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Note

The localization of rice prolamin species in protein body type I is determined by the temporal control of gene expression of the respective prolamin promoters

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Abstract Rice prolamin species form a layered structure in the protein body type I (PB-I) storage organelle. Rice prolamins are classified as 10 kDa, 13a-1, 13a-2, 13b-1, 13b-2 and 16 kDa prolamin. Prolamin species form layer structure in PB-I in order of 10 kDa core, 13b-1 layer, 13a (13a-1 and 13a-2) and 16 kDa middle layer and 13b-2 outer-most layer. In a previous study, we showed that the fusion proteins in 13b-2 prolamin-GFP, 13a-1 prolamin-GFP and 10 kDa prolamin-GFP were localized in the same layer of PB-I as the native prolamin, when they were expressed by their respective native prolamin promoters. Our preliminary study suggested that the temporal control of the native prolamin promoters was responsible for the localization of the respective prolamins. The aim of this study was to determine whether the use of a prolamin promoter other than the native prolamin promoter would change the localization of prolamin-GFP fusion proteins. For this purpose, we generated transgenic lines expressing 13b-2 prolamin-GFP and 13a-1 prolamin-GFP fusion proteins driven by each prolamin promoter other than the native prolamin promoter. As a result, the localization of the fusion protein in PB-I was changed. Based on our results, foreign protein localization in PB-I can be achieved by the temporal control of the different prolamin promoters.

Key words: green fluorescent protein, layer structure, prolamin, prolamin promoter, protein body type I, rice.

Rice prolamins, alcohol-soluble proteins in the rice seed, are synthesized in the endoplasmic reticulum (ER) and form protein body type I (PB-I) in endosperm cells (Tanaka et al. 1980; Yamagata and Tanaka 1986; Yamagata et al. 1982). Rice prolamins are classified by size as 10 kDa, 13 kDa and 16 kDa (Masumura et al. 1989; Mitsukawa et al. 1999a, b). In addition, 13 kDa prolamins are subdivided into Cys-rich 13a prolamin (13a-1 and 13a-2) and Cys-less 13b prolamin (13b-1 and 13b-2) (Mitsukawa et al. 1999a; Saito et al. 2012). Previously, we reported that these prolamin species form a layered structure in PB-Is, with the 10 kDa prolamin at the core of PB-I, the 13b-1 prolamin in the inner layer surrounding the 10 kDa prolamin core, 16 kDa and 13a prolamin in the middle layer, and 13b-2 prolamin in the outer-most layer of PB-I (Saito et al. 2012).

The formation of a layered structure in PB-Is was suggested to be dependent on the gene expression patterns of the respective prolamin species in rice seeds. We previously demonstrated that during development of rice seeds, the 10 kDa prolamin gene was expressed first, followed shortly by the 13b-1 prolamin gene, then the 16 kDa and 13a prolamin genes, and finally the 13b-2 prolamin gene (Saito et al. 2012). The location of the various respective prolamin polypeptides in PB-I coincided with the temporal expression patterns of their respective genes. We previously found that when the 13b-2 prolamin-GFP, 13a-1 prolamin-GFP and 10 kDa prolamin-GFP fusion proteins were expressed by their native promoters, each prolamin-GFP fusion protein was localized in the same layer in PB-Is as the native prolamin (Saito et al. 2012; Sasou et al. 2016). Based on these previous studies, the control of the respective prolamin promoters appears to be important for the localization of prolamin in PB-I.

Therefore, in this study, to investigate in more detail the relationship between the temporal control of prolamin promoters and the localization of prolamin species in PB-Is, we generated and analyzed transgenic rice seeds expressing 13b-2 prolamin-GFP fusion

Abbreviations: PB-I, protein body type I; GFP, green fluorescent protein; ER, endoplasmic reticulum.

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proteins under the control of the 13a-1 prolamin and 10 kDa prolamin promoters, and expressing 13a-1 prolamin-GFP fusion proteins under the control of the 13b-2 prolamin and 10 kDa prolamin promoter, respectively (Figure 1).

