Chapter 16

Analysis of Recombinant Proteins in Transgenic Rice Seeds: Identity, Localization, Tolerance to Digestion, and Plant Stress Response

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Abstract

Rice seeds are an ideal production platform for high-value recombinant proteins in terms of economy, scalability, safety, and stability. Strategies for the expression of large amounts of recombinant proteins in rice seeds have been established in the past decade and transgenic rice seeds that accumulate recombinant products such as bioactive peptides and proteins, which promote the health and quality of life of humans, have been generated in many laboratories worldwide. One of the most important advantages is the potential for direct oral delivery of transgenic rice seeds without the need for recombinant protein purification (downstream processing), which has been attributed to the high expression levels of recombinant products. Transgenic rice will be beneficial as a delivery system for pharmaceuticals and nutraceuticals in the future. This chapter introduces the strategy for producing recombinant protein in the edible part (endosperm) of the rice grain and describes methods for the analysis of transgenic rice seeds in detail.

Key words *Oryza sativa* L., Protein body, Recombinant protein, Seed storage protein, Transgenic rice

1 Introduction

Rice seeds are an ideal production platform for molecular farming, as recombinant proteins can accumulate at high levels in the rice endosperm through established production systems [1, 2]. Furthermore, recombinant proteins that accumulate in rice seeds can be stored stably for several years at room temperature (a cold-chain infrastructure is not required for transport or storage) and delivered orally without purification. The functions of these proteins can be maintained even when the rice is cooked due to high thermal tolerance [3]. Orally administered rice seed-based pharmaceuticals are effectively delivered to the intestinal tract because they can withstand harsh, acidic conditions and digestive enzymes due to bioencapsulation within the cell wall and protein

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bodies. In addition, the mechanisms used for the cultivation, harvesting, processing, and storage of rice have been well developed worldwide.

Rice endosperm cells have three organelles that are suitable for the accumulation of recombinant proteins: protein body I (PB-I), protein body II (PB-II), and the endoplasmic reticulum (ER) (Fig. 1). PB-I is an ER-derived protein body that contains prolamins; a group of seed storage proteins that include 10 kDa prolamin, 13 kDa cys-poor prolamin, 13 kDa cys-rich prolamin, and 16 kDa prolamin [4]. PB-II is a protein storage vacuole that contains 26 kDa globulin and glutelins [5]. Twenty-six kilodaltons globulin is encoded by a single gene, whereas glutelins constitute a multi-gene family that has been classified into four groups (subfamilies) including GluA (GluA1, GluA2, and GluA3), GluB (GluB1, GluB2, and GluB4), GluC, and GluD [6]. These storage proteins are exported to PB-II from the ER via two routes; through the Golgi apparatus via dense vesicles, or directly via precursor-accumulating vesicles.

To target recombinant proteins to PB-I, PB-II, or the ER, a suitable ER-targeting signal peptide must be attached to the N-terminus of the recombinant protein, in addition to a C-terminal retention signal such as Lys/His-Asp-Glu-Leu [(K/H)DEL], which is involved in the transport of proteins from the Golgi apparatus to the ER [7, 8] (Fig. 2a). ER retention signals can result in higher accumulation of recombinant proteins: a previous study reported that recombinant protein levels were two- to tenfold higher with the addition of an ER retention signal than without this signal [9].

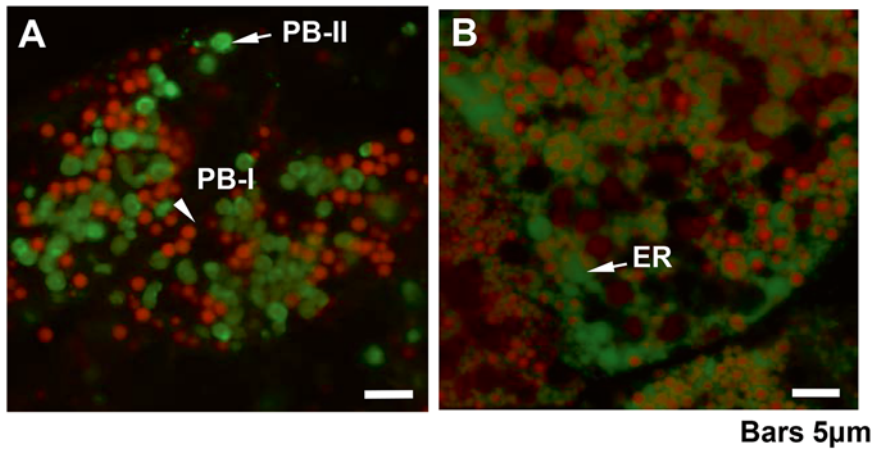

Fig. 1 Target organelles for the high accumulation of recombinant proteins. Confocal microscopy observations of transgenic rice seeds expressing GFP-fused glutelin A2 (**a**) and a GFP-attached signal peptide and ER retention signal to its 5′ and 3′ ends (**b**). Seed sections stained with rhodamine B to visualize PB-I are examined using confocal microscopy. (**a**) *Arrowhead* (*red signal*) shows PB-I, *arrow* (*green signal*) shows PB-II. (**b**) *Arrow* (*green signal*) shows ER

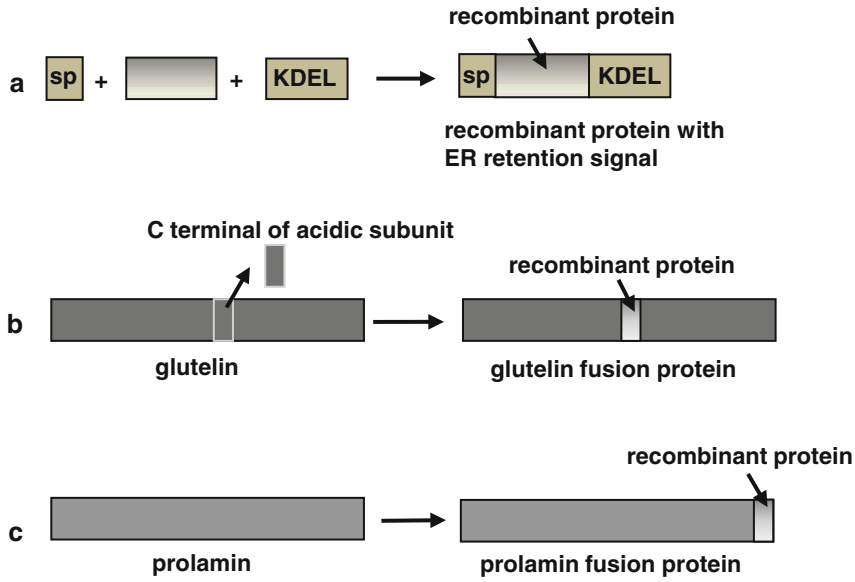

Fig. 2 Structures of coding regions required for the high accumulation of recombinant proteins. (**a**) Recombinant proteins carrying a heterologous signal peptide and ER retention signal accumulate in PB-I or the ER (Table 1). Recombinant peptides fused to full-length glutelin (**b**) or prolamin (**c**) accumulate in PB-II and PB-I (Table 1). This figure was reprinted with permission from Wiley

Recombinant proteins containing signal peptides and ER retention signals are generally sorted to PB-I or the ER lumen in transgenic rice seed. However, the use of these sequences alone does not guarantee the accurate trafficking of recombinant proteins to the desired organelle. The translational fusion of a recombinant protein to a seed storage protein ensures that the recombinant protein is deposited into the desired protein bodies. A recombinant protein is typically fused with prolamin or glutelin in order to transport it into PB-I or PB-II, respectively (Fig. 2b, c) [10]. An effective strategy has already been established for the accumulation of a small recombinant peptide (<200 amino acids) as a part of glutelin. Glutelin is synthesized as preproglutelin on the rough ER and is then posttranslationally processed into mature acidic and basic subunits in PB-II. The target recombinant peptides can be inserted into the C-terminal region of the acidic subunit of glutelin as part of a glutelin fusion protein (Fig. 2b). The glutelin-fused recombinant peptide then typically accumulates in PB-II. Soybean seed storage proteins such as glycinin can also be used as carrier proteins, similar to rice seed storage proteins [11].

