Plant Biotechnology 31, 67–70 (2014)
DOI: 10.5511/plantbiotechnology.13.1028a

Note

Localization of transgene-derived friabilins in rice endosperm cells

Misa Fujiwara[1], Go Suzuki[1,*], Daiki Kudo[1], Haruna Oba[1], Yukio Wada[1], Hideo Wada[1], Naoki Wada[2], Sadequr Rahman[3], Kiichi Fukui[2], Yasuhiko Mukai[1]

[1]Division of Natural Science, Osaka Kyoiku University, Kashiwara, Osaka 582-8582, Japan; [2]Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan; [3]School of Science, Monash University, Sunway Campus, Jalan Lagoon Selatan, Bandar Sunway, 46150 Petaling Jaya, Malaysia
*E-mail: gsuzuki@cc.osaka-kyoiku.ac.jp Tel & Fax: +81-72-978-3660

Received September 6, 2013; accepted October 28, 2013 (Edited by C. Matsukura)

Abstract Puroindoline-a (*Pina*), puroindoline-b (*Pinb*) genes in the wheat *hardness*-locus region encode 15-kDa friabilin proteins, whose accumulation in the endosperm leads to grain softness texture. In wheat, the PINA and PINB friabilins are associated with starch granules in the endosperm cell, while there is no friabilin in rice. The rice endosperm structure consisting of compound starch granules is fundamentally different from that in wheat. We previously produced two different lines of transgenic rice plants with the large genomic fragment including *Pina* or *Pinb* of *Aegilops tauschii*. However, localization of exogenous friabilins in the rice endosperm cell still remains to be determined. In the present study, we stacked the two different transgenic rice lines. The F_4 seeds of the stacked line, in which the homozygosity of the *Pina* and *Pinb* transgenes was checked by FISH analysis, were used for histochemical analysis of the endosperm cell. Immunodetection of PINA and PINB proteins using the Durotest antibody showed that wheat-derived friabilins were localized between compound starch granules as well as between starch granules in the rice endosperm cell. This suggests that such localization of the friabilins might prevent tight interaction between the compound starch granules and between the starch granules in the rice endosperm, leading to its soft texture.

Key words: Compound starch granule, friabilin, grain hardness, immunohistochemical analysis, *Oryza sativa*.

Grain hardness of wheat is an important agricultural trait, which is regulated by the *hardness* (*Ha*)-locus region (Bhave and Morris 2008; Pasha et al. 2010). There are three friabilin genes, *puroindoline-a* (*Pina*), *puroindoline-b* (*Pinb*), and *Grain Softness Protein-1* (*GSP-1*), in the *Ha* locus region on the chromosome arm 5DS. Friabilins are ca. 15-kDa lipid-binding proteins stored in seeds of soft wheat, and are considered to be associated with starch granules in seeds, resulting in the soft kernel texture of wheat. Molecular genetic studies of the friabilin genes in different types of wheat have suggested that PINA is a major contributor of grain softness (Chen et al. 2006; Giroux et al. 2000), and functions with PINB (Amoroso et al. 2004). *Puroindoline* genes are expressed in starchy endosperm cells, and their products are developmentally accumulated in the endosperm (Digeon et al. 1999; Turnbull et al. 2003a; Wiley et al. 2007). PINA and PINB proteins are co-localized to the starch granule surface in the wheat endosperm, possibly due to the association of positively charged friabilins with polar lipids on the surface of starch (Feiz et al. 2009).

Rice (*Oryza sativa*) with hard grain texture is best used as a host cereal plant for transgenic experiments with the friabilin-related genes. The puroindoline genes are absent in the rice genome, while genes at the boundaries of the *Ha* locus are conserved between rice and wheat (Chantret et al. 2005). It has been demonstrated that the *Pina* and *Pinb* transgenes derived from wheat are involved in the soft kernel texture in transgenic rice plants harboring wheat *Pina* and/or *Pinb* cDNA driven by the ubiquitin promoter (Krishnamurthy and Giroux 2001). We also produced the transgenic rice plants with the large genomic region containing *Pina* and *GSP-1* genes (a BAC 8 region) by *Agrobacterium*-mediated transformation (Nakano et al. 2005; Suzuki et al. 2011), or plants with a BAC 10 region containing *Pinb* and *GSP-1* genes by bioactive beads-mediated transformation (Wada et al. 2009, 2010). The BAC 8 and BAC 10 regions are derived from the *Ha*-locus of the D genome donor of wheat, *Aegilops tauschii*, which is a provider of soft texture in wheat (Chantret et al. 2005; Turnbull et al. 2003b).

