth (Figure 2-2B). However, the fertility of these plants was very low in the T_0 generation. Most of the transgenic plants did not produce kernels, the exceptions being transgenic plants 9-1-6 and 9-1-10, which produced 5 and 3 kernels, respectively. These T_1 kernels were tested for resistance toward hygromycin. Three out of five kernels from plant 9-1-6 and one out of three kernels from plant 9-1-10 exhibited hygromycin resistance. In the T_1 transgenic plants derived from these kernels, the fertility recovered to approximately 90% (Table 2-2). As a result, more than 100 T_2 kernels were obtained from each T_1 transgenic plant. There

was no difference in morphological characteristics between non-transgenic and transgenic kernels (Figure 2-2C). A segregation test according to hygromycin resistance revealed a 3:1 ratio for transgenic plants 9-1-6-1, 9-1-6-2 and 9-1-10-1, suggesting the integration of transgenes at a single locus (Table 2-2). For transgenic plant 9-1-6-3, all tested T_2 kernels exhibited hygromycin resistance. This result suggested that the *HPT* gene in T_1 transgenic plant 9-1-6-3 was homozygous.

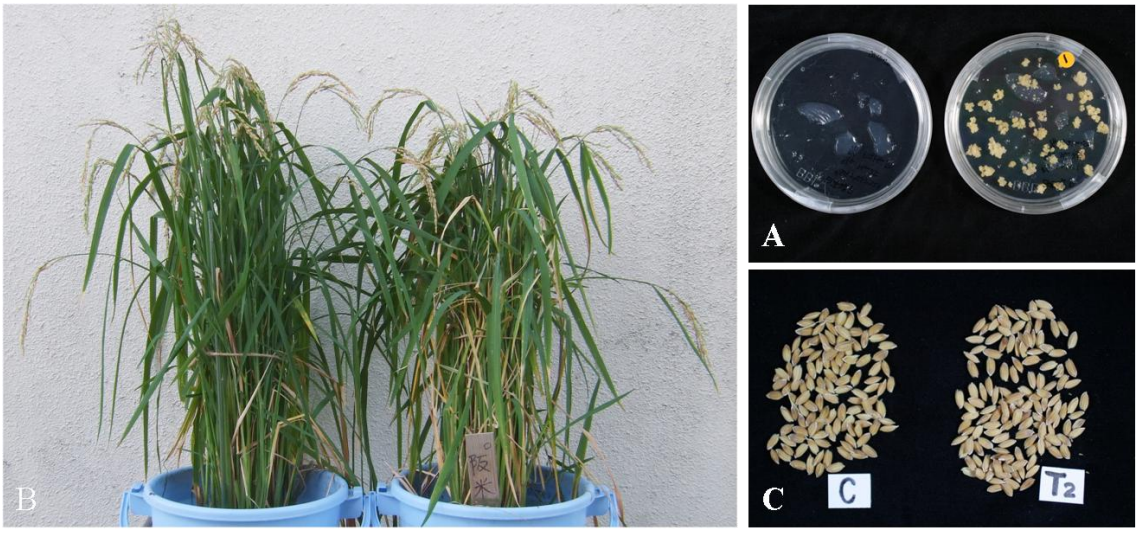


Figure 2-2 Transformation of rice. (**A**) Selection of transgenic plants with hygromycin; (left) Non-transgenic rice calli, (right) Transgenic rice calli. (**B**) T_0 transgenic rice plants; (left) Non-transgenic rice, (right) Transgenic rice. (**C**) Rice kernels of the T_2 generation; (left) Non-trasngenic kernels, (right) Transgenic kernels

Table 2-2. Fertility of T_1 transgenic plants and *HPT* gene segregation in their progeny

* + resistance / non resistance -

Transgenic plants	Fertility (%)	T_2 segregation*	Ratio tested	χ^2 value (P value)
Non-transgenic rice	98.4	+0/20-		
9-1-6-1	96.5	+17/3-	3:1	1.1 (0.30)
9-1-6-2	90.1	+15/5-	3:1	0 (1.0)
9-1-6-3	86.2	+20/0-		
9-1-10-1	84.5	+15/5-	3:1	0 (1.0)

2-3-2 DNA analysis of transgenic plants

The presence of transgenes in the genome of T_0 transgenic rice was examined by PCR with gene specific primers. The results of the PCR analysis are shown in Table 2-3. The insertion of multiple transgenes was confirmed in all the transgenic plants. The results demonstrated that large DNA fragments were integrated into the rice genome. On the other hand, the deletion of some transgenes was also observed. The *Pina* gene was not detected in all transgenic plants. In transgenic plants 9-1-1, 9-1-2, 9-1-6 and 9-1-10, the *NPTII* gene was not detected. In transgenic plant 9-1-10, only the *GSP-1* gene was detected. Although *HPT* gene also was not detected in this plant, the presence of *HPT* gene was confirmed by Southern blot analysis (Figure 2-3). These results suggest that some rearrangement of the integrated BACs had occurred during transformation.

Southern analysis was performed to check the copy number and integration of transgenes. The genomic DNA was digested with *Hin*dIII and hybridized with DIG-labeled probes for *HPT* gene and *Pinb* gene. As a result, all transgenic plants exhibited integration of the *HPT* gene (Figure 2-3A). The fragment size was the same as

size of digested pBI BAC DNA in all transgenic plants. Thus, the result suggests that intact insertion of this region may have occurred. Hybridization with the probe for *Pinb* gene also indicated that all transgenic plants contain the *Pinb* gene (Figure 2-3B). Their fragment sizes were the same as size of digested BAC DNA. This result suggests that the insert region containing *Pinb* gene was integrated as an intact fragment. In addition, the BAC DNA corresponding to 2 copies insertion into 5 μg rice genomic DNA was applied to electrophoresis in this experiment. Thus, based on their band intensities, the copy number of this region in these transgenic plants was presumed to be one. These results indicated that tested two fragments were introduced into rice plant as intact state.

Table 2-3. Profiles of T_0 transgenic plants as determined by PCR analysis
+: gene detected, – : gene not detected

	Lines	*NPTII* gene	*Pinb* gene	*Pina* gene	*GSP-1* gene	*HPT* gene
Non-transgenic plant		–	–	–	–	–
Transgenic plants with pBI BAC10-60	9-1-1	–	+	–	+	+
	9-1-2	–	+	–	+	+
	9-1-3	+	+	–	+	+
	9-1-4	+	+	–	+	+
	9-1-6	–	+	–	+	+
	9-1-7	+	+	–	+	+
	9-1-8	+	+	–	+	+
	9-1-9	+	+	–	+	+
	9-1-10	–	–	–	+	–

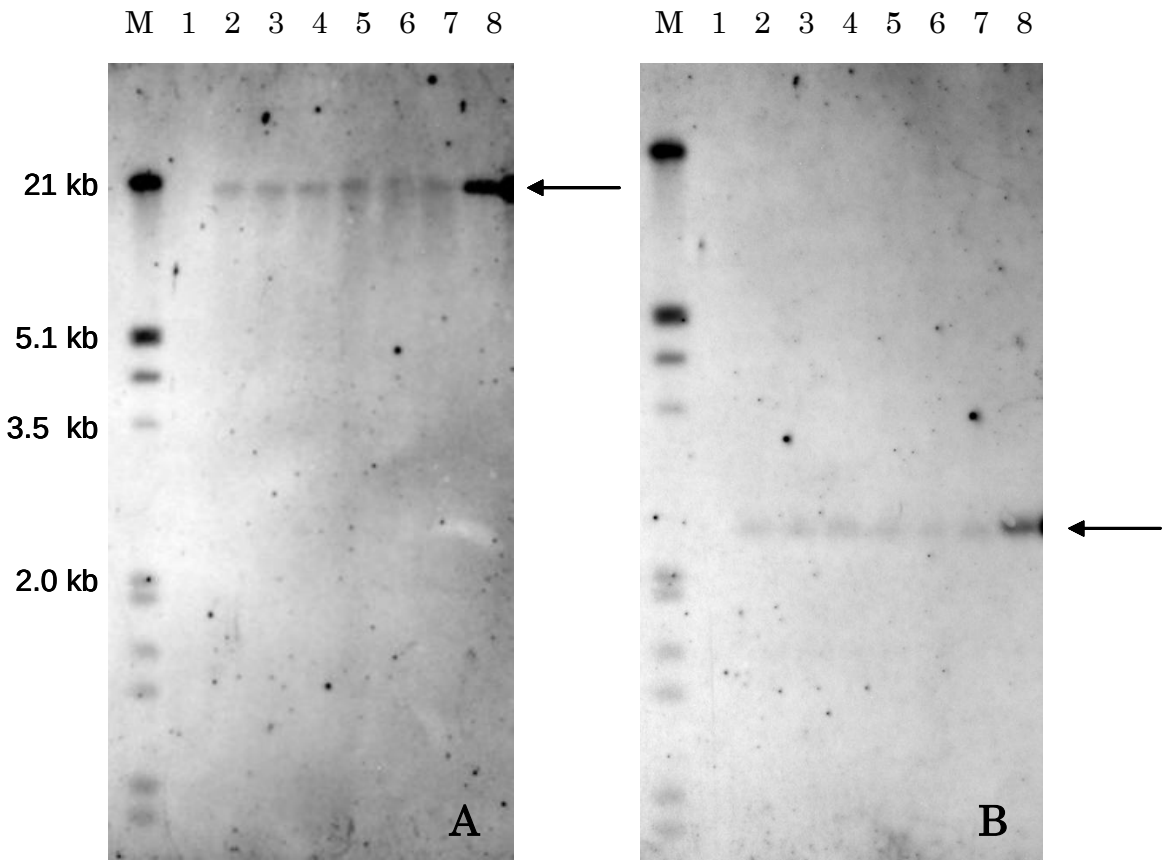


Figure 2-3 Southern blot analysis of T_0 transgenic plants. Total DNA of rice plants was digested with *Hin*dIII and probed with (A) the *HPT* gene and (B) *Pinb* gene. Lane 1 : control plant, Lane 2 : transgenic plant 9-1-3, Lanes 3 to 7 : transgenic plants 9-1-6 to 9-1-10, Lane 8 : pBI BAC DNA digested with *Hin*dIII. The amount of BAC DNA corresponds to two copies insertion of the transgene in rice genome. Arrows indicate the locations of the observed bands.