Promoters are also one of the most important factors that determine the level of accumulation of transgene products in rice seeds. Promoter strength and tissue specificity (seed-specific expression) are critical because they directly affect the accumulation of recombinant proteins. Several suitable promoters have been identified and used as seed-specific and high expression promoters

without detrimental effects on plant growth [12]. Some seed storage protein gene terminators have also been used successfully. Although a nopaline synthase (Nos) terminator is typically used in the field of plant molecular biology, it is not recommended for recombinant expression in rice seed tissue because its incomplete termination ability has been shown to reduce the accumulation of recombinant proteins [13].

Table 1 and Fig. 2 summarize suitable promoters, coding regions, and terminators for the high accumulation of recombinant proteins in transgenic rice seeds as well as the predicted deposit sites of target proteins. Several strategies are now available for the construction of plasmid vectors, including the conventional combination of restriction enzymes and ligases as well as the Gateway system (Invitrogen) and In-Fusion system (Clontech); therefore, vector plasmids can be easily constructed to express the desired gene.

Transgenic rice plants of both *japonica* and *indica* varieties are generally produced by *Agrobacterium*-mediated transformation. Useful information on rice transformation methods can be found in "Transgenic Plants: Methods and Protocols 2nd edition" (Chapters 5, 6, and 36), published by Springer [14], and in Chapter 2 of this volume.

Table 1
Preferable gene structure to express recombinant proteins in rice seeds

	Gene name	RAP locus[a]
Promoter	*2.3 kb GluB1* promoter	Os02g0249800
	1.4 kb GluB4 promoter	Os02g0268300
	2.0 kb GluC promoter	Os02g0453600
	1.0 kb Glb-1 promoter	Os05g0499100
	0.9 kb 10 kDa prolamin promoter	Os03g0766200
	0.9 kb 16 kDa prolamin promoter	Os06g0507200
	Expression type	Predicted localization in seed cells
Coding region	Signal peptide + KDEL	ER or PB-I
	Glutelin fusion	PB-II
	Prolamin fusion	PB-I
	Gene name	RAP locus[a]
Terminator	*0.65 kb GluB1* terminator	Os02g0249800
	0.6 kb GluB4 terminator	Os02g0268300
	0.3 kb 10 kDa prolamin terminator	Os03g0766200
	0.28 kb 16 kDa prolamin terminator	Os06g0507200

[a]Please refer to http://rapdb.dna.affrc.go.jp/

In this chapter, we describe methods for the analysis of recombinant proteins produced in rice seed. These include protein extraction, confirming recombinant protein identity and subcellular localization, determining tolerance to digestive enzymes, and assessing unwanted ER stress responses.

2 Materials

2.1 Extraction of Total Protein from Mature Rice Seeds

1. Total protein extraction buffer: 50 mM Tris–HCl, pH 6.8, 8 M urea, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol, 0.01 % bromophenol blue.
2. Vortex mixer.
3. Microcentrifuge.
4. Grinder.

2.2 SDS-PAGE (for the Mini-slab Gel)

1. 1.5 M Tris–HCl, pH 8.8.
2. 0.5 M Tris–HCl, pH 6.8.
3. Distilled water.
4. 30 % acrylamide stock solution: 29 % (w/v) acrylamide, 1 % (w/v) methylene bis acrylamide.
5. 10 % ammonium peroxodisulphate (APS).
6. TEMED (N,N,N′,N′-tetramethylethylenediamine).
7. SDS-PAGE running buffer: 25 mM Tris hydroxyaminomethane, 192 mM glycine, 0.1 % (w/v) SDS.
8. Coomassie brilliant blue (CBB) R250 staining solution.
9. Destaining solution: 5 % (v/v) methanol, 7 % (v/v) acetic acid.
10. Electrophoretic apparatus for SDS-PAGE.
11. Size marker proteins (10–150 kDa).
12. SDS-PAGE loading buffer.
13. 10 % SDS.
14. 2-propanol.
15. Filter paper.
16. Kimwipes.
17. Shaker.

2.3 Immunoblot Analysis (for the Mini-slab Gel)

1. 10× Tris-buffered saline (TBS): 20 mM Tris–HCl, pH 7.5, 9 % NaCl.
2. Polyvinyl difluoride (PVDF) or nitrocellulose membrane (pore size 0.22–0.45 μm, 5.5 cm × 9 cm).
3. Filter paper (7.5 cm × 10.5 cm).

4. Blotting buffer: 25 mM Tris hydroxymethyl aminomethane, 192 mM glycine, 20 % methanol.
5. TTBS: 1× TBS, 0.05 % Tween 20.
6. Blocking buffer: 5 % skim milk powder in TTBS.
7. Primary antibody.
8. Horseradish peroxidase (HRP)-labeled secondary antibody (including a rabbit IgG antibody and HRP-linked antibodies).
9. ECL detection reagent.
10. X-ray film.
11. Devices for blotting.
12. Ice pack.
13. Shaker.
14. Methanol.
15. Darkroom.

2.4 Confocal Laser Scanning Microscopy

1. 10× phosphate buffered saline (PBS): 1.37 M NaCl, 81 mM Na_2HPO_4, 26.8 mM KCl, 14.7 mM KH_2PO_4.
2. Fixation solution: 3.7 % formaldehyde in 1× PBS.
3. Cell wall digestion solution: 1 % cellulase, 0.1 % pectolyase, in 1× PBS.
4. Permeabilization solution: 0.1 % Triton X-100 in 1× PBS.
5. Blocking solution: 1 % BSA, 0.01 % Triton X-100, in 1× PBS.
6. Primary antibody.
7. Secondary antibody with Alexa 488 conjugate.
8. 10 μg/mL rhodamine B (1× solution).
9. Slide glass.
10. Cover glass (e.g., 24×45 mm).
11. Razor blade.
12. Nail polish (inexpensive).
13. Confocal laser scanning microscope.
14. Low-melt agarose.
15. Gel tray.
16. Manicure pen.
17. Vibratome with accessories.

2.5 Digestibility Analysis of Recombinant Proteins

1. Grinder.
2. Microcentrifuge.
3. Pepsin dissolution buffer: 30 mM NaCl, pH 1.2 with HCl.

4. Pancreatin dissolution buffer: 50 mM K_2HPO_4–KH_2PO_4, pH 7.5.
5. Artificial gastric juice: 0.1 % pepsin in dissolution buffer.
6. Artificial intestinal juice: 1 % pancreatin in dissolution buffer.
7. Stop solution for the pepsin reaction: 160 mM Na_2CO_3.
8. Total protein extraction buffer (*see* **item 1** in Subheading 2.1).
9. Methanol.
10. Chloroform.
11. Distilled water.
12. Heat block or incubator.

2.6 Stepwise Extraction of Seed Storage Proteins

1. Globulin extraction buffer: 20 mM Tris–HCl, pH 6.8, 0.5 M NaCl.
2. Cysteine-rich prolamin extraction buffer: 60 % *n*-propanol.
3. Cysteine-poor prolamin extraction buffer: 60 % *n*-propanol, 5 % 2-mercaptoethanol.
4. Glutelin extraction buffer: 1 % lactic acid.
5. Total protein extraction buffer (*see* **item 1** in Subheading 2.1).
6. Litmus paper (pH 1–8).
7. 1 M NaOH.
8. Microcentrifuge.
9. Sonicator (the handheld type is easy to use).
10. Grinder.

2.7 Investigation of Undesirable Effects of Recombinant Proteins on Transgenic Rice Seeds

1. Liquid N_2.
2. Water-saturated phenol:chloroform:isoamyl alcohol (25:24:1).
3. RNA extraction buffer: 100 mM Tris–HCl, pH 9.0, 1 % SDS, 100 mM NaCl, 5 mM EDTA.
4. 99 % ethanol.
5. 3 M sodium acetate buffer, pH 5.2 with acetic acid.
6. 70 % ethanol.
7. DEPC-treated water (RNase-free water).
8. 8 M LiCl.
9. 2 M LiCl.
10. Vortex mixer.
11. Microcentrifuge.
12. Reagents for RT-PCR (reverse transcriptase, DNA polymerase, buffers, primers, dNTPs).
13. Thermal cycler or real-time PCR machine.
14. Grinder.