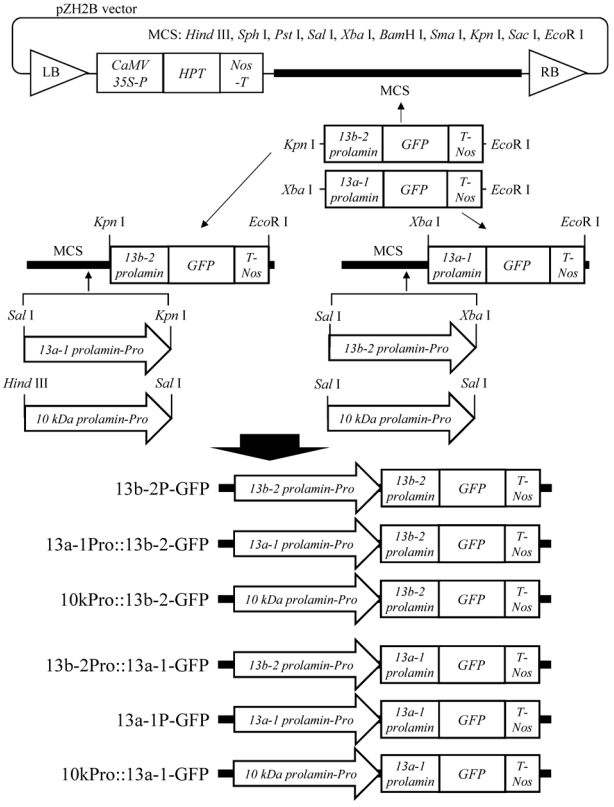

Figure 1. The flow diagram of constructions expressing 13b-2 prolamin-GFP fusion protein and 13a-1 prolamin-GFP fusion protein under the control of various prolamin promoters. 13b-2P-GFP and 13a-1P-GFP are transgenic lines expressing 13b-2 prolamin-GFP and 13a-1 prolamin-GFP fusion prolamin respectively, which were generated previously (Sasou et al. 2016). MCS, multiple cloning site; CaMV35S-P, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase; Nos-T, nopaline synthase terminator; 13b-2 prolamin-Pro, 13b-2 prolamin promoter; 13a-1 prolamin-Pro, 13a-1 prolamin promoter; 10 kDa prolamin-Pro, 10 kDa prolamin promoter; GFP, green fluorescent protein.

Constructions expressing 13b-2 prolamin-GFP fusion protein and 13a-1 prolamin-GFP fusion protein driven by the respective native promoters were generated previously (Sasou et al. 2016). Constructions expressing 13b-2 prolamin-GFP fusion protein and 13a-1 prolamin-GFP fusion protein driven by other, non-native prolamin promoters were generated based on the pZH2B vector (Kuroda et al. 2010). Respective sequences of the promoter region (1500 bp from transcriptional initiation point) were amplified from genomic DNA. The coding regions for 13b-2 prolamin (*λRM4*), 13a-1 prolamin (*λRM1*) and 10 kDa prolamin (*λRP10*) were amplified from the 13b-2P-GFP, 13a-1P-GFP and 10kP-GFP vectors constructed previously (Sasou et al. 2016).