In order to examine the segregation of transgenes in the T_2 transgenic plants, PCR analysis was performed to determine the presence of transgenes in the T_2 transgenic plants being homozygous for the *HPT* gene. Since PCR analysis of T_0 transgenic plant 9-1-6 revealed that the *Pinb* and *GSP-1* genes were integrated in this plant, its progenies were investigated for segregation of the genes. The results are shown in Figure 2-4. Both genes were detected in all the tested T_2 kernels harvested from T_1 plant 9-1-6-3. These results indicate that both the *Pinb* and *GSP-1* genes coexist with the *HPT* gene being homozygous in T_1 transgenic plant 9-1-6-3. Furthermore, these results demonstrated that the large DNA fragment was maintained in transgenic plants and was stably inherited through several generations.

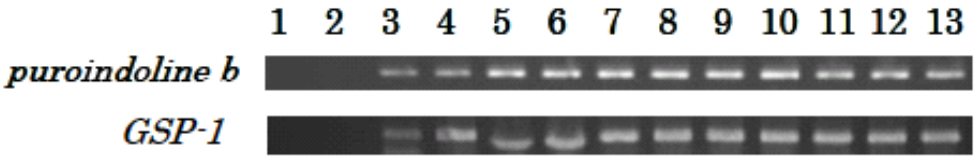


Figure 2-4 PCR analysis of segregation of transgenes among T_2 kernels of plant 9-1-6-3. *Pinb* gene and *GSP-1* gene were amplified with primer sets specific for each gene. The templates used for each sample were as follows: lane 1 ; No template, lane 2 ; Non-transgenic rice, lane 3 ; Transgene donor plant, *Ae. tauschii*, lanes 4~13 ; T_2 Transgenic plants 9-1-6-3-1~10.

The presence of transgenes in the T_2 transgenic plants was also investigated by FISH analysis. Chromosome specimens were prepared from the root tips obtained from the kernels of the T_1 transgenic plant 9-1-6-3 and subjected to FISH analysis. PBI BAC 10-60 was labeled with digoxigenin and used as a probe. Two pairs of green signals were detected at the telomeric region of homologous chromosomes that was predicted to be chromosome 2 or 3 based on their morophology (Figure 2-5) (Fukui and Iijima 1991). This result is consistent with the segregation results mentioned above (Table 2-2, Figure 2-4), which indicate homozygosity of these transgenes in the T_2 trangenic plants. It also suggests that the transgenes might be integrated at a single locus in the genome at the T_0 generation, although there might have been the other DNA insertions which were eliminated during T_0 and T_2 generations.

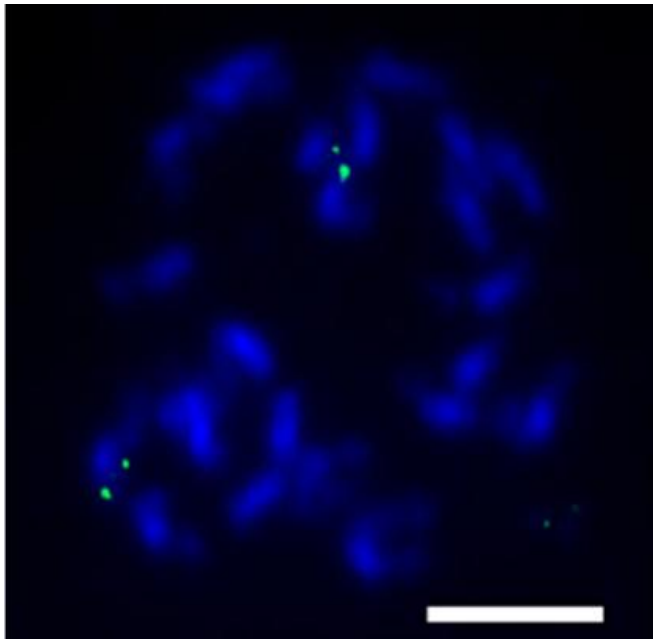


Figure 2-5 FISH analysis of the kernels of the T_1 transgenic plant 9-1-6-3. The two paired green signals indicate the integration sites of pBI BAC 10-60. Bar = 10 μm

2-3-3 Transgene expression analysis

In order to analyze the expression of transgenes at the mRNA level, total RNA was extracted from the T_2 kernels of plant 9-1-6-3 containing homozygous transgenes. The extracted RNA was subjected to RT-PCR. The expression of the *Pinb* and *GSP-1* genes was investigated because the presence of both genes was detected by PCR. The *actin* gene was used as a positive control for the RT-PCR. A band of the expected size (446 bp) was obtained using *Pinb* gene primers when cDNA from plant 9-1-6-3 were used as a template (Figure 2-6). This indicates that *Pinb* was expressed in the transgenic plants. On the other hand, no RT-PCR products of *GSP-1* were observed in the transgenic plants. This result implies that the *GSP-1* gene was not expressed probably due to deletion of the promoter region or gene silencing.

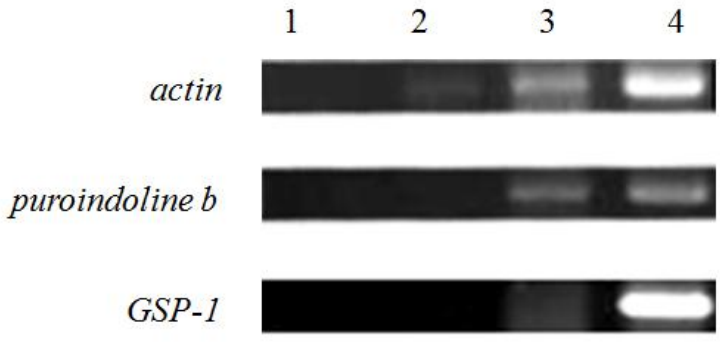


Figure 2-6 RT-PCR analysis of the transgene expressions. Total RNA was extracted from frozen T_2 transgenic kernels and subjected to DNase treatment. cDNA was synthesized from the solution. The templates used for RT-PCR were as follows: lane 1 ; No template, lane 2 ; cDNA of non-transgenic rice, lane 3 ; cDNA of transgenic plant, lane4 ; Total DNA of transgenic rice. The primer sets used are shown on the left.

In order to analyze the expression of the transgene at the protein level, Triton X-114-soluble proteins were extracted from T_2 kernels of plant 9-1-6-3 and were subjected to SDS-PAGE. Similar loadings of protein in each lane were confirmed by Coomassie brilliant blue (CBB) staining (data not shown). With silver staining, an approximately 15-kDa specific band was observed in the transgenic plant, but not in non-transgenic plant (Figure 2-7). The molecular weight of this protein was consistent with that of PINB. To confirm that this band is the product of transgenes, mass spectrometry analysis was performed. Three peptide sequences were obtained and their sequences, LGGFFGIWR, DFPFTWPTK, QLSQIAPQCR, were matched with that of PINB. The mass spectra are shown in Figure 2-8. The obtained Mowse score was 161 (in this experiment, individual ion scores >31 indicate identity or extensive homology (p<0.05)). Thus, the mass spectrometry confirmed that the obtained band was the product of one of transgenes, PINB.These results revealed that PINB was expressed in transgenic plant 9-1-6-3. The introduced BAC contains the native promoters and regulator regions of the target genes. Thus, these results also suggest that the promoter of the *Pinb* gene from *Ae. tauschii* is active in rice kernels.