3 Method

3.1 Extraction of Total Protein from Matured Rice Seeds

When transgenic rice seeds are obtained, the first step is to prepare total seed protein for SDS-PAGE and immunoblot analyses. An adequate amount of seed protein can be extracted from one rice seed for SDS-PAGE or immunoblotting.

1. Grind mature seeds (*see* **Note 1**) individually into a fine powder.
2. Add 500 μL of total protein extraction buffer to the seed powder (approximately 20 mg) and vigorously vortex for 1 h at room temperature.
3. Centrifuge the mixture in a microcentrifuge at maximum speed (14,000 rpm; 10,000 × *g*) for 10 min at room temperature and collect the supernatant in a new tube by decantation. Typically, only 2–3 μL of supernatant is needed for SDS-PAGE and immunoblot analysis (Subheadings 3.2 and 3.3).

3.2 SDS-PAGE

To analyze total rice seed proteins, a small (e.g., 5.5 cm height × 9 cm width) 12 % acrylamide gel is suitable for the separation of these proteins. Thus, the SDS-PAGE method using a 12 % mini-slab gel is summarized in this section.

1. Assemble a mini-slab gel device, glass plate, comb, clip, and other necessary equipment (Fig. 3a).
2. Mark the level of the separation (running) gel on the glass plate (Fig. 3b).
3. Prepare a separation gel (Table 2) and mix gently without generating bubbles.
4. Immediately transfer the separating gel solution into the gap between the glass plates.
5. Add 2-propanol on top of the separation gel and keep the glass plate at room temperature for at least 30 min, until gel polymerization (*see* **Note 2**).
6. Completely remove 2-propanol from the gel plate by absorption with filter paper and prepare the stacking gel (Table 2).
7. After transferring the stacking gel to the top of the separation gel, put the comb in the stacking gel without generating bubbles. The stacking gel will completely polymerize within a few minutes.
8. Set the SDS-PAGE gel and running buffer in an electrophoresis gel tank.
9. After removing the comb, apply the samples (2–5 μL) and size marker proteins, mixed with loading buffer, to the wells of the SDS-PAGE gel.

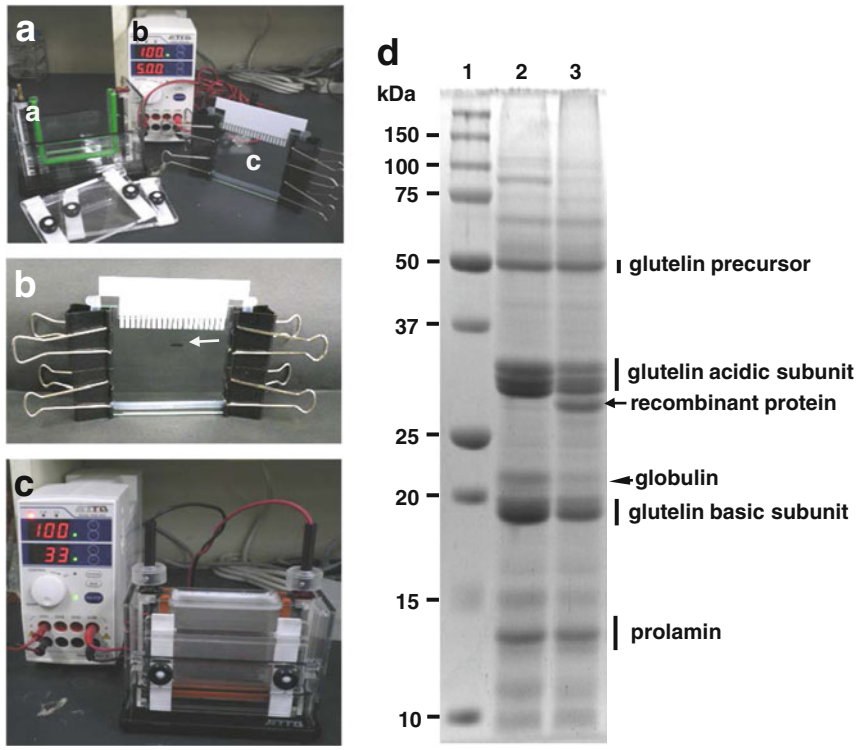

Fig. 3 SDS-PAGE. **a**, Assembly for SDS-PAGE, the electrophoretic apparatus (a), power supply (b), glass plate (c). **b**, Determine the amounts of separation (running) gel on the gel plate (*white arrow*). **c**, Electrophoresis. Electrophoresis generally takes approximately 1 h (200 V) ~ 2 h (100 V) to complete. **d**, CBB-stained SDS-PAGE gel. Lane 1, size marker; Lane 2, total rice seed protein (cv. Kitaake); Lane 3, total rice seed protein (transgenic rice expressing one recombinant protein). The positions of the glutelin precursor, acidic or basic glutelin, globulin, prolamin, and recombinant proteins on the SDS-PAGE gel are indicated on the right side of the panel. If a clear recombinant protein signal is detected by CBB staining such as in lane 3, the accumulation of the recombinant protein is considered to be high. If no signal derived from the recombinant protein is detected at this step, proceed to immunoblot analysis

10. Perform electrophoresis according to standard methods (Fig. 3c). Electrophoresis is completed when the tracking dye moves to the lower edge of the PAGE gel.
11. After electrophoresis, stain the SDS-PAGE gel with CBB or by immunoblotting (*see* Subheading 3.3 for immunoblotting).
12. In the case of CBB staining, transfer the SDS-PAGE gel to a small container. Add CBB R250 solution to the container and gently shake for at least 15 min at room temperature (*see* **Note 3**).
13. Wash the stained SDS-PAGE gel with destaining solution for at least 2 h at room temperature by gently shaking (*see* **Note 4**).
14. Ensure the signal pattern of the seed protein samples (Fig. 3d).

Table 2
Components of separation (12 %) and stacking gels for SDS-PAGE

	Final concentration	Mini-slab gel × 1	Mini-slab gel × 2	Mini-slab gel × 4
Separation (Running) gel		Total approx. 8 mL	Total approx. 16 mL	Total approx. 32 mL
Water	–	2.8 mL	5.6 mL	11.2 mL
30 % acrylamide	12 %	3.2 mL	6.4 mL	12.8 mL
1.5 M Tris–HCl pH 8.8	375 mM	2 mL	4 mL	8 mL
10 % SDS	0.1 %	80 µL	160 µL	320 µL
10 % APS	–	30 µL	60 µL	120 µL
TEMED	–	4 µL	8 µL	16 µL
Stacking gel	–	Total approx. 2.5 mL	Total approx. 5.0 mL	Total approx. 10.0 mL
Water	–	1.44 mL	2.88 mL	5.76 mL
30 % acrylamide	4.8 %	400 µL	800 µL	1.6 mL
0.5 M Tris–HCl pH 6.8	125 mM	625 µL	1.25 mL	2.5 mL
10 % SDS	0.1 %	25 µL	50 µL	100 µL
10 % APS	–	30 µL	60 µL	120 µL
TEMED	–	2 µL	4 µL	8 µL

3.3 Immunoblot Analysis

Described here is a typical immunoblotting method using immersion (wet)-type transfer, primary and secondary antibodies, an enhanced chemiluminescence (ECL) detection system, and X-ray films. These methods remove the need for a commercial kit and specific machines, thereby reducing costs.

In immunoblot analysis, a specific antibody is required as a primary antibody for the target protein. Prepare the primary antibody needed or fuse a tag (e.g., His-tag) to the recombinant protein beforehand.

1. Prepare the blotting apparatus (blotter, membrane, filter paper, ice pack, and blotting buffer) (Fig. 4a). If a PVDF membrane is used for blotting, immerse the PVDF membrane in 100 % methanol before use and then transfer to the blotting buffer.
2. Perform blotting according to standard conditions (e.g., 80 V for 1 h) (Fig. 4b, c). Use an ice pack to prevent excessive temperature increases in the blotting buffer due to electrification (Fig. 4c).