The DNA fragments of 13b-2 prolamin-GFP-NOS (containing *Kpn*I and *Eco*RI site) and 13a-1 prolamin-GFP-NOS (containing *Xba*I and *Eco*RI site) were amplified from 13b-2P-GFP and 13a-1P-GFP (Sasou et al. 2016) as the template using the primer set shown in Table 1. PCR fragments were digested with *Kpn*I/*Xba*I and *Eco*RI, and inserted in the multiple cloning site of a pZH2B vector (Figure 1). The promoter regions of the 13b-2 prolamin, 13a-1 prolamin and 10 kDa prolamin were also amplified from 13b-2P-GFP, 13a-1P-GFP and 10 kP-GFP (Sasou et al. 2016) using the primer set in Table 1. PCR fragments of the 13b-2 prolamin promoter (containing the *Sal*I and *Xba*I sites) and 13a-1 prolamin promoter (containing the *Sal*I and *Kpn*I sites) were digested with an adequate combination of restriction endonucleases and inserted in the upstream of the each prolamin-GFP-NOS sequence in the pZH2B vector (Figure 1). The PCR fragment of 10 kDa prolamin promoter (containing the *Hind*III and *Sal*I sites) was digested with an adequate combination restriction endonucleases and inserted in the upstream of the 13b-2 prolamin-GFP in pZH2B vector (Figure 1). Besides, The PCR fragment of 10 kDa prolamin promoter (containing the *Sal*I and *Sal*I sites) was also digested with *sal*I and inserted the upstream of 13a-1 prolamin-GFP in pZH2B vector because there is the *Hind*III site in the cording region of 13a-1 prolamin (Figure 1). The

Table 1. Primer sequences used to generate the constructs expressing the prolamin-GFP fusion proteins under the control of other prolamin promoters.

Primer name	Upstream and downstream primer sequences
13b-2-GFP T-Nos fw+*Kpn*I	5′-aggggtaccgcattatacagcaaaataga-3′
13a-1-GFP T-Nos fw+*Xba*I	5′-atatctagagttgaagcatagtagtagaatcc-3′
T-Nos rv+*Eco*RI	5′-agagaattcccgatctagtaacatagatg-3′
13b-2 prolamin promoter fw+*Sal*I	5′-gtcgactgtccatcattcctaacaagagg-3′
13b-2 prolamin promoter rv+*Xba*I	5′-atatctagactgtgtgaacgatgaggagg-3′
13a-1 prolamin promoter fw+*Sal*I	5′-aaagtcgacgaggagggtcagctgtggc-3′
13a-1 prolamin promoter rv+*Kpn*I	5′-ataggtacctgtgtgaaggatgaggaggg-3′
10 kDa prolamin promoter fw+*Hind*III	5′-ataaagcttgacgggatcacgatctggg-3′
10 kDa prolamin promoter fw+*Sal*I	5′-atagtcgacgacgggatcacgatctggg-3′
10 kDa prolamin promoter rv+*Sal*I	5′-atagtcgacggaataatggttgcctaggg-3′

orientation of 10 kDa prolamin promoter (containing the *Sal*I and *Sal*I sites) was confirmed using the forward primer which binds to the interior of 10 kDa prolamin promoter (5′-CGG TCT AGA TGA CTA GAT TG-3′) and the reverse primer which binds to the interior of GFP (5′-GTA GGT GAA GGT GGT CAC GA-3′) by PCR, after then confirmed by sequence analysis. We named the resulting binary vectors 13a-1Pro::13b-2-GFP, 10kPro::13b-2-GFP, 13b-2Pro::13a-1-GFP, and 10kPro::13a-1-GFP (Figure 1). These binary vectors were introduced into rice calli using an *Agrobacterium*-mediated method (Hiei et al. 1994). The rice calli containing the transgene were selected by hygromycin B (Nacalai Tesque, Kyoto, Japan) and transferred to redifferentiation medium. Then, the shoots from selected calli were transferred to plant pots containing soil in a naturally illuminated temperature-controlled (28°C) greenhouse of the Biotechnology Research Department, Kyoto Prefectural Agriculture, Forestry, and Fisheries Technology Research Center, Japan.

To clarify the localization of prolamin-GFP fusion proteins in each transgenic rice line, the observation of frozen sections prepared with mature transgenic rice seeds was performed by confocal laser microscopic analysis. The fluorescent observation was performed as previously described (Saito et al. 2008; Sasou et al. 2016). Briefly, frozen sections (5-μm thick) of each transgenic rice seed were prepared by the method developed by Saito et al. (2008), and were stained with 10-nM rhodamine B, which is commonly used to stain the peripheral region of PB-Is, for 10 min at room temperature. The sections stained with rhodamine B were observed using a confocal laser scanning microscope (A1 confocal imaging system; Nikon Instech, Tokyo).