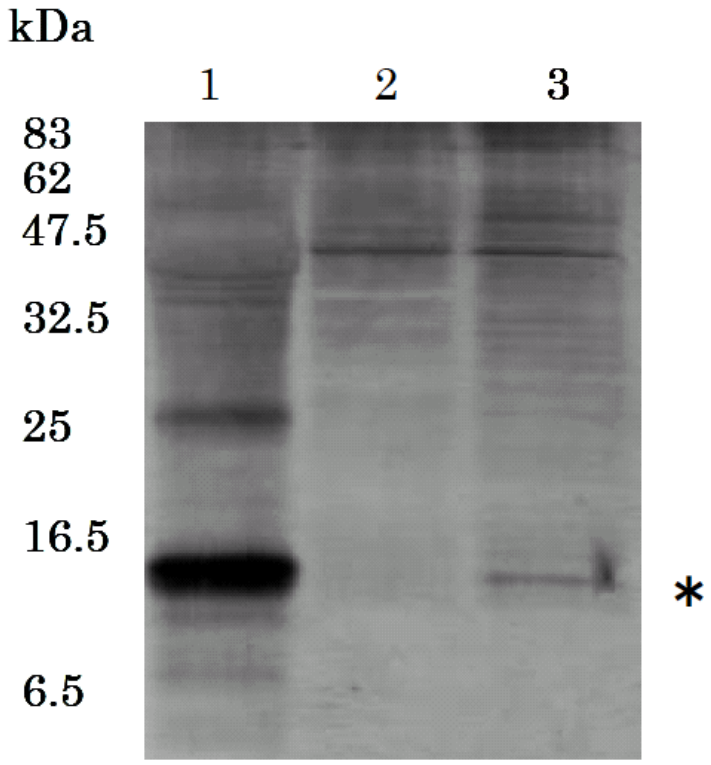
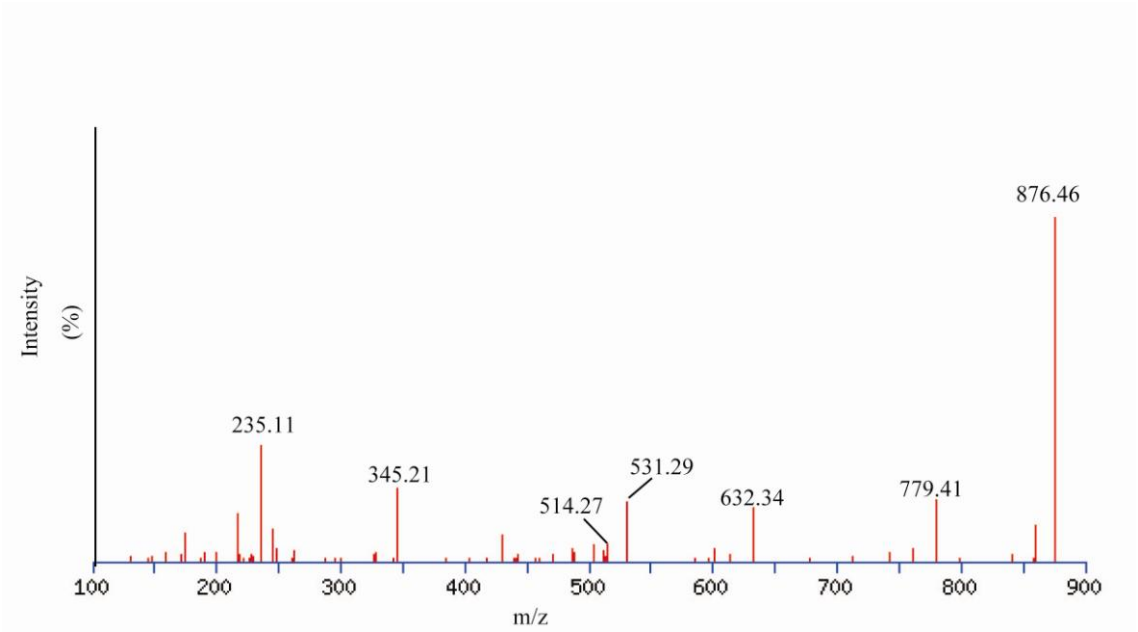


Figure 2-7 Analysis of transgene expression at the protein level. Triton X-114-soluble proteins were extracted from kernels and were subjected to SDS-PAGE. The gel was staind by silver staining. lane 1: *Ae. tauschii* kernels, lane 2 : Non-transgenic rice kernels, lane 3 : T_2 kernels of plant 9-1-6-3. Asterisk indicates the expected size of PINB and GSP-1. PINB was identified from an in-gel digested sample of the 15-kDa band (*) in lane 3.

(A)


Intensity
(%)
876.46
235.11
345.21
531.29
514.27
632.34
779.41
100
200
300
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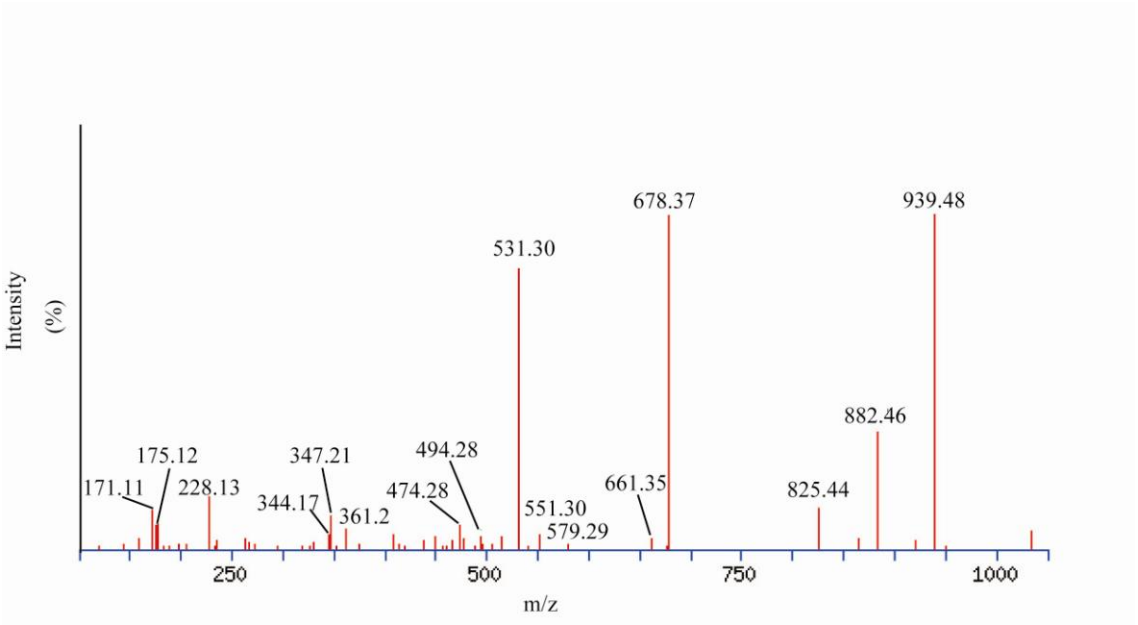
700
800
900
m/z

(B)


Intensity
(%)
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531.30
882.46
175.12
347.21
494.28
171.11
228.13
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474.28
661.35
825.44
361.2
551.30
579.29
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500
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1000
m/z

(C)

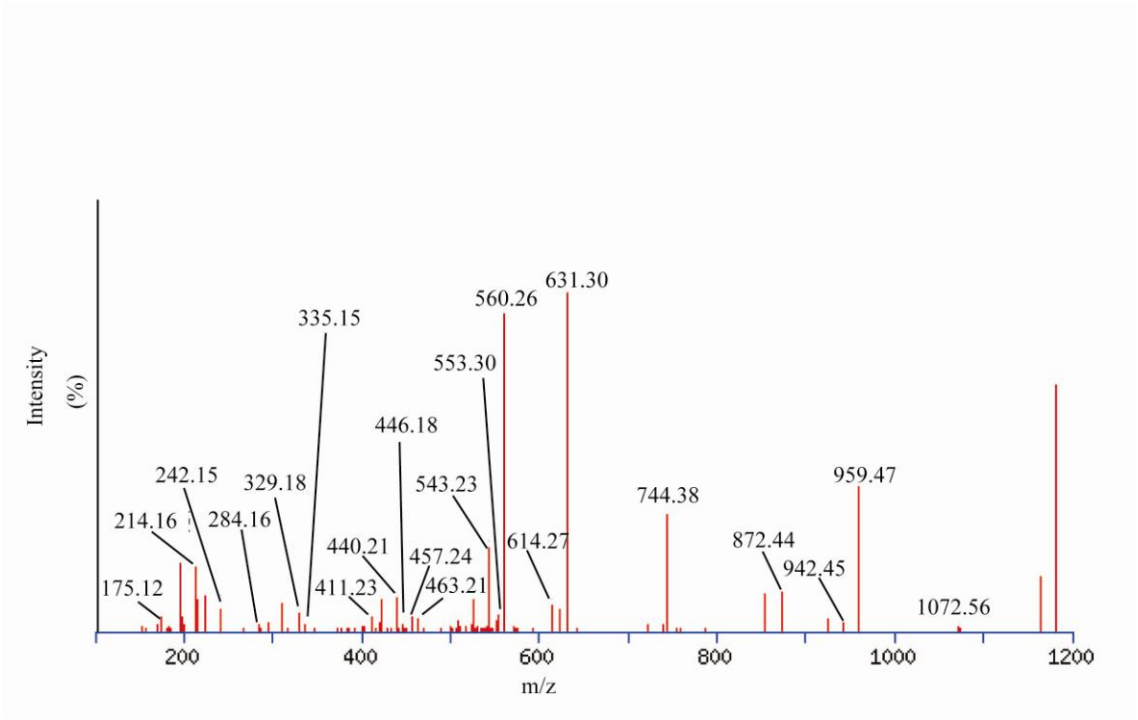

Figure 2-8 Mass spectra of three peptides, (A) DFPFTWPTK, (B) LGGFFGIWR, and (C) QLSQIAPQCR.

2-4 Discussion

The bioactive beads method was developed in 2002 (Sone et al. 2002; Mizukami et al. 2003; Liu et al. 2004b). This method was successfully utilized to deliver chromosomal DNA of up to 468 kb into yeast cells (Mizukami et al. 2003). In the present study, large DNA fragments were introduced into rice plants using the bioactive beads method. Although we have previously demonstrated the transient transformation of several plant species by the bioactive beads method, the present report is the first demonstration of producing stable transgenic plants transformed with large DNA fragments. Furthermore, Liu et al. (2004a) succeeded in co-transformation of two kinds of plasmid DNA into tobacco SR-1, which indicates the possibility of co-transformation with large DNA fragments by the method. Co-transformation allows simultaneous introduction of multiple genes without exceeding the maximum size of transgenes allowed for each plasmid, and its application facilitates genetic engineering of plants. Co-transformation with several kinds of BAC DNAs is in progress.

PCR, segregation, and FISH analyses revealed that plant 9-1-6-3 contained homozygous transgenes at a single locus. These results also indicated that transgenes were stably inherited in the successive generations. Furthermore, FISH analysis also indicated that pBI BAC 10-60 was integrated into the telomeric region of a chromosome. This result is consistent with the general observation that the integration of transgenes occurs preferentially at subtelomeric and telomeric region, which are typically gene-rich (Szabados et al. 2002; Alonso et al. 2003; Chen et al. 2003; Sallaud et al. 2004). On the other hand, Kim et al. (2007) reported that insertion of transgenes occurred randomly, but the selection pressure might shift the recovery of insertions into gene-rich or

transcriptionally active regions of a genome. Our results might reflect selective pressure during cell growth.