3. Transfer the membrane to a container filled with 10–20 mL of 1× TTBS containing 5 % skim milk powder. Shake gently for at least 1 h at room temperature (Fig. 4d).
4. Add the appropriate primary antibody (1000–10,000× dilution) and react by gently shaking for 3 h at room temperature or overnight at 4 °C.
5. Wash the membrane with 1× TTBS for 10 min at room temperature.
6. Repeat the wash step two times. Prepare 1× TTBS containing 0.5 % skim milk powder while washing the primary antibody.
7. After washing, add 1× TTBS containing 0.5 % skim milk powder to the container. Add appropriate amounts of the HRP-labeled secondary antibody (e.g., Rabbit IgG antibody or HRP-linked antibody) according to the manufacturer's protocol (5000–10,000× dilution).
8. React by shaking gently for 1–3 h at room temperature.
9. Wash the membrane with 1× TTBS for 10 min at room temperature three times.
10. Transfer the membrane to a new container and prepare the ECL detection reagent, X-ray film, and cassette.
11. Detect signals using the ECL detection reagent in a dark room according to the manufacturer's protocol (Fig. 4e) (*see* **Notes 5** and **6**).

3.4 Confocal Laser Scanning Microscopy

This experiment is conducted to determine the intracellular localization of recombinant proteins in rice seed cells using confocal microscopy and an antibody to the recombinant protein (confocal immunohistochemical microscopy). Seeds collected approximately 10–15 days after flowering (DAF) are suitable as samples for confocal microscopy. Sample preparation becomes difficult at more than 20 DAF due to seed firmness.

1. Harvest premature seeds (10–15 DAF) and carefully remove the hull (lemma and palea) from the seeds (Fig. 5a).
2. Cut seeds in half using a razor blade (Fig. 5a).
3. Suspend 4 g of low melting agarose in 100 mL water. Dissolve the agarose well in a microwave oven.
4. Pour liquefied agarose into a gel tray and cool the agarose 50 °C at room temperature (Fig. 5b).
5. Place the rice seeds, with the cut surface facing down, into the liquefied agarose with fine-tipped tweezers (Fig. 5c).
6. Leave the samples at room temperature for at least 15–20 min until gelling is complete.
7. While waiting for gelling, prepare the vibratome and its accessories.

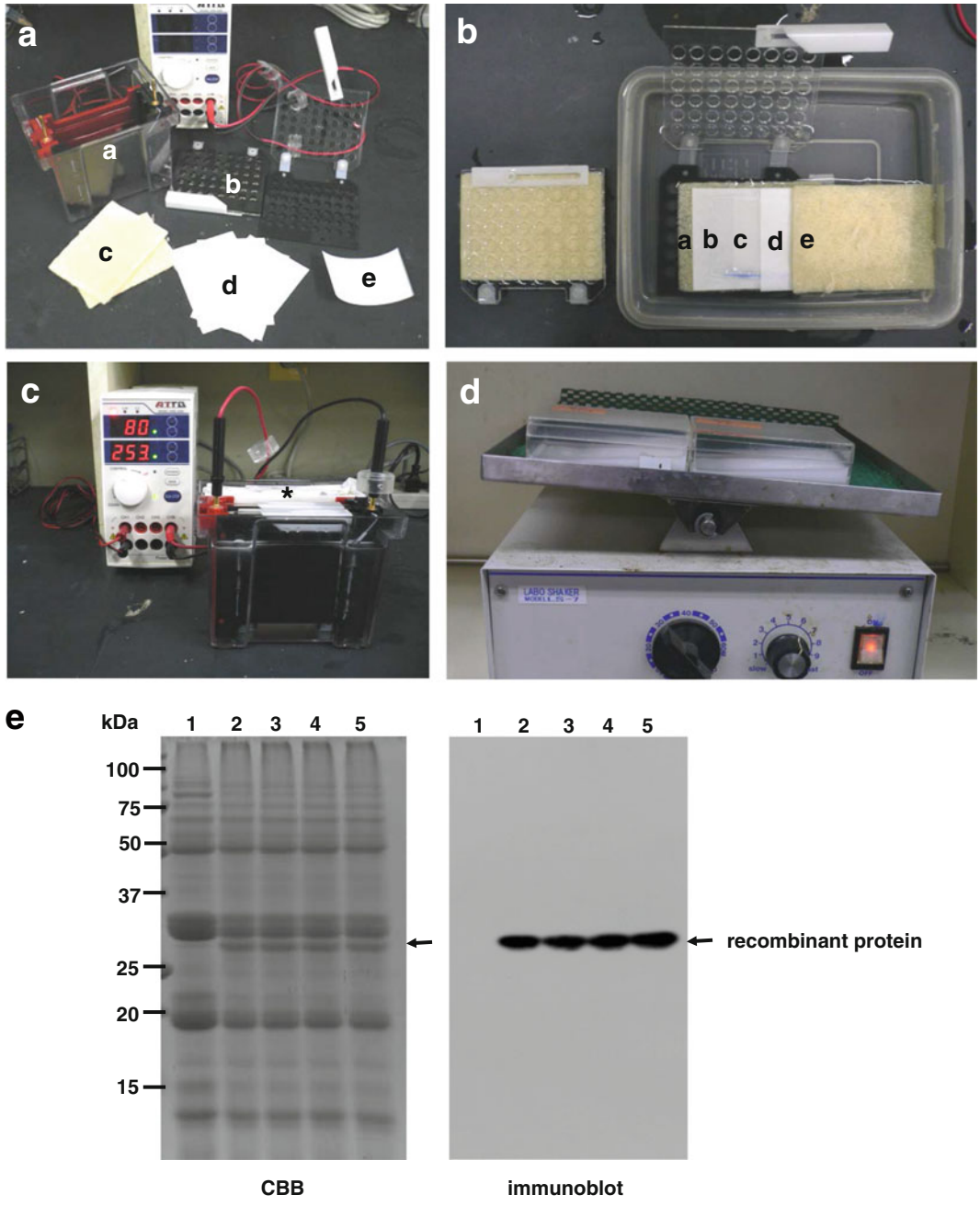

Fig. 4 Immunoblotting. **a**, Blotting apparatus for wet (immersion) type transfer. a, buffer tank; b, Sandwich plate; c, urethane pads; d, filter paper; e, PVDF membrane. **b**, Assembly of the sandwich plate. (a) Urethane pad, (b) filter paper, (c) PAGE gel, (d) filter paper, (e) urethane pad. **c**, Blotting. An *asterisk* indicates an ice pack. **d**, Gentry shaking of membranes. **e**, CBB-stained SDS-PAGE gel (CBB) and X-ray film after signal detection (immunoblot). Lane 1, nontransgenic rice seed; lanes 2–5, transgenic rice seed expressing a recombinant protein. An *arrow* indicates the signal of a recombinant protein