Abbreviations: EM, electron microscopy; FISH, fluorescence in situ hybridization; *GSP-1*, *Grain Softness Protein-1*; *Ha*, *hardness*; HRP, horseradish peroxidase; *Pina*, *puroindoline-a*; *Pinb*, *puroindoline-b*; PCR, Polymerase Chain Reaction.

This article can be found at http://www.jspcmb.jp/
Published online December 12, 2013

Protein electrophoresis and electron microscopy (EM) analyses of endosperms of these transgenic rice plants with BAC 8 or BAC 10 have suggested that the transgenes-derived friabilins might be localized between compound starch granules, and more space might be created between them (Suzuki et al. 2011; Wada et al. 2010). However, differences of endosperm structure between the transgenic and non-transgenic rice plants were less obvious than those reported between hard and soft wheat plants (Turnbull et al. 2003a). The structure of the rice endosperm is fundamentally different from that of wheat. In the rice endosperm cell, dozens of starch granules with a polyhedral shape are tightly packed in the amyloplast and form an ellipsoidal compound starch granule (Zhou et al. 2002), while individual starch granules with spherical and disc shapes are found within the protein matrix in the mature wheat endosperm cell. Therefore, the precise localization of wheat friabilins in the rice endosperm cell is of interest. Its localization can be determined by histochemical analysis which would help to understand their functions in the transgenic rice plants. Here we performed immunohistochemical analysis using plastic-embedded sections of the transgenic rice endosperm cells to determine whether exogenous friabilins are localized between starch granules and/or compound starch granules.

Producing homozygous plants with both Pina and Pinb transgenes

First, we stacked the two transgenic rice lines, N and 9-1-6, which possess *Pina* and *Pinb*, respectively (Figure 1). The N-derived T_4 plant is homozygous for the BAC 8 region with cv. Yamahoushi background (Nakano et al. 2005; Suzuki et al. 2011), while the 9-1-6-derived T_4 plant is homozygous for the BAC 10 region with cv. Nipponbare background (Wada et al. 2009, 2010). The N-derived T_4 plant was crossed with pollen from the 9-1-6-derived T_4 plant, and a F_1 plant hemizygous for both *Pina* and *Pinb* loci was obtained (Figure 1). Selfing of the F_1 plant resulted in production of segregated F_2 plants. We selected a F_2 plant (F_2homo) homozygous for both *Pina* and *Pinb*, determined by polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) analyses (data not shown). In the selfed progeny of F_2homo, two F_3 plants, F_3homo-6 and F_3homo-11, were confirmed to be homozygous for *Pina* and *Pinb* by FISH analysis (data not shown). PCR analysis demonstrated that F_3homo-6 and F_3homo-11 possessed both *Pina* and *Pinb* genes (Figure 2A). For the further experiments in this study, we used two F_4 seeds, F_4homo-6 and F_4homo-11, derived from the F_3homo-6 and F_3homo-11 plants, respectively (Figure 1). The embryo part of the seeds was cut off and grown on the MS medium (Murashige and Skoog 1962), and the roots were subjected to FISH analysis, which demonstrated

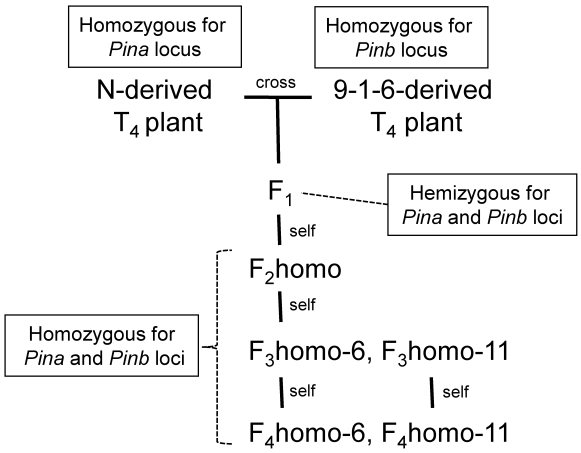

Figure 1. Breeding scheme of the rice lines used in this study.

that the two transgenes were actually homozygous in F_4homo-6 and F_4homo-11 (Figure 2B). The remaining endosperm part of the seeds was fixed with 4% paraformaldehyde/PBS at 4°C and used for further histochemical analysis.