In transgenic rice of the 13b-2P-GFP line, the 13b-2 prolamin-GFP fusion protein was observed in the outer-most layer of PB-Is (Figure 2A–C). In the 13a-1Pro::13b-2-GFP line, the prolamin-GFP fusion proteins were observed in the middle layer of PB-I (Figure 2D–F). In the 10kPro::13b-2-GFP line, the prolamin-GFP fusion proteins were mainly observed in the core region of PB-Is, as small spots, although a small number were also observed in the layer surrounding the core region of PB-Is (Figure 2G–I). Although the same fusion protein was expressed in each transgenic rice line, the localization of the fusion protein was changed by the type of prolamin promoter.

Similar results were obtained with transgenic rice lines expressing the 13a-1 prolamin-GFP fusion protein. In the 13b-2Pro::13a-1-GFP line, the 13a-1 prolamin-GFP fusion proteins were observed in the outer-most layer of PB-Is (Figure 3A–C). In the 13a-1P-GFP line, the GFP signal was observed as the ring shape in PB-I and the prolamin-GFP fusion proteins were seemed to

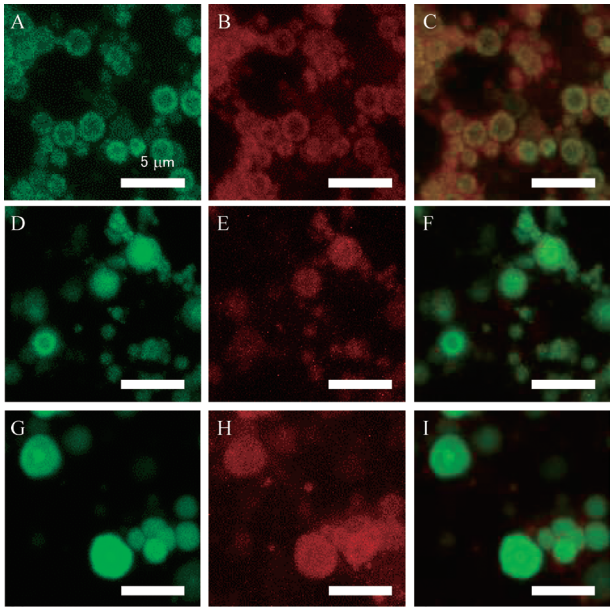

Figure 2. Localization of 13b-2 prolamin-GFP fusion proteins under the control of promoters derived from 13b-2 prolamin (A–C), 13a-1 prolamin (D–F) and 10 kDa prolamin (G–I). Green indicates the GFP signal (A, D, G), and red indicates the signal of rhodamine B, which stains the peripheral region of PB-Is (B, E, H). C, F, I indicate the merged images. Bars=5 μm.