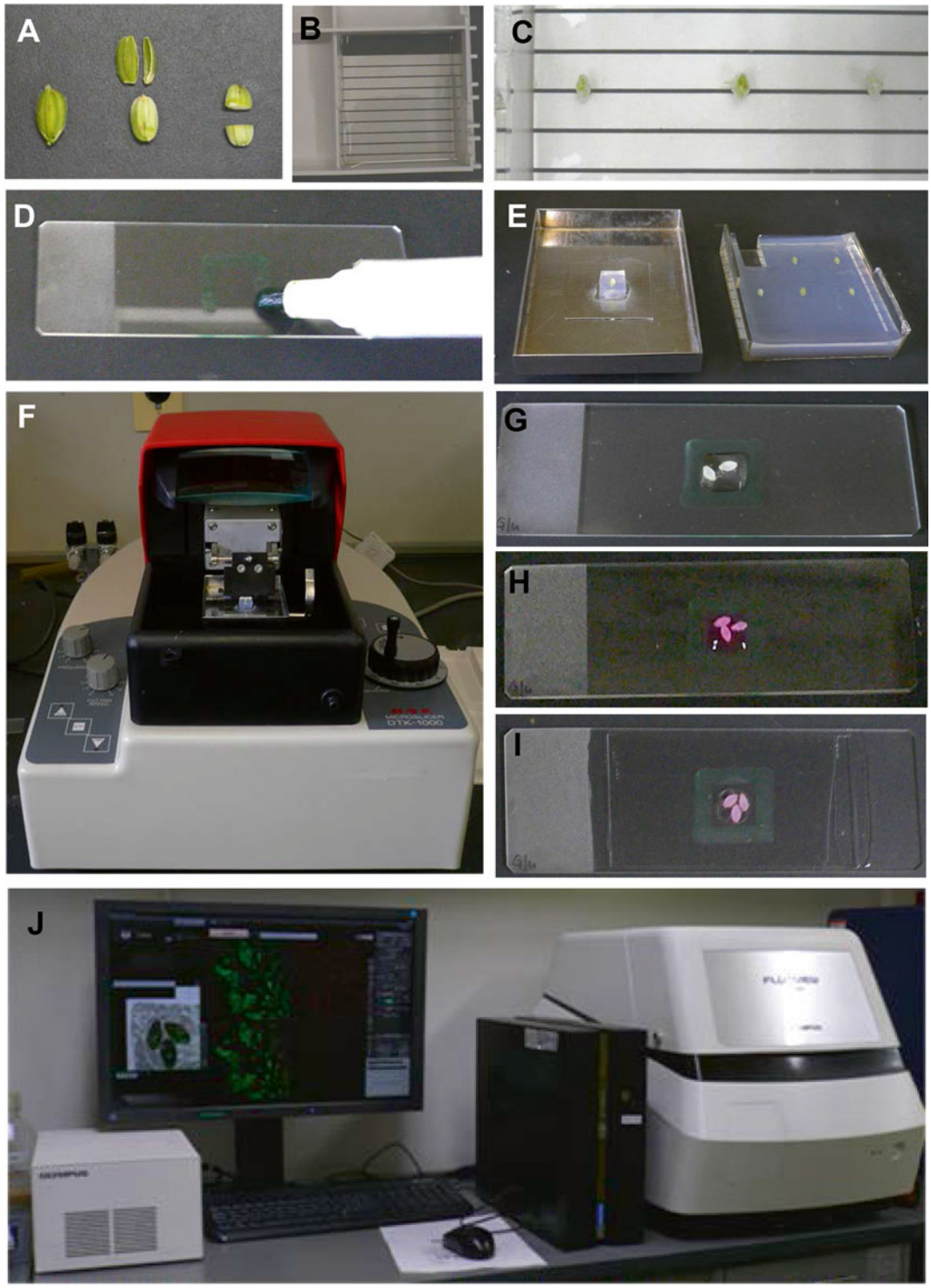

Fig. 5 Confocal immunohistochemical microscopy analysis. (**a**) Premature seed (*left*), hulled premature rice (*middle*), and cut premature seed (*right*). (**b**) Agarose gel polymerization on the gel plate. (**c**) Agarose gel-embedded samples. (**d**) Making a square frame on a slide glass. (**e**) Agarose block on a steel dish. (**f**) Setting of the steel dish on a vibratome. (**g**) Sample sections on slide glass. (**h**) Rhodamine staining of samples. (**i**) Preparation for observations using confocal microscopy. (**j**) Confocal laser scanning microscopy

8. Draw an approximately 1-cm square frame on a slide glass with a manicure pen (Fig. 5d).
9. Use a razor blade to cut blocks of agarose containing the samples.
10. After excising the agarose containing the seed samples, adhere the gel block onto the steel dish of the vibratome (Fig. 5e).
11. Set the steel dish on the vibratome (Fig. 5f).
12. Use the vibratome to cut approximately 100–200 μm-thick slices from the agarose gel block. Place the sections (one to six sections) in distilled water within the square frame of the slide glass (Fig. 5g).
13. Remove the distilled water, apply 3.7 % formaldehyde in 1× PBS to the sections, and incubate at room temperature for 1 h (fixation).
14. Wash three times with 1× PBS at intervals of 10 min at room temperature.
15. Apply cell wall digestion solution to the sections and incubate for 10 min at room temperature.
16. Wash three times with 1× PBS at intervals of 10 min at room temperature.
17. Apply permeabilization solution to the sections and incubate for 15 min at room temperature.
18. Wash three times with 1× PBS at intervals of 10 min at room temperature.
19. Apply blocking solution to the sections and incubate for 1 h at room temperature.
20. Wash three times with 1× PBS at intervals of 10 min at room temperature.
21. Dilute the primary antibody (100–1000× dilution) in blocking solution and apply the primary antibody solution to the sections. Incubate for 3 h at room temperature or overnight at 4 °C (*see* **Note 7**).
22. Wash three times with 1× PBS at intervals of 10 min.
23. Dilute the secondary antibody (100–500× dilution) in blocking solution and apply the secondary antibody solution to the sections. Incubate for 3 h at room temperature.
24. Use rhodamine B to stain PB-I. Apply 1× rhodamine B solution to samples and incubate for 15 min at room temperature (Fig. 5h).
25. Wash three times with 1× PBS at intervals of 10 min at room temperature.
26. Mount the cover glass on sections and seal the cover glass by using nail polish (Fig. 5i). Large cover glasses (e.g., 24 × 45 mm) may be more easy to use than normal cover glasses (18 × 18 mm).

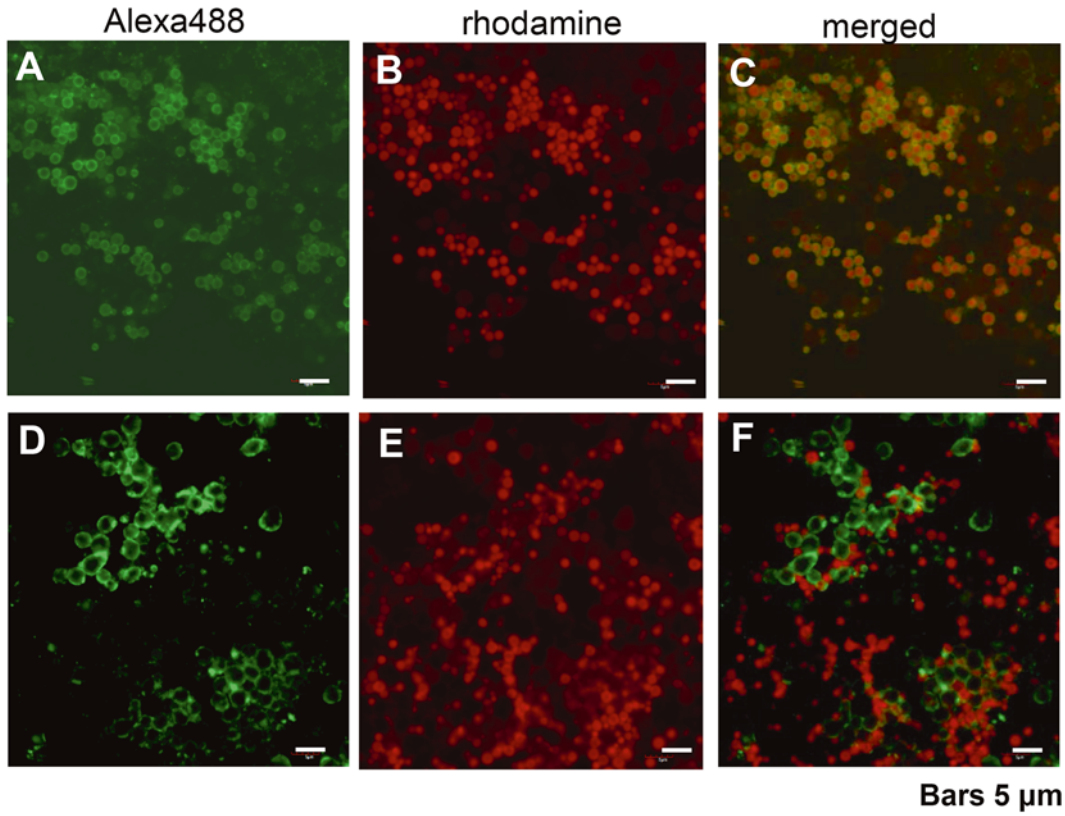

Fig. 6 Indirect immunohistochemical analysis using a confocal laser scanning microscope. *Left panels* show the localization of recombinant proteins (*green*, Alexa 488), *middle panels* show the localization of PB-I (*red*, rhodamine), and *right panels* show merged images. (**a–c**) This recombinant protein accumulated in PB-I. (**d–f**) This recombinant protein accumulated in PB-II (*see* **Note 17**)

27. View the preparation with a confocal laser scanning microscope (e.g., FV10i, Olympus) according to the manufacturer's protocol (Figs. 5j and 6). Magnification of 60–300× is usually suitable for rice endosperm cells. Recommended excitation wavelengths are around 499 nm (Alexa 488, emission 520 nm) and 558 nm (rhodamine B, emission 575 nm).