Immunolocalization of PINA and PINB proteins in the rice endosperm cell

After dehydration, the fixed seeds were embedded in Technovit 8100 (Heraeus Kulzer). The embedded seeds were cut, and sections of 5 μm thickness were used for Lugol's iodine staining and/or immunohistochemical analysis (Figures 2C–F). The structure of the compound starch granules in the rice endosperm cell was clearly observed in the Lugol's iodine staining (Figure 2C). The structure of the compound starch granules in the transformant (F_4homo-11) was not significantly different from that in untransformants (Nipponbare and Yamahoushi are cultivars of the parent donors of the transgenic line), although the slightly additional space between starch granules and compound starch granules was observed in some endosperm cells of F_4homo-11 (Figure 2C). Previously, we found more space between compound starch granules in the transgenic plants in EM analyses (Suzuki et al. 2011; Wada et al. 2010), whose resolution is much higher than that determined by light microscopy analysis in this study.

In immunohistochemical analysis, we used a Durotest monoclonal antibody (R-Biopharm Rhone Ltd.) to detect both PINA and PINB proteins. Because this mouse anti-friabilin antibody is labeled with horseradish peroxidase (HRP), FITC-conjugated AffiniPure Rabbit Anti-Horseradish Peroxidase (Jackson ImmunoResearch) or Alexa Flour 555 goat anti-mouse IgG (Invitrogen) was used as a secondary antibody to detect it. Figure 2D shows the results of immunodetection of PINA and PINB in the rice endosperm cells using the FITC Anti-HRP antibody as the secondary antibody. The strong FITC signals were obviously observed in the endosperm cells