FISH analysis is the preferred tool to confirm insertion of large DNA fragments since large DNA fragments are more easily detected than small fragments by FISH. Moreover, FISH results can provide the information as to whether the transgene inserted into the chromosome is homozygous or heterozygous. That is, in the case of large DNA insertion, segregation analysis for confirmation of homozygous status is not required, transgenic breeding program can be shortened by omitting one generation of cultivation for the segregation test.

With the exception of low fertility in the T_0 generation, the transgenic plants exhibited no other abnormal phenotypes through T_0 to T_2 generations. Given that a decrease in fertility has previously been observed in transgenic rice plants generated by tissue culture (Bhat and Srinivasan 2002; Larkin and Scowcroft 1981), this is thought to be due to somaclonal mutation during the regeneration process. Transgene expression might also influence the fertility of transgenic plants since introduced genes are expressed in an endosperm-specific manner in *Ae. tauschii*. However, in the present study, fertility was recovered in the T_2 plants, which expressed the *Pinb* gene. It indicates that the sterility was not caused by the introduced transgenes.

The expression of the *Pinb* gene was also confirmed in the T_2 generation by RT-PCR and mass spectrometry. The introduced BAC contained the native promoters and regulator regions of the target genes; therefore, this result indicates that the promoter of the *Pinb* gene derived from *Ae. tauschii* can function in rice kernels. It has also been reported that 388 bp of the upstream region of the *Pinb* gene derived from *Triticum aestivum* can also function as a promoter that directs tissue-specific expression

in rice kernels (Digeon et al. 1999). Given that the sequence of 600 bp of the upstream region of the *Ae. tauschii Pinb* gene is 93% identical to that of the *T. aestivum Pinb* gene (Turnbull et al. 2003), this result is not unexpected. Kubo et al. (2005) have demonstrated that the promoter of the *Ae. tauschii isoamylase1* (*ISA1*) gene can function in rice kernels. However, the expression of the *GSP-1* gene could not be demonstrated despite the presence of the *GSP-1* gene being detected by PCR analysis. It could be due to a random breakage in promoter region or transgene silencing (Iyer et al. 2000). More detailed analysis of the introduced DNA fragments will be required.

Southern analysis using whole BAC DNA as probe was also performed to get the additional information about the rearrangement level of introduced BAC DNA. However, clear bands could not be obtained. When the whole BAC DNA was used as probe, smear signals were observed not only in transgenic plants, but also in non-transgenic plants. It suggests that the repetitive sequences in BAC DNA hybridized to the sequences in rice genome. More detailed Southern analysis, extended DNA fiber FISH, and sequencing analysis will be required to investigate the rearrangement of introduced DNA fragments.

In this study, transgenic plants which contain *Pina* gene were not obtained. However, the transgenic plants which contain *Pina* gene have been obtained in co-transformation experiments with two kinds of BAC DNAs (data not shown). Thus, the *Pina* gene can be transferred into rice cells by bioactive beads method. It remains unknown if the deletion of *Pina* gene in this study occurred as the result of random deletion or the specific structure of genomic DNA near *Pina* gene, which made it easy to remove the *Pina a* gene during the integration into plant genomes. To increase the number of transgenic lines will be necessary in order to check if the phenomenon

observed in this study was accidental, i.e., the result of random deletion.

Northern analysis gives the detailed information on the mRNA structure. However, Northern analysis was not performed in this study because of the following three reasons. First, the expression of mRNA was confirmed by RT-PCR. Second, the full length *Pinb* mRNA was detected by using the primer sets designed at the terminal sequences of *Pinb* gene. Third, the result of mass spectrometry indicated the expected molecular weight of PINB. However, there is still possibility that the length of *Pinb* gene mRNA was different from the expected length. In fact, only a part of *GSP-1* gene mRNA could also be expressed in transgenic rice. Northern analysis would have been needed to investigate the expression of transgenes in more detail.

Based on the aforementioned results, the author has indicated that the bioactive beads method has a potential to transform rice plants with large DNA fragments. However, the further improvements and trials will be necessary to achieve the intact transfer of large DNA fragments into plant cells. The capacity to deliver large DNA fragments will facilitate the engineering of plants by multiple gene transfer. This approach is likely to be useful for the introduction of families of genes related to phenotypic properties such as metabolic pathways. Furthermore, this method should contribute to the development of plant artificial chromosomes. The development of new transformation method is considered to be necessary for the introduction of long centromeric repetitive DNA sequences. In addition, alternative transformation methods will be required to introduce constructed autonomous plant artificial chromosomes into plant cells because *Agrobacterium*-mediated method will not transfer T-DNA without integrating it into the plant genome. The bioactive beads method can be a powerful tool for this purpose. Autonomous plant artificial chromosome delivering large DNA

fragment will offer simultaneous multiple gene transfer, and enable us to overcome position effects and the rearrangement of transgenes in plant genomes.

2-5 Summary

Transformation with large DNA molecules enables multiple genes to be introduced into plants simultaneously to produce transgenic plants with complex phenotypes. In this study, a large DNA fragment (*ca.* 100 kb) containing a set of *Ae. tauschii* hardness genes was introduced into rice plants using a novel transformation method, called bioactive beads-mediated transformation. Nine transgenic rice plants were obtained and the presence of transgenes in the rice genome was confirmed by PCR and FISH analyses. The results suggested that multiple transgenes were successfully integrated in all transgenic plants. The expression of one of the transgenes, *Pinb*, was confirmed at the mRNA and protein levels in the T_2 generation. Our study indicates that the bioactive bead method is capable of producing transgenic rice plants carrying large DNA fragments. This method will facilitate the production of useful transgenic plants by introducing multiple genes simultaneously.

Chapter 3

The effects of puroindoline b on the ultrastructure of endosperm cells and physicochemical properties of transgenic rice

3-1 Introduction

Wheat grain hardness is controlled by the hardness locus (*Ha*) on the short arm of chromosome 5D (Law et al. 1978). A 15 kDa complex of the *Ha* termed friabilin was found to be related with endosperm texture, which was more abundant on soft wheat starch than on hard wheat starch (Greenwell and Schofield 1986). PINA and PINB have been identified as the components of friabilin based on their N terminal sequences (Rahman et al. 1994). The *Pina* and *Pinb* genes have been cloned and were shown to encode wheat endosperm-specific lipid binding proteins with a unique tryptophan-rich domain (Gautier et al. 1994). This tryptophan rich domain has been considered as being responsible for the strong affinity of PINs to polar lipids (Marion et al. 1994). It has been suggested that these lipid/protein interfaces play an important role in the texture of wheat endosperm by preventing the adhesion between the starch granules and surrounding protein matrix (Morris 2002). Promoter analysis has shown that *Pina* and *Pinb* genes are expressed only in the endosperm (Wiley et al. 2007) and immunostaining studies have shown that PINs are localized on the starch granule surface (Wiley et al. 2007; Feiz et al. 2009). Based on these studies, it has been considered that PINs bound to lipid on the starch granule surfaces prevent the starch granules from being packed tightly, resulting in the soft grain texture.

PINA and PINB have 60% homology in their amino acid sequence. Some studies showed that PINA has a greater role for grain hardness than PINB (Corona et al. 2001; Capparelli et al. 2003). Some studies showed that addition of PINB was more effective at reducing the grain hardness (Hogg et al. 2004) and that PINB was the limiting factor in a sense because it assists PINA in binding to starch (Swan et al. 2006).

Recently, Wanjugi et al. (2007) has indicated that PINA or PINB can act independently leading to intermediate-textured grain or can function together to give a soft grain texture.

Rice (*Oryza sativa* L.) is an important cereal and is also a model plant among monocotyledons. In addition, rice does not contain *PinA* and *PinB* homologs. These characteristics make it a good model cereal to investigate the effect of PINs on the other cereals. Krishnamurthy and Giroux (2001) have already reported that expression of wheat *Pins* in transgenic rice enhances grain softness. However, the ultrastructure of transgenic rice endosperm cells has not been investigated. Examining the effect of PINs on the structure of plant endosperm cells will be useful to understand the functions of PINs in more detail. Furthermore, it will also help to collect additional knowledge to manipulate the grain hardness in other cereals.

As described in Chapter 2, I have introduced the *Ae. tauschii* genomic region containing the hardness genes into japonica rice cv. Nipponbare using a bioactive beads method and obtained the homozygous transgenic rice stably expressing the *Pinb* gene. In this study, the homozygous transgenic rice expressing *Pinb* gene in T_4 generation was used to investigate the effect of the *Pinb* gene on the ultrastructure of the rice endosperm cells. In addition, I also assessed the physicochemical properties of transgenic rice flour to characterize the transgenic rice.

3-2 Materials and Methods

3-2-1 Plant materials

The embryogenic rice calli induced from mature kernels (*O. sativa* L. ssp. *japonica* cv. Nipponbare) were transformed as described in Wada et al. (2009). The T_4 kernels derived from the homozygous transgenic rice (line 9-1-6-3) expressing *Ae. tauschii Pinb* gene were used throughout the study. Three transgenic and three non-transgenic plants were grown in pots containing fertilized granulated soils (Kureha, Tokyo, Japan) in the greenhouse at 30°C at same time in the summer of 2008. The non-transgenic plants which did not go through tissue culture but were nearly identical to transgenic plants in terms of their seed size, protein content (data not shown), amylose content, the shape and the size of isolated starch granules (as described in Results section) were used as control in this study.

