Localization of transgene-derived friabilins in rice endosperm cells

Misa Fujiwara1, Go Suzuki1,*, Daiki Kudo1, Haruna Oba1, Yukio Wada1, Hideo Wada1, Naoki Wada2, Sadequr Rahman3, Kiichi Fukui2, Yasuhiko Mukai1

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 F1 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
Friabilin localization in rice endosperm

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 T4 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 T4 plant is homozygous for the BAC 10 region with cv. Nipponbare background (Wada et al. 2009, 2010). The N-derived T4 plant was crossed with pollen from the 9-1-6-derived T4 plant, and a F1 plant hemizygous for both *Pina* and *Pinb* loci was obtained (Figure 1). Selfing of the F1 plant resulted in production of segregated F2 plants. We selected a F2 plant (F2homo) 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 F2homo, two F3 plants, F3homo-6 and F3homo-11, were confirmed to be homozygous for *Pina* and *Pinb* by FISH analysis (data not shown). PCR analysis demonstrated that F3homo-6 and F3homo-11 possessed both *Pina* and *Pinb* genes (Figure 2A). For the further experiments in this study, we used two F3 seeds, F3homo-6 and F3homo-11, derived from the F2homo-6 and F2homo-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 that the two transgenes were actually homozygous in F3homo-6 and F3homo-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 (F3homo-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 F3homo-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 ImmunolResearch) 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.
of F4homo-6 and F4homo-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.

Copyright © 2014 The Japanese Society for Plant Cell and Molecular Biology
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$_2$homo-11. Thus, wheat PINA and PINB proteins might be visualized 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., 19380194 to Y.M.).

References