3.5 Stepwise Extraction of Seed Storage Proteins

Unexpected subcellular localization of recombinant proteins has been reported in transgenic rice seeds. For example, a protein expressed as a glutelin fusion was sequestered to PB-I, but was not detected in PB-II [15, 16]. This was attributed to free cysteine residues in the recombinant protein, which formed intermolecular disulfide bonds with cysteine-rich prolamins that accumulate mainly in PB-I, such as 10 kDa prolamin, 13 kDa cys-rich prolamin, and 16 kDa prolamin. However, the accumulation of recombinant proteins in PB-I has several advantages. Human pharmaceutical proteins deposited in PB-I are more effectively

delivered to the small intestine without being degraded than those targeted to PB-II or to the ER.

Interactions between cysteine-rich prolamins and recombinant proteins expressed as glutelin fusion proteins can be confirmed experimentally. This section describes a stepwise seed protein extraction method, consisting of three separate stepwise extractions, to determine colocalization of recombinant glutelin fusions with endogenous seed storage proteins. An outline of this experiment is shown in Table 3 and Fig. 7a.

1. Grind individual mature transgenic rice seeds into fine powder. Three samples are needed: one for each of the three extractions.
2. Add 500 μL of globulin extraction buffer to each sample and sonicate for 2 min on ice.
3. Centrifuge in a microcentrifuge at maximum speed (20,000 × *g*) for 10 min. Remove the supernatant (*see* **Note 8**) and wash the pellet three times by resuspending in 500 μL of globulin extraction buffer and centrifuging at maximum speed for 10 min. For one of the samples, proceed to **step 8** (Extraction 1). For the other two samples, continue with **step 4**.
4. Resuspend the pellet in 500 μL of cys-poor prolamin extraction buffer and sonicate for 2 min on ice.
5. Centrifuge at maximum speed (14,000–20,000 × *g*) for 10 min. Remove the supernatant (*see* **Note 9**). Wash the pellet three times by resuspending in 500 μL of the cys-poor prolamin extraction buffer and centrifuging at maximum speed for 10 min. For one of the samples, proceed to **step 8** (Extraction 2). For the remaining sample, continue with **step 6** (Extraction 3).

Table 3
Extraction order of the stepwise extraction

	Buffer composition	Extraction 1	Extraction 2	Extraction 3
Globulin extraction buffer	0.5 M NaCl, 10 mM Tris–HCl (pH 6.8)	+	+	+
Cys-poor extraction buffer	60 % *n*-propanol	–	+	+
Cys-rich extraction buffer	60 % *n*-propanol, 5 % 2-mercaptoethanol	–	–	+
Glutelin extraction buffer	1 % lactic acid	+	+	+
Extraction steps in Subheading 3.5		**1–3, 8–12**	**1–5, 8–12**	Perform all steps

+, with pre-extraction; –, without pre-extraction

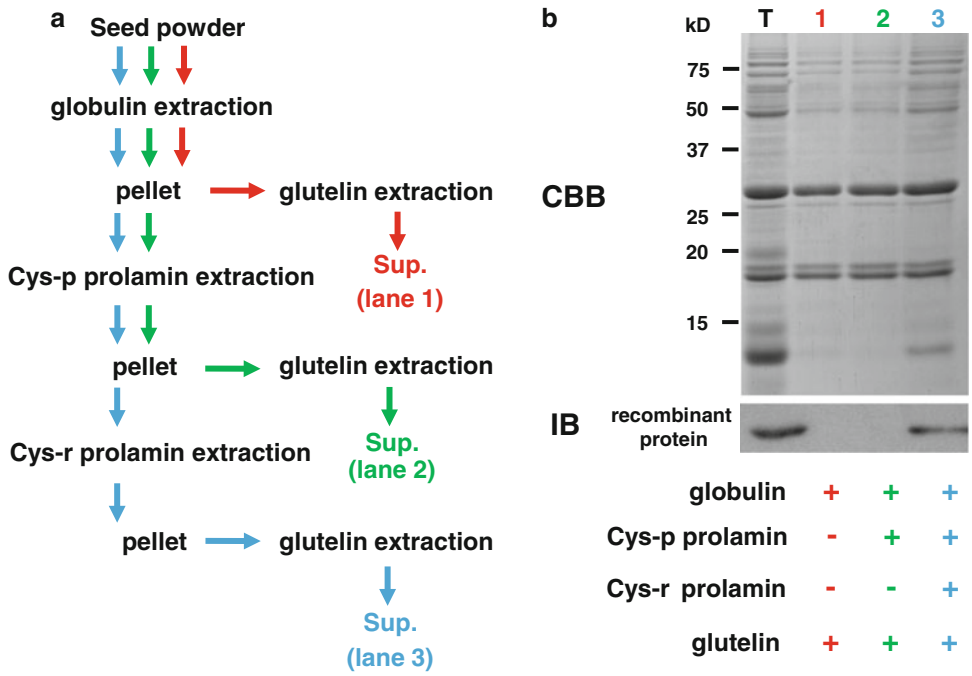

Fig. 7 Stepwise extraction of seed storage proteins. (**a**) Flow chart of this experiment. (**b**) SDS-PAGE (CBB) and immunoblot (IB) analyses are shown. Proteins were extracted with 1 % lactic acid following a pre-extraction with different solvents. Total seed proteins; +, with pre-extraction; –, without pre-extraction; Cys-p, cysteine-poor; Cys-r, cysteine-rich. This figure was reprinted with permission from Wiley. *Red*, *green*, and *blue arrows* indicate extraction 1, extraction 2, and extraction 3 in Table 3, respectively

6. Resuspend the pellet in the cys-rich prolamin extraction buffer and sonicate for 2 min on ice.
7. Centrifuge at the maximum speed (14,000–20,000 × *g*) for 10 min. Remove the supernatant (*see* **Note 9**). Wash the pellet three times by resuspending in 500 μL of the cys-rich prolamin extraction buffer and centrifuging at maximum speed for 10 min.
8. Resuspend the pellet in the glutelin extraction buffer and sonicate for 2 min on ice.
9. Centrifuge at the maximum speed (14,000–20,000 × *g*) for 10 min. Collect the supernatant.
10. Adjust the pH of samples to pH 7.0 with 1 M NaOH (*see* **Note 10**).
11. Centrifuge at the maximum speed (14,000–20,000 × *g*) for 10 min. Remove the supernatant and dry the pellet in air or by using a vacuum pump.

12. Resuspend the pellet in 500 μL of total protein extraction buffer and subject the samples to SDS-PAGE and immunoblotting according to Subheadings 3.2 and 3.3 (Fig. 7). If the recombinant protein is observed in the sample from Extraction 3, this indicates that the protein interacts with cysteine-rich prolamins and accumulates in PB-I (*see* **Note 11**).

3.6 Digestibility Analysis of Recombinant Protein

This experiment determines whether recombinant proteins in rice seed cells exhibit higher resistance to digestive enzymes than bare proteins. When recombinant proteins for oral intake are expressed in transgenic rice seeds, this experiment should be conducted to validate their resistance to gastric and intestinal juices. Control proteins are needed for a comparison of resistance to digestive enzymes. Although purified recombinant proteins derived from *E. coli* are often available, the use of proteins extracted from rice seeds is preferable, since identical glycosylation patterns are assumed. This section explains the methodologies used in the digestion experiment with an artificial gastric juice (pepsin; Subheading 3.6.1) or artificial intestinal juice (pancreatin; Subheading 3.6.2) as well as the preparation of control proteins from rice seeds (Subheading 3.6.3).