3-2-2 RT-PCR analysis

RT-PCR was carried out as mentioned in Wada et al. (2009). RT-PCR was performed with primers for the *actin* and *Pinb* genes. The *actin* primers were used to confirm the cDNA synthesis. The nucleotide sequences of the primers are as follows: *actin* F, ACATCGCCCTGGACTATGAC; *actin* Re, TGGAATGTGCTGAGAGATGC; *Pinb* F, ATGAAGACCTTATTCCTCCTA; *Pinb* Re, TCACCAGTAATAGCCACTAGG GAA. The thermal cycle conditions for the RT-PCR were as follows: 95°C for 2 min, and then 35 cycles of 95°C for 15 s, 50°C (for *actin* primer) or 55°C (*Pinb* primer) for 30 s, and 72°C for 40 s.

3-2-3 Isolation of Triton X-114 soluble proteins, SDS-PAGE

Triton-soluble proteins were isolated by phase partitioning of Triton X-114 as described in Wada et al. (2009). The pellet was dissolved in SDS sample buffer (4% SDS, 10% sucrose in 125 mM Tris-HCl, pH 6.8). SDS-PAGE was performed by standard method (Laemmli method) using a 20% gel and visualized by silver staining using Sil-Best Stain-Neo (NACALAI TESQUE, Inc., Kyoto, Japan) according to the manufacturer's instructions.

3-2-4 Scanning electron microscopy (SEM)

Two kinds of samples were prepared; one was the whole grains of milled rice and another was isolated starch granules. For the whole grains, individual grains were fractured by the razor blade with a slight pressure on the top of the grain. Fractured rice grains were immediately mounted on the specimen stage, and the fractured surface of rice grains was directed upward. The samples were then coated with osmium (3 nm in thickness) in an osmium plasma coater, HPC-15 (Vacuum Device Inc., Mito, Japan) and were observed under a SEM, S-5200 (Hitachi, Tokyo, Japan) at 15 kV. Seven seeds were used for SEM observation. The isolated starch granules were prepared according to the protocol described by Fujita et al. (2003). Dried seeds of non-transgenic and transgenic plants were dehulled, and the outer layer of the seed was removed using a rice polisher (Twinbird corporation, Niigata, Japan). And then the milled rice was ground into powder with mortar and pestle. The morphology of starch granules was examined under a SEM. The projected surface areas of each granule were measured using the Image J program (http://rsb.info.nih.gov/ij/) to analyze the size distribution of isolated starch granules (Abramoff et al. 2004).

3-2-5 Transmission electron microscopy (TEM)

Using a razor blade, individual mature grains were first cut transversely at the mid region of endosperm, and then were cut longitudinally. These blocks were fixed with gas from 5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for a week at room temperature, and postfixed by 2% osmium tetraoxide gas overnight at room temperature. The blocks were then dehydrated through the graded ethanol series, followed by n-butyl glycidyl ether (QY-1) and embedded in Qutol-651 (NISSHIN EM Co., Ltd. Tokyo, Japan). Sections were cut with a diamond knife on an ultramicrotome, ULTRACUT E (Leica Biosystems Nussloch GmbH, NuBloch, Germany), and examined under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 100 kV. Three seeds were used for TEM observation.

3-2-6 Damaged starch assay

The quantity of damaged starch from rice flour was measured using the Megazyme starch damage assay kit (Megazyme Int'l Ltd., Bray, Ireland) according to the manufacturer's protocol. The analysis was performed in duplicate and the values are averages of three independent experiments.

3-2-7 Flour size distribution assay

The milled rice was ground to flour using the vibrating sample mill, model TI-100 (CMT Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The flour particle size distribution was measured with a laser diffraction system, HELOS & RODOS (Sympatec GmbH, Clausthal-Zellerfeld, Germany) according to the

manufacturer's protocol. The median diameter calculated was chosen to characterize the flour particle size. The analysis was performed in duplicate.

3-2-8 Quantification of apparent amylose content

The apparent amylose content was measured based on an iodine colorimetric assay (Juliano 1971) using an Auto Analyzer II (Bran+Luebbe, Norderstedt, Germany). The standard curve was constructed using different amounts of potato amylose (Sigma Chemical Co., St. Louis, USA) and starch extracted from glutinous rice. Starch extraction was performed as described in Yamamoto et al. (1981). The reference sample Nipponbare whose amylose content is 19.2% (supplied by Bran+Luebbe, Norderstedt, Germany) was used to correct the errors derived from rice components other than the starch. The sample analyses were repeated in triplicate.

3-2-9 Analysis of pasting properties

The pasting properties of the flour were measured using the Rapid Visco-Analyzer (RVA) model 3D (Newport, Sydney, Australia). The aqueous rice flour suspensions (14% w/w) were prepared using 3.5 g of rice flour and 25 ml of distilled water. The applied temperature program was as follows: (1) hold at 50 °C for 1 min; (2) from 50 to 95 °C for 4 min; (3) hold at 95 °C for 7 min; (4) from 95 to 50 °C for 4 min; and (5) hold at 50 °C for 3 min. The program was initiated by mixing at 960 rpm at 50 °C for 10 s and 160 rpm was used for the rest of the program. The parameters recorded were initial gelatinization temperature, peak viscosity, hot paste viscosity, final viscosity, breakdown and setback. Rice flour samples were analyzed in triplicate.

3-3 Results

3-3-1 Expression analysis of the transgenic rice in T_4 generation

Expression of *Pinb* gene was confirmed at the RNA and protein levels, respectively. At the RNA level, the expression of *Pinb* gene under the *Ae. tauschii PinB* promoter was detected only in kernels, not in leaves (Fig. 3-1A). At the protein level, an approximately 15 kDa specific band was observed in transgenic rice by silver staining (Fig. 3-1B). This result was consistent with the previous result obtained with the transgenic rice in T_2 generation, in which the PINB was identified from the 15 kDa specific band (Wada et al. 2009). These results indicated that PINB was expressed stably in the transgenic rice kernels used in this study.

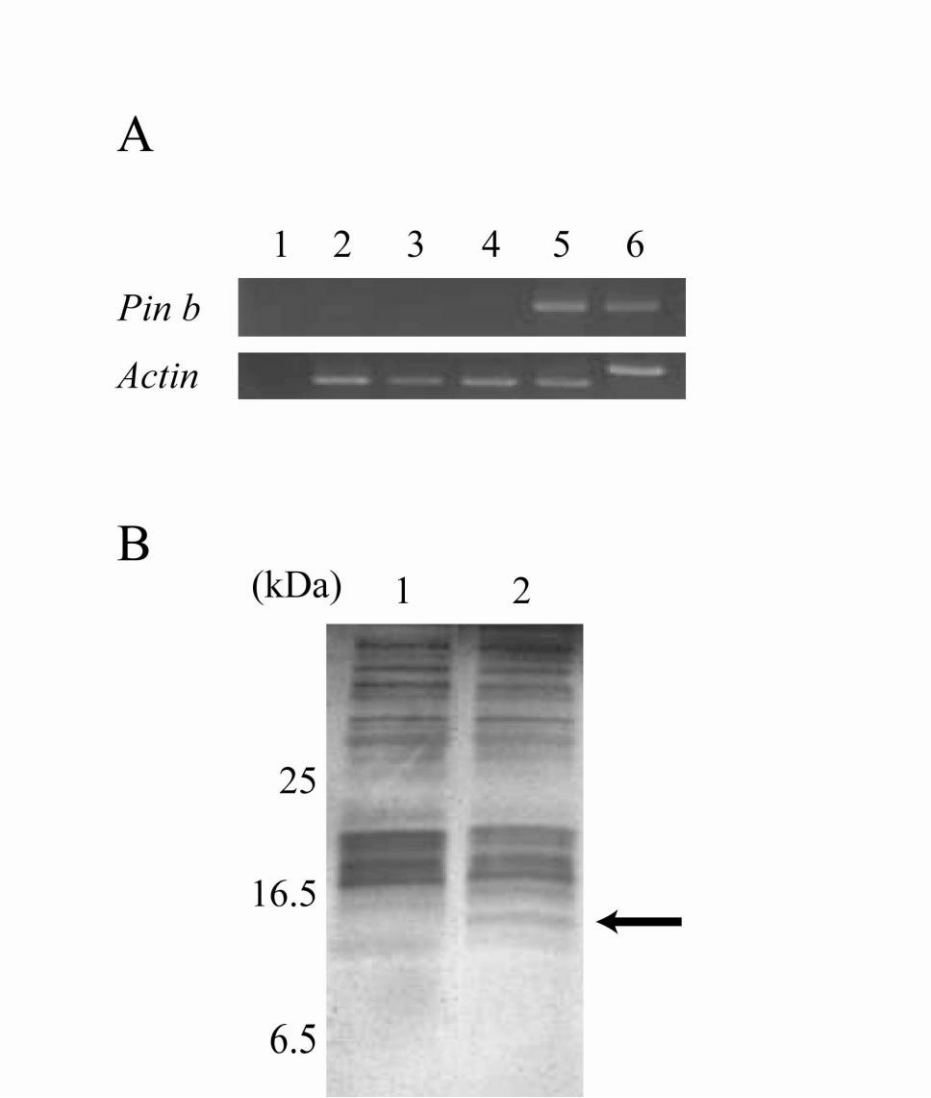

Figure 3-1. Expression analysis of *Pinb* RNA **(A)** and PINB protein **(B)**. **(A)** Total RNA was extracted from the frozen T_4 transgenic leaves or kernels and subjected to DNase treatment. cDNA was synthesized from the solution. The templates used for RT-PCR reaction were as follows: lane 1: No template, lane 2: Non-transgenic rice leaf cDNA, lane 3: Non-transgenic rice kernel cDNA, lane 4: Transgenic rice leaf cDNA, lane 5: Transgenic rice kernel cDNA, lane 6: Transgenic rice genomic DNA. The primer sets used are shown on the left. **(B)** Triton X-114-soluble proteins were extracted from kernels and were subjected to SDS-PAGE and the gel was silver stained. lane 1: Non-transgenic rice kernels, lane 2 : Transgenic rice kernels. Arrow indicates the expected size of PINB.