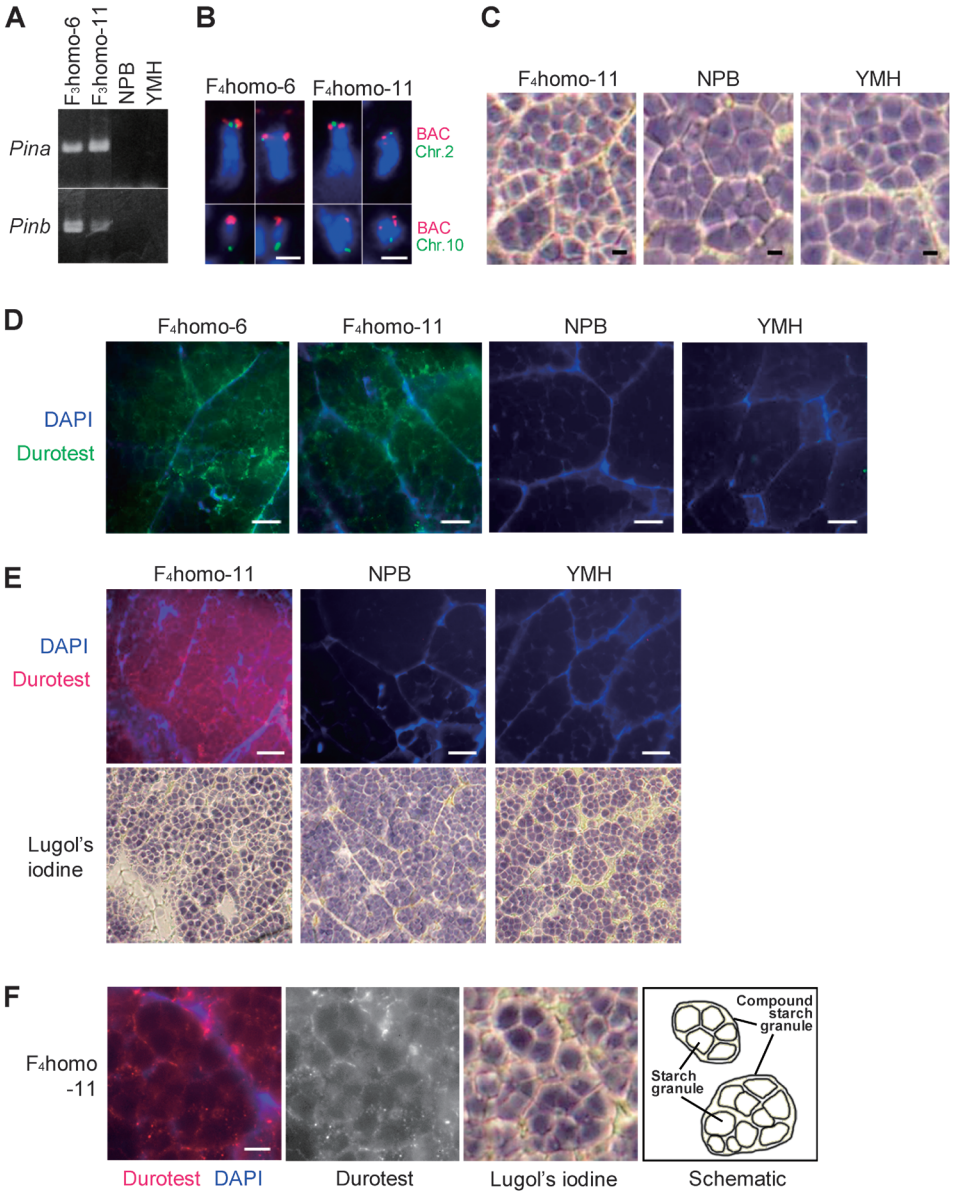

Figure 2. Characterization of the stacked transgenic rice plants homozygous for both *Pina* and *Pinb* transgenes. (A) Existence of *Pina* and *Pinb* transgenes in the F_3 plants. Genomic PCR was performed with a Pina primer set (5′-ATGAAGGCCCTCTTCCTCATAGG-3′, 5′-TCACCAGTAATA GGCAATAGTGCC-3′) or with a Pinb primer set (5′-ATGAAGACCTTATTCCTCCTA-3′, 5′-TCACCAGTAATAGCCACTAGGGAA-3′) for 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C followed by a final extension for 5 min. About 450-bp amplified fragments of *Pina* or *Pinb* were observed in transgenic lines (F_3homo-6 and F_3homo-11) and not in untransformants (NPB, Nipponbare; YMH, Yamahoushi); (B) FISH detection of the homozygous BAC 8 and BAC 10 fragments integrated into different chromosomes in the F_4 plants (F_4homo-6 and F_4homo-11). Chromosomal preparation, probe labeling, and FISH analysis were performed according to standard protocols as described in Suzuki et al. (2012). BAC 8 and BAC 10 were detected as red rhodamine signals, and chromosome markers were detected as green FITC signals. The bars represent 2 μm; (C) Lugol's iodine staining of the Technovit 8100 section of the endosperm of the transgenic rice plant (F_4homo-11) and untransformants (NPB, Nipponbare; YMH, Yamahoushi). The bars represent 3 μm; (D) Immunodetection of friabilins (green) using the Technovit 8100 sections of the rice endosperm in the F_4 plants (F_4homo-6 and F_4homo-11) and untransformants (NPB, Nipponbare; YMH, Yamahoushi). Durotest monoclonal antibody raised against friabilins was detected as green fluorescence using the FITC Anti-HRP. DAPI staining (blue) was used for visualizing the cell shape. The bars represent 20 μm; (E) Comparison of immunostaining and Lugol's iodine staining in the same field of the same section. Durotest monoclonal antibody was detected as red fluorescence using the Alexa Flour 555 anti-mouse IgG. DAPI staining (blue) was used for visualizing the cell shape. The bars represent 20 μm. F_4homo-11, the F_4 plant; NPB, Nipponbare; YMH, Yamahoushi; (F) The enlarged picture showing localization of friabilins (red) in F_4homo-11. The Durotest signal only is indicated as a black-and-white data in the same field. Starch was stained with Lugol's iodine to visualize a structure of the rice endosperm. Schematic representation of compound starch granules with starch granules is also shown. The bar represents 2 μm.

of F_4homo-6 and F_4homo-11, but not in untransformed plants. Similar results were obtained when using Alexa Flour 555 anti-mouse IgG (Figure 2E). These results indicated that PINA and PINB proteins were highly accumulated in the endosperm cells of the transgenic rice seed.

To understand additional details about localization of PINA and PINB proteins in the endosperm cell, we compared the results of immunostaining and Lugol's iodine staining in the same field of the microscope (Figures 2E, F). The enlarged Figure 2F clearly shows that Durotest signals were observed between compound starch granules as well as between starch granules in the endosperm of F_4homo-11. Thus, wheat PINA and PINB proteins might be localized on surfaces of both starch granules and compound starch granules in the rice endosperm. Positively charged PINA and PINB could be associated with the negatively charged membrane surface of the amyloplast, which forms the compound starch granule. This finding of possible localization of friabilins on the surface of compound starch granules is consistent with our previous EM observation of additional space between the compound starch granules in the transgenic rice plants (Suzuki et al. 2011; Wada et al. 2010). Hence, the mechanism of reduction of grain hardness in the transgenic rice plants harboring the *Pina* and/or *Pinb* cDNA (Krishnamurthy and Giroux 2001) might be as follows: the exogenous friabilins localized on the surface of compound starch granules as well as on starch granules prevent tight interaction between them, leading to the soft texture of the rice endosperms.