3.6.1 Pepsin Digestion

1. Set a heat block or incubator to 37 °C.
2. Grind five to ten dry seeds.
3. Prepare seven microcentrifuge tubes per sample. Six tubes are needed for the pepsin time course treatment (for, e.g., 0, 1, 5, 10, 20, and 30 min), and one is for a negative control.
4. Transfer 5 mg of seed powder into each microcentrifuge tube.
5. Add 150 μL of artificial gastric juice (pepsin solution) to each tube and immediately mix well. Incubate the tubes at 37 °C for 0–30 min, removing each tube at a different time point. For the negative control, add the same volume of pepsin dissolution buffer without pepsin and incubate at 37 °C for 30 min.
6. After this incubation, immediately add 100 μL of the stop solution and mix well.
7. Add 750 μL of the total protein extraction buffer and mix well (*see* **Note 12**).
8. Centrifuge in a microcentrifuge at maximum speed for 10 min and collect the supernatant.
9. Subject 8 μL of the supernatant to SDS-PAGE and immunoblotting according to Subheadings 3.2 and 3.3 (Fig. 8).

3.6.2 Pancreatin Digestion

1. Follow **steps 1–4** of Subheading 3.6.1 (pepsin digestion).
2. Add 200 μL of artificial intestinal juice (pancreatin solution) into each microcentrifuge tube and immediately mix well.

Incubate the tubes at 37 °C for 0–120 min, removing each tube at a different time point. As a negative control, add the same volume of pancreatin dissolution buffer without pancreatin and incubate at 37 °C for 120 min.

3. After this incubation, add 800 μL of total protein extraction buffer and mix well (*see* **Note 13**).
4. Boil the samples for 5 min to inactivate the pancreatin enzyme.
5. Mix the samples again. Centrifuge at maximum speed for 10 min and collect the supernatant.
6. Subject 8 μL of samples to SDS-PAGE and immunoblotting according to Subheadings 3.2 and 3.3. Results of the pancreatin digestion would be similar to the pepsin digestion (Fig. 8).

3.6.3 Preparation of Control Proteins

1. Extract proteins from transgenic rice as described in Subheading 3.1. Approximately 400–450 μL of total protein solution can be obtained from one seed.
2. Transfer 200 μL of the total protein sample to a new microcentrifuge tube.
3. Add 600 μL of methanol and mix well.
4. Add 200 μL of chloroform and vortex vigorously for 1 min.
5. Add 600 μL of distilled water and mix well.

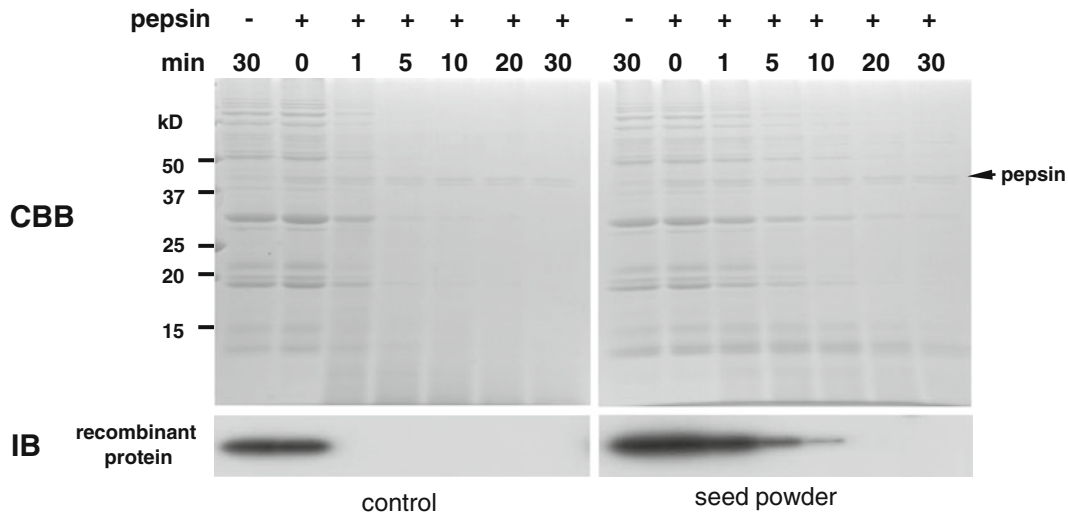

Fig. 8 Digestibility analysis of recombinant protein. The digestibility of recombinant proteins by 0.1 % pepsin at 37 °C for up to 30 min (0, 0.5, 1, 5, 10, 20, and 30 min). The *left panel* shows recombinant proteins after extraction from transgenic rice seeds (control). The *right panel* shows transgenic seed powder (seed powder). SDS-PAGE (CBB) and immunoblot (IB) analyses are shown. +, with 0.1 % pepsin; –, without 0.1 % pepsin. An *arrowhead* indicates pepsin

6. Centrifuge the sample in a microcentrifuge at maximum speed (14,000–20,000 × *g*) for 1 min.
7. Remove the aqueous phase carefully, ensuring not to touch the middle layer (protein layer).
8. Add 600 μL of methanol and mix well.
9. Centrifuge the sample at maximum speed for 1 min.
10. After air-drying the pellet, grind the pellet into a fine powder.
11. Use the powder for control samples for comparison to rice seed powder in the pepsin and pancreatin digests (Fig. 8) (*see* **Note 14**).

3.7 Investigation of Undesirable Effects of Recombinant Proteins on Transgenic Rice Seeds

When recombinant proteins or peptides are accumulated as secretory proteins in rice seeds for oral intake, grain qualities such as yield, appearance, and flavor are important. Some recombinant proteins perturb homeostasis in rice seed cells, resulting in ER stress responses. ER stress responses maintain ER homeostasis by balancing the folding capacity and folding demands imposed on the ER [17]. Transgenic rice seeds under ER stress conditions have shrunken and floury phenotypes that lead to reductions in grain quality and yield [18]. Low grain quality due to ER stress conditions during seed development in transgenic rice is an issue that needs to be addressed. A clearer understanding of the molecular mechanisms underlying ER stress is needed in the near future. Here, we describe a method to estimate relative ER stress levels in transgenic rice seeds using RT-PCR of ER stress marker genes (Table 4). The expression of these genes is closely associated with ER stress levels in rice [19].

Table 4
Primer sets for amplification of ER stress responsive genes in rice

Gene name	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
OsBiP2	Os03g0710500	TACTCCTCTGTTCAGGGTG	GCTTGATGTCGAAGATGGTG
OsBiP3	Os05g0367800	GGCGTCAAGAACGCCGTGGTGGG	AAATAAATTCTAGCTAGCCGGCGG
OsBiP4	Os05g0428600	CAAGGAGGAGTACGAGGAGAAG	CACACTTTCGATCGAATCCAAAC
OsBiP5	Os08g0197700	AACATCAAGAACACGCTCGGCG	GCAGCTAGCACCTAGCAGCGAC
SAR1B-like	Os06g0225000	TTCTACATGCTCTCACAAG	ACCACGTAAACTACTGCATC

3.7.1 Total RNA Extraction from Rice Seed Tissue

1. Freeze premature seed samples using liquid N_2 (1–3 seeds per tube).
2. Grind the frozen samples into a fine powder and add 400 μL of water-saturated phenol:chloroform:isoamyl alcohol and 400 μL of RNA extraction buffer immediately to the powder.
3. Vortex for at least 15 min at room temperature
4. Centrifuge in a microcentrifuge at maximum speed (14,000–20,000 × *g*)
5. Collect the supernatant in a new tube. Add 400 μL of water-saturated phenol:chloroform:isoamyl alcohol.
6. Mix well and centrifuge at the maximum speed.
7. Collect the supernatant in a new tube.
8. Add 40 mL of 3 M sodium acetate buffer and 1 mL of 99 % ethanol and mix well.
9. Incubate for at least 10 min on ice.
10. Centrifuge samples at maximum speed for 10 min.
11. Discard the supernatant and dry the pellet in air or by using a vacuum pump.
12. Dissolve the pellet in 60 μL of DEPC-treated water (or RNase-free water).
13. Add 20 μL of 8 M LiCl to the sample and mix well.
14. Incubate samples for at least 1 h or overnight at 4 °C.
15. Centrifuge samples at maximum speed for 10 min.
16. Discard the supernatant and rinse the pellet with 2 mM LiCl.
17. Rinse the pellet well with 70 % ethanol (*see* **Note 15**)
18. Dry the pellet in air or by using a vacuum pump and dissolve in 30–100 μL of DEPC-treated water (or RNase-free water) (*see* **Note 16**)

3.7.2 RT-PCR

Perform RT-PCR or quantitative real-time RT-PCR using equal amounts of RNA template according to the manufacturer's protocols (Fig. 9). The RT reaction can be primed with a mixture of oligo-dT and random hexamers. Some candidate maker genes and their primer sets for the amplification of these genes are shown in Table 4. Thirty cycles of PCR reaction will give preferable results. No amplification signal should be detected in normal seed RNA, but a clear amplification signal is detected in RNA from an ER-stressed seed. To normalize for differences in starting RNA concentrations, use a reference RNA such as *17S ribosomal* RNA (Forward primer 5′-tccatcttggcatctctcag-3′ and reverse primer 5′-gtacccgcatcaggcatctg-3′). ER stress levels are estimated based on the accumulation of mRNA from genes in Table 4 and matured seed phenotypes [18].