3-3-2 SEM observation of the fractured surface

To investigate the effects of *Pinb* gene on the structure of rice endosperm cells, the transversely fractured surface of milled rice was observed under SEM. As shown in Fig. 3-2A and 3-2B, two types of endosperm cell morphology were observed depending on the position where the cleavage occurred. In the area where the cleavage occurred between cells, the surface was smooth and individual cells could be identified by angled shape (Fig. 3-2C and 3-2D). In the area where the cleavage occurred within cells, the surface was rough and the individual cells could not be identified because of the disruption of cell morphology (Fig. 3-2E and 3-2F). In this area, partially split compound starch granules exposing individual starch granules with sharp angles and edges were observed in various shapes and sizes.

In non-transgenic rice, the tightly packed compound starch granules were observed in the area where the cleavage occurred between cells (Fig. 3-2C). All of the spaces between compound starch granules were filled with matrix material. In contrast, the spaces between each compound starch granules were observed in the transgenic rice expressing *Pinb* gene (Fig. 3-2D). The compound starch granules varied in sizes and were not tightly packed. The matrix material did not fill the spaces between compound starch granules. Instead, air spaces surrounded the compound starch granules. In the place where the cleavage occurred within cells, no difference was observed between non-transgenic and the transgenic rice (Fig. 3-2E and 3-2F).

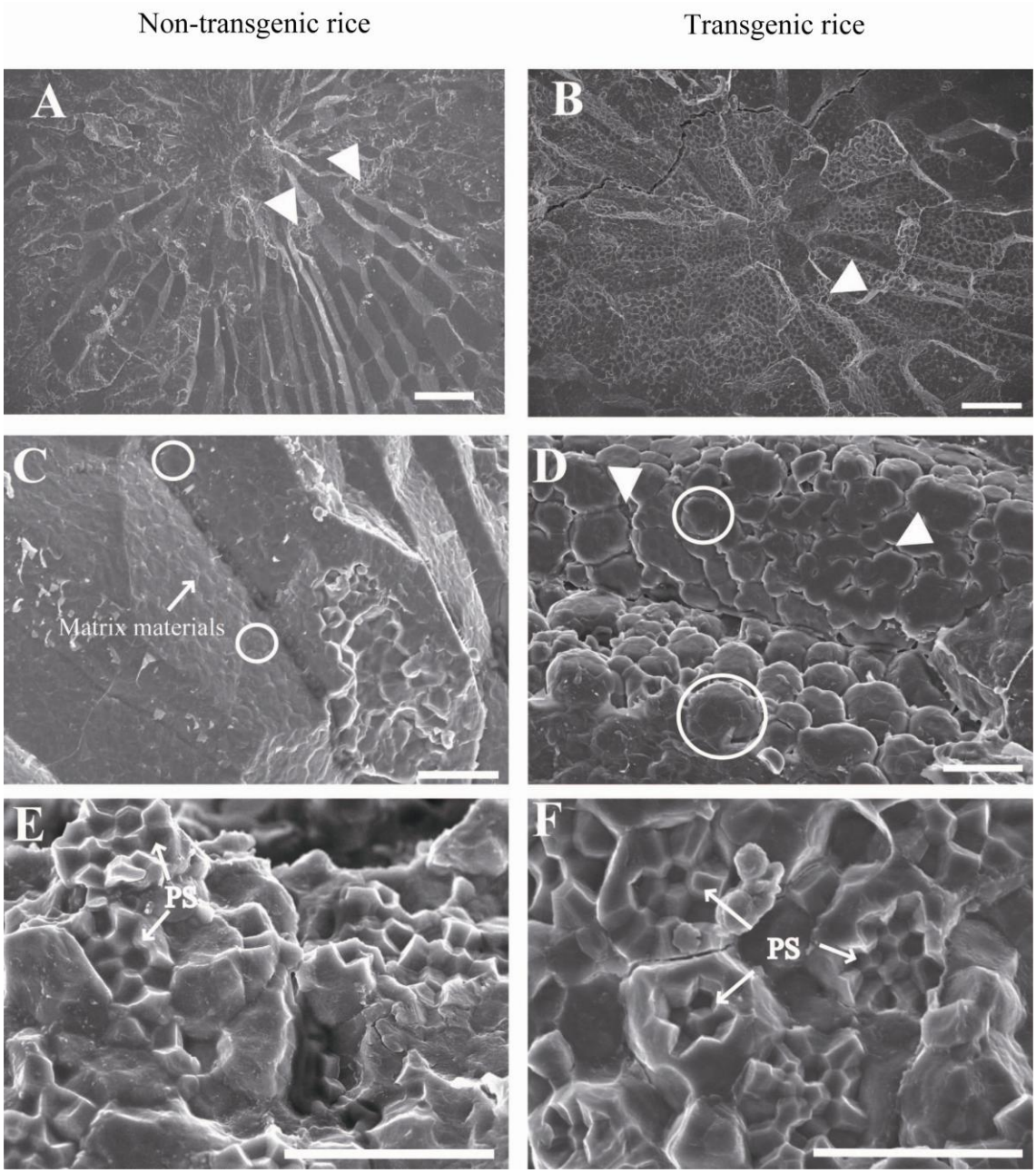

Figure 3-2. SEM observation of rice endosperm cells. **(A,B)** Low magnification view of transversely fractured surface of milled rice of **(A**) Non-transgenic rice and **(B)** Transgenic rice. Arrowheads indicate the intracellularly cleaved site. Bars: 100 μm. **(C,D)** Higher maginification view of intercellularly cleaved site of **(C)** Non-transgenic rice and **(D)** Transgenic rice. **(C)** Compound starch granules (circles) are embedded within matrix material in non-transgenic rice. Intracellularly cleaved sites are also observed. **(D)** Starch compound granules (circles) are surrounded by airspaces (arrowhead). Bars: 20 μm. **(E,F)** Higher magnification view of intracellularly cleaved

site of **(E)** Non-transgenic rice and **(F)** Transgenic rice. Partially split compound starch granules (PS) exposing individual starch granules with sharp angles and edges are observed. Bars: 20μm.

3-3-3 SEM observation of isolated starch granules

The morphology and size of isolated starch granules were investigated by SEM. As shown in Fig. 3-3, no clear difference was observed between non-transgenic rice and the transgenic rice. Both of them consisted of polygonal starch granules with sharp angles and edges. To analyze the size distribution of isolated starch granules, the projected area of each starch granule was calculated. The result indicated that their differences were not significant statistically ($p = 0.11$, *t*-test).

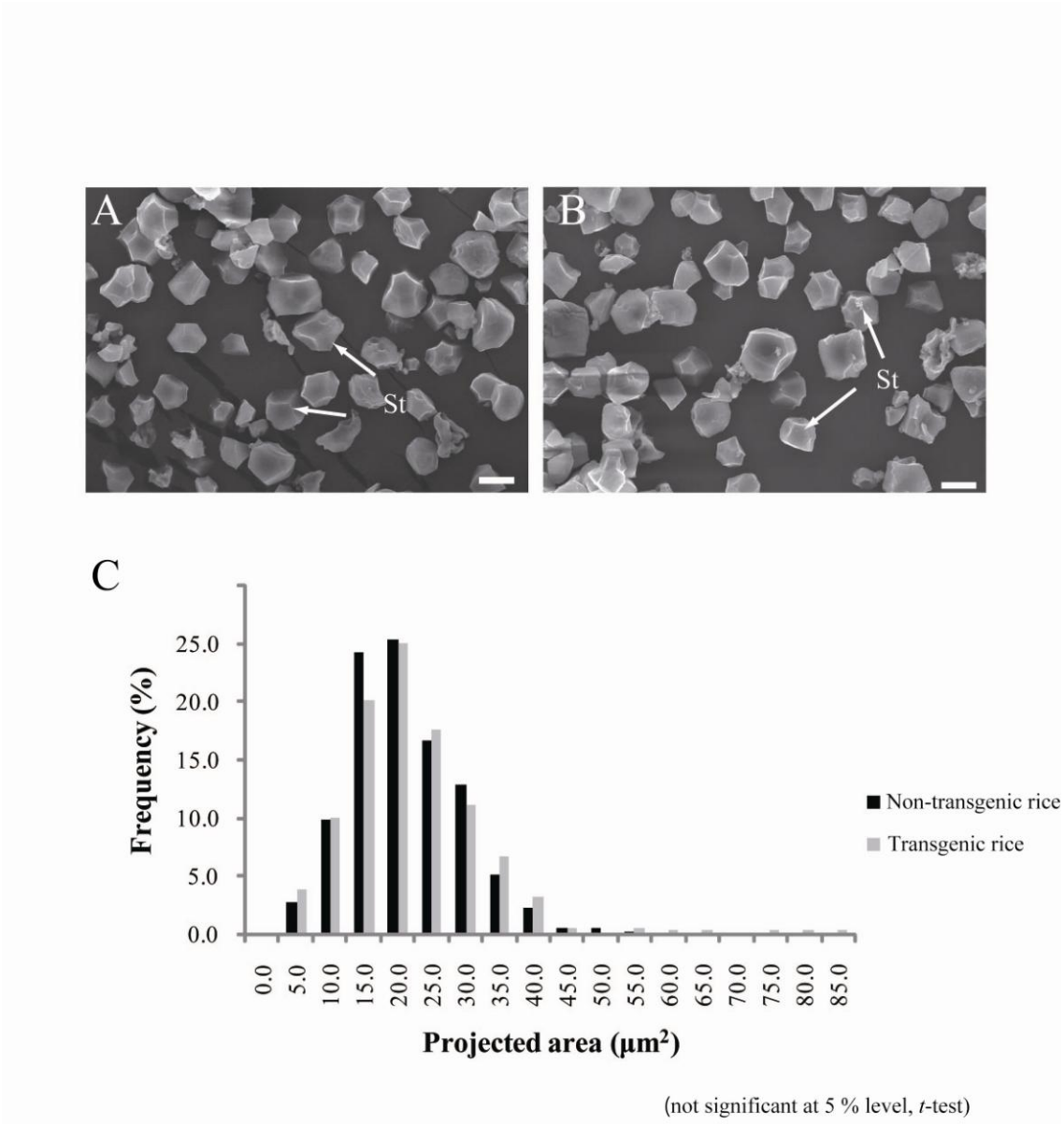

Figure 3-3. Morphology and size of isolated starch granules. **(A,B)** The morphology of isolated starch granules from **(A)** Non-transgenic rice and **(B)** Transgenic rice. Both are polygonal in shape with sharp angles and edges, indicating no differences between them. St indicates starch granules. Bars: 5 μm. **(C)** Size distribution of isolated starch granules. The projected surface area of each granule was measured with Image J program (http://rsb.info.nih.gov/ij/download.html). The projected surface areas of approximately 400 starch granules were measured. Non-transgenic rice and the transgenic rice showed no significant difference (p = 0.11, t-test)