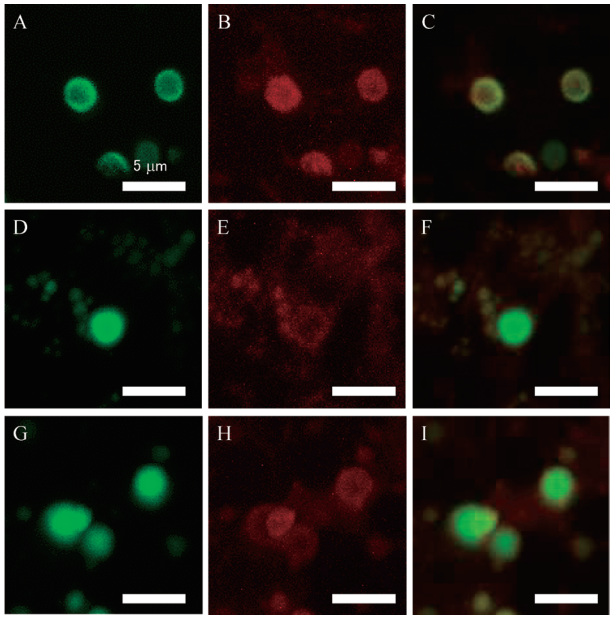

Figure 3. Localization of 13a-1 prolamin-GFP fusion proteins under the control of the promoter derived from 13b-2 prolamin (A–C), 13a-1 prolamin (D–F) and 10 kDa prolamin (G–I). Green indicates the GFP signal (A, D, G), and red indicates the signal of rhodamine B, which stains the peripheral region of PB-Is (B, E, H). C, F, I indicate the merged images. Bars=5 μm.

localize in the middle layer of PB-Is (Figure 3D–F). In the 10kPro::13a-1-GFP line, the GFP signal was observed as the small spots in PB-I and the prolamin-GFP fusion proteins were localized in the core region of PB-Is

(Figure 3G–I).

The results of the fluorescence microscopic analysis thus suggested that the localization of the respective prolamin species was determined by the expression of the respective prolamin promoter, regardless of the type of prolamin polypeptides being expressed. We previously showed that the 13b-2 prolamin-GFP, 13a-1 prolamin-GFP and 10 kDa prolamin-GFP fusion proteins expressed under the control of their native promoters were localized in the same layer as the respective endogenous prolamins in PB-I (Sasou et al. 2016). Our preliminary study suggested that the temporal control of the respective prolamin promoters determined the localization of each prolamin in its specific layer of PB-Is (Sasou et al. 2016). Moreover, we reported that the prolamin signal peptide and GFP fusion protein expressed by the native prolamin promoter were not localized in PB-Is, while the fusion proteins containing hydrophobic portion sequence in mature prolamin polypeptides was localized in PB-Is (Sasou et al. 2017). In this study, the localization of the 13b-2 prolamin-GFP fusion protein and 13a-1 prolamin-GFP fusion protein were both regulated by the expression pattern of the each prolamin promoter. The results of this study suggested that the prolamin promoter is important for the localization of GFP-tagged prolamins in a particular layer of the PB-I.

Cys-rich 13a-1 prolamin is thought to be able to form disulfide bonds with other Cys-rich prolamin (e.g., 10 kDa prolamin). However, 13b-2 prolamin is the Cys-less type prolamin, so 13b-2 prolamin may interact with other prolamin by hydrophobic interaction alone. 13a-1 Prolamin and 13b-2 prolamin may be accumulated in PB-I by different properties in interaction of other prolamin species, but it is difficult to perform experiments for interaction analysis such as co-immunoprecipitation (co-IP) because of the hydrophobicity of prolamin polypeptides in their entirety. Some reports have shown the possibility of analyzing the formation of PBs by expressing respective prolamin polypeptides in heterologous cells without other complicated factors (Masumura et al. 2015; Shigemitsu et al. 2013); hence, it may be possible to analyze the interaction between respective prolamin species by co-expressing some kind of prolamin polypeptides in the same transgenic rice calli or the yeasts.

We conclude altogether the previous report and this research as followed. The temporal control of the respective prolamin promoter is a key factor determining the localization of the respective prolamin polypeptides within the layered structure of the PB-I. The prolamin signal peptides were considered to help the fusion protein transfer into the ER for entering secretory pathway rather than for accumulating into PB-Is. Then, mature prolamin polypeptides are thought to be important to retain prolamin-GFP fusion proteins in PB-Is.

Our finding in this study will provide a novel technique for the expression of useful proteins such as oral vaccine antigens in PB-Is because of their indigestibility (Sasou et al. 2016). If we can control the localization of a vaccine protein in a specific layer of PB-Is by linking it with the appropriate prolamin promoter, it may be possible to develop techniques to encapsulate vaccines that can be released in the small intestine without undergoing exposure to the harsh environment of the stomach.

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Conflict of interest

The authors have no conflicts of interest to declare.

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