Acknowledgements

We thank Hiromi Masuko-Suzuki (Tohoku University) for her helpful advice on preparation of Technovit sections. This work was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) and Japan Society for the Promotion of Science (JSPS) grants, KAKENHI (20780240, 23113006 and 25450515 to G.S., 22580004 and 19380194 to Y.M.).

References

Amoroso MG, Longobardo L, Capparelli R (2004) Real time PCR and flow cytometry to investigate wheat kernel hardness: role of puroindoline genes and proteins. *Biotechnol Lett* 26: 1731–1737

Bhave M, Morris CF (2008) Molecular genetics of puroindolines and related genes: allelic diversity in wheat and other grasses. *Plant Mol Biol* 66: 205–219

Chantret N, Salse J, Sabot F, Rahman S, Bellec A, Laubin B, Dubois I, Dossat C, Sourdille P, Joudrier P, et al. (2005) Molecular basis of evolutionary events that shaped the *hardness* locus in diploid and polyploid wheat species (Triticum and Aegilops). *Plant Cell* 17: 1033–1045

Chen F, He Z, Xia XC, Xia LQ, Zhang XY, Lillemo M, Morris CF (2006) Molecular and biochemical characterisation of puroindoline a and b alleles in Chinese landraces and historical cultivars. *Theor Appl Genet* 112: 400–409

Digeon JF, Guiderdoni E, Alary R, Michaux-Ferrière N, Joudrier P, Gautier MF (1999) Cloning of a wheat puroindoline gene promoter by IPCR and analysis of promoter regions required for tissue-specific expression in transgenic rice seeds. *Plant Mol Biol* 39: 1101–1112

Feiz L, Wanjugi HW, Melnyk CW, Altosaar I, Martin JM, Giroux MJ (2009) Puroindolines co-localize to the starch granule surface and increase seed bound polar lipid content. *J Cereal Sci* 50: 91–98

Giroux MJ, Talbert L, Habernicht DK, Lanning S, Hemphill A, Martin JM (2000) Association of puroindoline sequence type and grain hardness in hard red spring wheat. *Crop Sci* 30: 370–374

Krishnamurthy K, Giroux M (2001) Expression of wheat puroindoline genes in transgenic rice confers grain softness. *Nat Biotechnol* 19: 162–166

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Nakano A, Suzuki G, Yamamoto M, Turnbull K, Rahman S, Mukai Y (2005) Rearrangements of large-insert T-DNAs in transgenic rice. *Mol Genet Genomics* 273: 123–129

Pasha I, Anjum FM, Morris CF (2010) Grain hardness: a major determinant of wheat quality. *Food Sci Technol Int* 16: 511–522

Suzuki G, Ogaki Y, Hokimoto N, Xiao L, Kikuchi-Taura A, Harada C, Okayama R, Tsuru A, Onishi M, Saito N, et al. (2012) Random BAC FISH of monocot plants reveals differential distribution of repetitive DNA elements in small and large chromosome species. *Plant Cell Rep* 31: 621–628

Suzuki G, Wada H, Goto H, Nakano A, Oba H, Deno T, Rahman S, Mukai Y (2011) Transgenic rice plants harboring the grain hardness-locus region of *Aegilops tauschii*. *Plant Cell Rep* 30: 2293–2301

Turnbull K-M, Marion D, Gaborit T, Appels R, Rahman S (2003a) Early expression of grain hardness in the developing wheat endosperm. *Planta* 216: 699–706

Turnbull K-M, Turner M, Mukai Y, Yamamoto M, Morell MK, Appels R, Rahman S (2003b) The organization of genes tightly linked to the *Ha* locus in *Aegilops tauschii*, the D-genome donor to wheat. *Genome* 46: 330–338

Wada N, Kajiyama S, Akiyama Y, Kawakami S, No D, Uchiyama S, Otani M, Shimada T, Nose N, Suzuki G, et al. (2009) Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes. *Plant Cell Rep* 28: 759–768

Wada N, Kajiyama S, Cartagena JA, Lin L, Akiyama Y, Otani M, Suzuki G, Mukai Y, Aoki N, Fukui K (2010) The effects of puroindoline b on the ultrastructure of endosperm cells and physicochemical properties of transgenic rice plant. *J Cereal Sci* 51: 182–188

Wiley PR, Tosi P, Evrard A, Lovegrove A, Jones HD, Shewry PR (2007) Promoter analysis and immunolocalisation show that puroindoline genes are exclusively expressed in starchy endosperm cells of wheat grain. *Plant Mol Biol* 64: 125–136

Zhou Z, Robards K, Helliwell S, Blanchard C (2002) Composition and functional properties of rice. *Int J Food Sci Technol* 37: 849–868