3-3-4 TEM observation of rice endosperm cells

To facilitate a more detailed observation of the ultrastructure of endosperm cells in the transgenic rice, the central region of rice grain was observed under a TEM. Endosperm cells were typically occupied by compound starch granules (Fig. 3-4A and 3-4B). In non-transgenic rice, compound starch granules were compacted together, making it difficult to distinguish the individual compound starch granules (Fig. 3-4A). Most of the compound starch granules were surrounded by the space in the transgenic rice and individual compound starch granules could be identified even when numerous compound starch granules were present within a small area (Fig. 3-4B).

Each compound starch granules consisted of a number of starch granules. In both non-transgenic and the transgenic rice, the spaces between starch granules were sometimes observed.

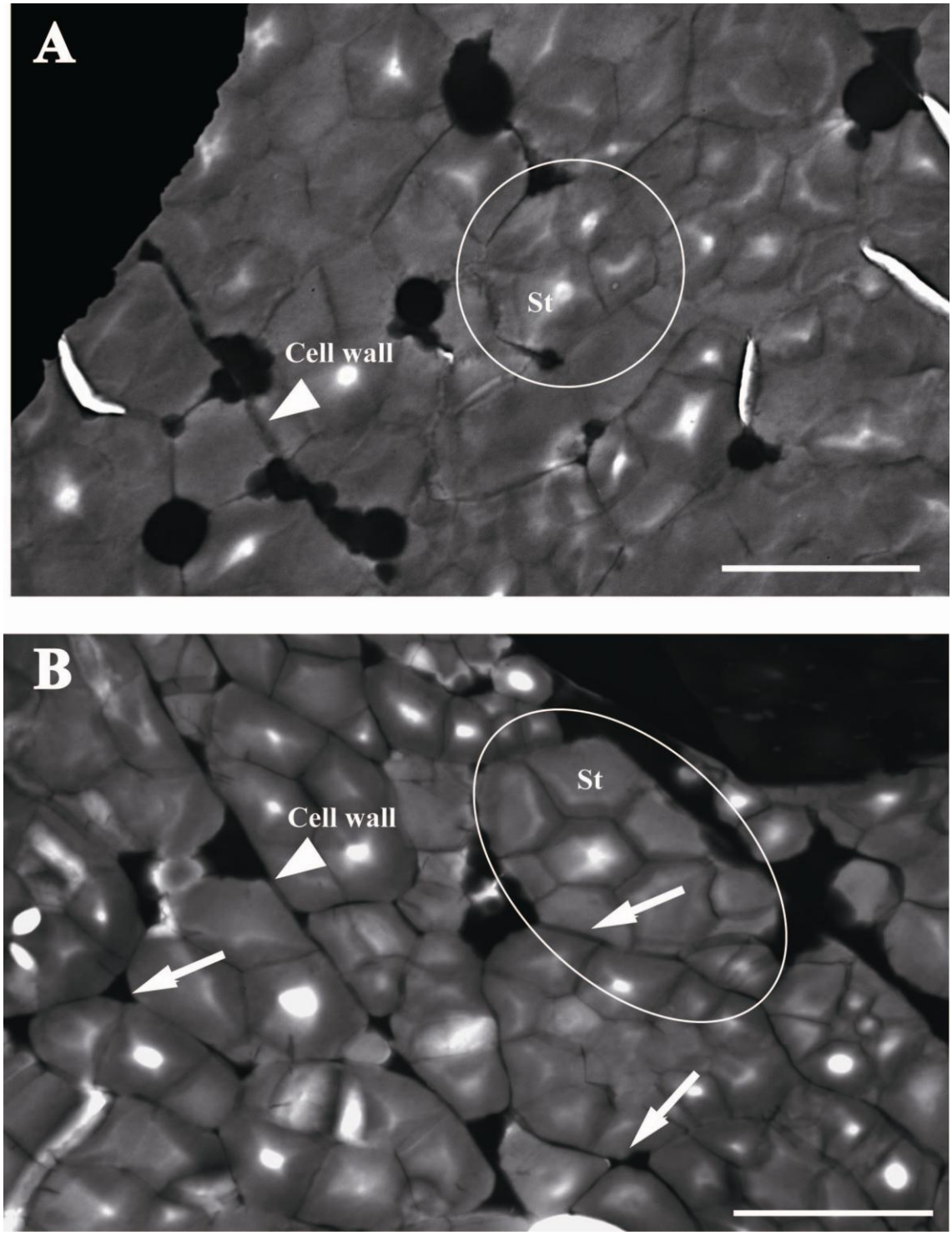

Figure 3-4. TEM observation of starch endosperm cells from (A) Non-transgenic rice and (B) Transgenic rice. The endosperm cells are filled with compound starch granules (circle). (A) In non-transgenic rice, compound starch granules are tightly packed and sometimes fused together. (B) In transgenic rice, compound starch granules have spaces

between each other. They are not fused together, allowing us to distinguish the shape of each compound starch granule. Arrows indicate the spaces between compound starch granules. St indicates starch granules. Bars: 10 μm.

3-3-5 Flour particle size, starch damage and apparent amylose content

Many studies have reported that PINB confers grain softness which correlates with flour size distribution and starch damage (Beecher et al. 2002; Brites et al. 2008; Martin et al. 2008; Martin et al. 2007). Therefore, the flour size distribution and starch damage of the transgenic rice were analyzed. As shown in Table 3-1, the transgenic rice showed smaller particle size (Median value: 74 ± 2.4 μm) than non-transgenic rice (100.7 ± 5.1 μm). The starch damage was higher in the transgenic rice (8.6 ± 0.2%) than in non-transgenic rice (7.9 ± 0.1%). The apparent amylose content was not different between them, 14.2% in non-transgenic rice and 14.3% in the transgenic rice.

Table 3-1. Differences in median flour particle size, starch damage and apparent amylose content between non-transgenic and transgenic rice

	Median particle size (μm)[b]	Starch damage (%)[c]	Apparent amylose content (%)[d]
Non-transgenic rice	100.7 ± 5.1	7.9 ± 0.1	14.2 ± 0.1
Transgenic rice	74.9 ± 2.4	8.6 ± 0.2	14.3 ± 0.1
Difference[a]	- 25.8**	0.7*	0.1

[a] Difference between non-transgenic and transgenic rice. [b] Brown rice kernels were ground into flour in duplicate and particle size was calculated. [c] Starch damage was measured in duplicate and the values are averages of three independent experiments. [d] The analysis was repeated in triplicate. *, ** Significantly different at 0.05 and 0.01 levels, respectively (*t*-test).

3-3-6 Pasting properties of transgenic rice flour

Table 3-2 shows the pasting viscosity profile of non-transgenic and the transgenic rice. The transgenic rice showed lower viscosity during gelatinization than non-transgenic rice. The statistically significant differences are as follows; -73 RVU (Peak viscosity), -29 RVU (Hot past viscosity), -43 RVU (Breakdown), -35 RVU (Final viscosity). The setback, peak time and initial gelatinization temperature values were not different between non-transgenic and the transgenic rice. The pasting viscosity profile was also analyzed in T_3 generation, which showed the similar differences in pasting viscosity profile between non-transgenic and the transgenic rice (data not shown).

Table 3-2. Pasting properties of non-transgenic and the transgenic rice

	Median particle size (μm)[b]	Starch damage (%)[c]	Apparent amylose content (%)[d]
Non-transgenic rice	100.7 ± 5.1	7.9 ± 0.1	14.2 ± 0.1
Transgenic rice	74.9 ± 2.4	8.6 ± 0.2	14.3 ± 0.1
Difference[a]	- 25.8**	0.7*	0.1

Pasting properties were measured in triplicate. [a] Difference between non-transgenic and the transgenic rice. *, **, Significantly different at 0.05 and 0.005 levels, respectively (*t*-test).

3-4 Discussion

In this study, the homozygous transgenic rice in T_4 generation was used to investigate the effect of PINB on the ultrastructure of the endosperm cells and physicochemical properties of the rice flour. The expression of *Pinb* gene was confirmed by RT-PCR and SDS-PAGE in T_4 generation as well as in T_2 generation as reported in Wada et al. (2009). The results indicated that the *Ae. tauschii* authentic promoter of *Pinb* gene can function in rice as a seed-specific promoter.

The transgenic rice endosperm exhibited a loosely packed structure under SEM. The spaces between compound starch granules were observed at the intercellularly cleaved site. In wheat, PINs have been reported to localize on the protein matrix and the surface of starch granules. They prevent the adhesion between starch granules, resulting in a softer grain texture (Morris 2002). Therefore, the loosely packed structure in the transgenic rice may suggest that PINB also functions in rice in the similar way as in wheat, preventing each compound starch granules from being packed tightly. However, the intracellularly cleaved site exposing the polygonal starch granules showed no clear differences between non-transgenic and the transgenic rice.

PINs also influence the sizes of each starch granule in wheat. Studies of different types of wheat granules indicated that softer textured flours have larger granules than hard textured flours (Gaines et al. 2000). In this study, the morphology and size distribution of isolated starch granules in the transgenic rice were found to be not different from those of non-transgenic rice. The differences of the effect of PINB on the sizes and morphology of isolated starch granules can be explained by the developmental differences between rice and wheat endosperm. In rice endosperm,

multiple polygonal granules develop within a single amyloplast. They are compressed together to form compound starch granules which have the appearance of a single granule. In wheat endosperm, each granule develops into individual amyloplasts. This structural difference could give the difference in PINB localization, providing the different effect on the isolated starch granules.

TEM observation clearly supports the results of SEM observation. The shape of each compound starch granules could easily be identified because the boundary of compound starch granules was clear. This would be the effect of PINB on the surface of the compound starch granules, preventing the adhesion between compound starch granules.

The difference of viscosity profile also could be attributed to the interaction of PINB with rice starch granules. The starch granule structure, lipid and protein in rice endosperm have been reported to affect the pasting property (Hamaker and Griffin 1993; Xie et al. 2008).The apparent amylose content was not different between non-transgenic and the transgenic rice, which indicates that the viscosity changes were not due to the change of apparent amylose content. Instead, the decrease of peak viscosity in the transgenic rice might be due to the association of PINB with the surface of compound starch granules. PINB localized to the starch surface could inhibit the access of water to starch or swelling of starch, which would lead to the decrease in viscosity during gelatinization in the transgenic rice.

The other well known characteristics of soft textured grain are the smaller flour particle size and less starch damage compared with hard textured grain (Bhave and Morris 2008; Brites et al. 2008). As expected, the transgenic rice showed smaller median flour particle size than non-transgenic rice. However, the starch damage was

similar between non-transgenic and transgenic rice. Usually flours with the smaller flour particle size have higher starch damage under the same milling conditions (Sun et al. 2007). In this study, the difference of flour particle sizes was significant (25.8 μm). Therefore, it could be possible that PINB suppressed the increase of starch damage in the transgenic rice otherwise the starch damage might be higher.

The observed characteristics of the transgenic rice mentioned above suggest that (1) the expression of *PinB* gene reduces the grain hardness in japonica rice and (2) PINB functions at the surface of compound starch granules, not of polygonal starch granules in rice endosperm.

The similar relationship between hardness and endosperm structure has been reported in wheat (Xia et al. 2008) and barley (Brennan et al. 1996). In wheat, knock-out of *PinA* resulted in hard texture, in which the starch granules have rougher appearance with more protein matrix adhered to surface than soft textured wheat. In barley, soft textured cultivars, which show good malting quality, have a lower degree of starch-protein binding than hard textured cultivar. Thus, the observed relationship between hardness and endosperm structure is common in these three grains.

Feiz et al. (2009) reported that PINs overexpression in wheat resulted in increased seed-bound polar lipids and hypothesized that PINs stabilize bound polar lipids on the surface of starch granule membranes preventing breakdown during seed desiccation and maturation. The polar lipids are considered to be from the ripened remnants of amyloplast. Thus, it suggests that PINs are related to the degradation of amyloplast membrane. Our results support their hypothesis because the association of PINs with lipids derived from amyloplasts should occur on the surface of compound starch granules in rice which has compressed multiple starch granules within a single

amyloplast. However, further experiments such as immunohistochemical staining using anti-PINB antibody will be necessary to confirm the localization of PINB in rice endosperm.

This is the first report that investigates the ultrastructure of endosperm and physicochemical properties of japonica rice expressing *Pinb* gene. Krishnamurthy and Giroux (2001) introduced *Pinb* gene into japonica rice cultivar M202 and observed differences in flour particle size distributions and starch damages. Comparing their work with our present study, differences in the results could be attributed to the differences in the promoter used and the grinding method employed. In this study, I used the *Ae. tauschii Pinb* promoter, while Krishnamurthy's group used the maize ubiquitin promoter, giving a different expression level of *Pinb* gene. The maize ubiquitin promoter is the promoter which shows high-level gene expression in monocot cells (Christensen and Quail 1996). Unlike the maize ubiquitin promoter, the *Ae. tauschii Pinb* promoter showed the endosperm-specific expression as well as *T. aestivum Pinb* promoter did (Digeon et al. 1999). Furthermore, I applied a stronger grinding method than the method they used, resulting in smaller flour particle sizes and higher starch damage.

The japonica rice mutant, Suweon 464 (Kim et al. 2004), a waxy rice variety (Ibanez et al. 2007), and a brewer's rice (Tamaki et al. 2007) also have the spaces between starch granules as in the transgenic rice obtained in this study. However, some properties such as the morphology of isolated starch granules, ultrastructure of endosperm cells and viscosity profile during gelatinization are distinct to this transgenic rice obtained. Thus, the transgenic rice reported here has the novel characteristics and could possess different processing properties from the cultivated rice reported so far. The creation of such variations in rice should make it possible to develop new end

products and food uses. Furthermore, the same strategy could be applied to manipulate the endosperm texture of other cereals. The softer texture and smaller flour particle sizes in cereals would have the advantages in such as saving the milling costs and increasing the feed efficiency in cattle and broiler chickens (Carr et al. 2002; Swan et al. 2006). The successful manipulation of endosperm texture of cereals would contribute to yield numerous benefits for the future agriculture and food industry.

3-5 Summary

Endosperm texture is an important factor to determine the end-product quality. The texture of wheat (*Triticum aestivum* L.) endosperm is controlled by *Pina* and *Pinb* genes which are both absent in rice (*Oryza sativa* L.). It has been reported that the endosperm texture of rice can be modified by *Pin* genes. The mechanism, however, by which puroindolines affect the ultrastructure of rice endosperm cells remains to be investigated. In this study, we observed the ultrastructure of endosperm cells and the morphology of isolated starch granules of the transgenic rice expressing the *Pinb* gene. SEM and TEM observations indicated that compound starch granules were embedded within the matrix material in non-transgenic rice, Nipponbare, whereas they were surrounded by spaces in the transgenic rice. The morphology and size of each starch granule were not different between non-transgenic and the transgenic rice. However, the transgenic rice flour showed smaller particle size, higher starch damage, and lower viscosity during gelatinization than that of non-transgenic rice. These results confirm that PINB reduces the grain hardness in rice. Moreover, the results also suggest that PINB functions at the surface of compound starch granules, and not of polygonal starch granules in rice endosperm.

Chapter 4

General conclusion

In this study, a transgenic rice which has a softer endosperm texture has been produced using the bioactive beads-mediated transformation. The large DNA fragments containing a set of *Ae. tauschii* hardness genes (*Pina*, *Pinb*, *GSP-1*) were introduced into rice genome and the effects of the *Pinb* gene on the ultrastructure of endosperm cells and rice flour were investigated.

In Chapter 2, it was mentioned that the bioactive beads method has a potential to be a powerful tool to introduce large DNA fragments into rice. The PCR, Southern blot, and FISH analyses indicated that the multiple transgenes were introduced simultaneously and inherited into the next generations successfully. Segregation analysis revealed that the homozygous transgenic rice (plant 9-1-6-3) was obtained. In the homozygous transgenic rice, the BAC DNA was integrated at the telomeric region of chromosomes. The expression of *Pinb* gene was confirmed by RT-PCR and mass spectrometry. The results mentioned above indicate that the bioactive beads method can be used to transfer the large DNA fragments into rice. The endosperm-specific expression of *Pinb* gene was shown in the T_4 generation as described in Chapter 3. The strategy applied in this study is to introduce the authentic genomic region concerned with the grain hardness into plants. This genomic region also contains the native promoter region for each gene. The endosperm-specific expression of transgene indicates that the strategy is applicable to produce the transgenic plants with tissue-specific expression of transgenes.

In Chapter 3, the endosperm structure of transgenic rice expressing *Pinb* was observed under SEM and TEM. The transgenic plants showed the loosely packed endosperm structure in which the compound starch granules were surrounded by air space instead of protein matrix. The results suggest that PINB functions at the surface of

compound starch granules, thus, preventing the adhesion between each compound starch granules. The studied characteristics of transgenic rice flour (smaller flour particles than those of Nipponbare, suppression of starch damage increase in transgenic rice) showed that the transgenic plants have a softer grain texture than Nipponbare. Based on the SEM and TEM observations, the soft texture has been considered to be the result of weak adhesion between compound starch granules.

Due to the lack of variation in rice grain texture compared with wheat grain, the application of rice flour has been limited. The introduction of hardness genes is one of the good approaches to make more variation in rice grain texture because non-transgenic rice does not contain hardness genes. The soft textured rice would have different processing properties and the higher quality as animal feed. In addition, the successful manipulation of endosperm texture in cereals using hardness genes would contribute to yield and benefit for the future agriculture.

My study has raised the possibility that the bioactive beads method facilitates the production of transgenic plants with large DNA fragments. The bioactive beads method does not require the construction of binary vectors which are required for *Agrobacterium*-mediated transformation. Therefore, the BAC clones in the libraries which have been established already can directly be used for bioactive bead-mediated transformation.

Therefore, it is concluded that the bioactive beads method has a potential to be a powerful tool to introduce large DNA fragments into plants and it can produce a novel transgenic plants, such as the soft textured rice which would possess different processing properties and applications, as indicated by the results obtained in this study.

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