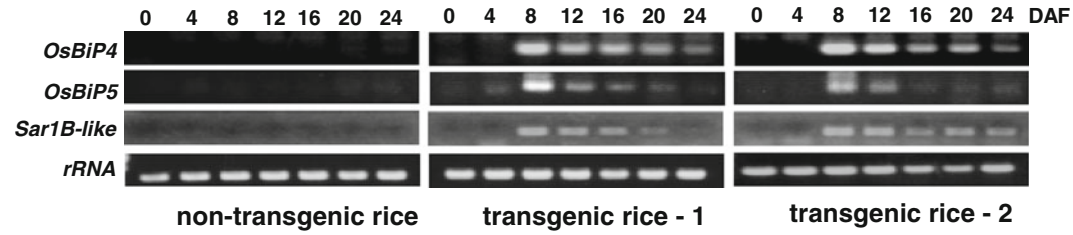

Fig. 9 RT-PCR analysis of ER stress marker genes in nontransgenic rice or transgenic rice seeds. Total RNA was extracted from 0, 4, 8, 12, 16, 20, and 24 DAF seed tissues. The expression of *OsBiP4*, *OsBiP5*, and *Sar1B-like* genes is shown as examples. *17S ribosomal RNA* (*rRNA*) was used as a control. Transgenic rice 1 and transgenic rice 2 exhibit ER stress in response to recombinant protein accumulation, as revealed by the expression of the stress marker genes

4 Notes

1. If the aim is to extract total protein from premature seeds, the seed sample should be frozen by liquid N_2 before grinding.
2. Once the separation gel is polymerized, a clear boundary line can be observed between the gel and 2-propanol.
3. CBB-G250 is also available for gel staining. If you use CBB-G250, washing buffer should be replaced with distilled water.
4. Placing Kimwipes in the destaining solution helps decolorize the stained gel by adsorbing the released dye.
5. If a high background and/or nonspecific signals are detected, use high stringency washing buffer (0.2 % SDS, 0.5 % Triton X-100 in 1× TBS) instead of TTBS.
6. After ECL signal detection, the membrane can be stained with CBB to ensure blotting efficiency. Transfer the membrane to CBB-R250 and shake gently for 1–2 min. Wash the membrane with distilled water and dry at room temperature.
7. When the sample is reacted for a long time with the primary antibody on glass slides, the reaction should be performed in an airtight plastic container (such as Tupperware) together with wet Kimwipes to prevent the primary antibody solution from drying.
8. To ensure that globulin dissolved adequately, add an equal volume of total protein extraction buffer to the supernatant and perform SDS-PAGE. You can see a visible band of globulin together with water and saline soluble proteins (Fig. 10, lane 1).
9. To ensure that cys-poor or cys-rich prolamins dissolved adequately, add an equal volume of distilled water to the supernatant and incubate for at least 1 h on ice. After centrifuging at the maximum speed (14,000–20,000×*g*), dissolve the pellet in total protein extraction buffer. Subject the sample to SDS-PAGE. You can see visible bands of cys-poor or cys-rich

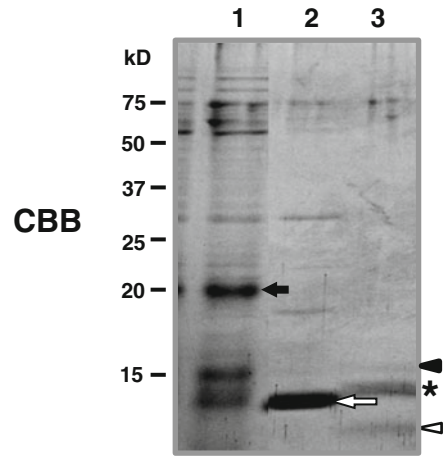

Fig. 10 SDS-PAGE of globulin, cys-poor prolamin, and cys-rich prolamin fractions. Lane 1, globulin fraction; Lane 2, cys-poor prolamin fraction; Lane 3, cys-rich prolamin fraction. Bands corresponding to globulin (*black arrow*), 13 kDa cys-poor prolamins (*white arrow*), 16 kDa cys-rich prolamin (*black arrowhead*), 13 kDa cys-rich prolamins (*asterisk*), and 10 kDa cys-rich prolamins (*white arrowhead*) are indicated

prolamin (Fig. 10, lanes 2 and 3, respectively). Because cys-rich prolamins accumulate at lower levels than the other seed storage proteins, the entire cys-rich prolamin fraction sample should be subjected to SDS-PAGE.

10. Use litmus paper (wide range) to measure approximate pH. Glutelins are precipitated as insoluble matter as pH levels approach 7.0. Glutelins can redissolve if the pH increases above 8.0, for example, if too much NaOH is added. In this case, the pH can be lowered to around 7.0 by adding more glutelin extraction buffer.
11. Endogenous glutelins can typically be extracted after the removal of globulins from the seed powder (extraction 1), or the removal of cysteine-poor prolamins after globulin has been removed (extraction 2). However, recombinant proteins are sometimes extracted by lactic acid only after cysteine-rich prolamins have been removed by a 2-propanol and 2-mercaptoethanol treatment following th extraction of globulin (extraction 3). In this case, the necessity for the removal of cysteine-rich prolamins before the extraction of recombinant proteins suggests that the free-cys of recombinant proteins aggregates with cysteine-rich prolamins via intermolecular disulfide bonds.
12. Bromophenol blue in the total extraction buffer is used as a pH indicator. The color of the solution, after adding the extraction buffer, should be blue. An orange or yellow color indicates that the solution is too acidic. In such a case, slowly add more of the stop solution (which is basic) until the color changes to blue.

13. The addition of total protein extraction buffer to samples is not sufficient to inactivate pancreatin. Samples should be immediately boiled after adding the total protein extraction buffer or stored at −30 °C until the boiling step.
14. Since only a small amount of seed protein is lost during these steps, the amount of protein collected should be adequate. Because seeds weigh approximately 17–20 mg per seed, control proteins equivalent to approximately 6.8–8 mg of seed powder can be obtained from 200 μL of the total protein extract. Control proteins are typically digested less than 1–2 min after pepsin or pancreatin treatment.
15. Li^+ remaining in the samples may inhibit the activity of reverse transcriptase in subsequent steps (RT-PCR).
16. A DNase I treatment should be performed before RT-PCR. On the other hand, an RT-PCR kit in which a separate DNase I treatment is unnecessary is also commercially available (e.g., ReverTra Ace qPCR RT Master Mix with gDNA Remover).
17. Signals of recombinant proteins that accumulate in PB-I completely merge with rhodamine signals. When recombinant proteins accumulate in PB-II or the ER, a double immunostaining experiment using two antibodies [e.g., an anti-recombinant protein antibody (rabbit) and anti-marker protein (mouse)] is required to confirm the correct localization of recombinant proteins. OsTiP3 and calnexin are generally used as PB-II and ER marker proteins, respectively. On the other hand, not only PB-I but also PB-II can be stained with treatment of rhodamine (for more than 1 h) but PB-II may be stained pale red (Fig. 11) [20].

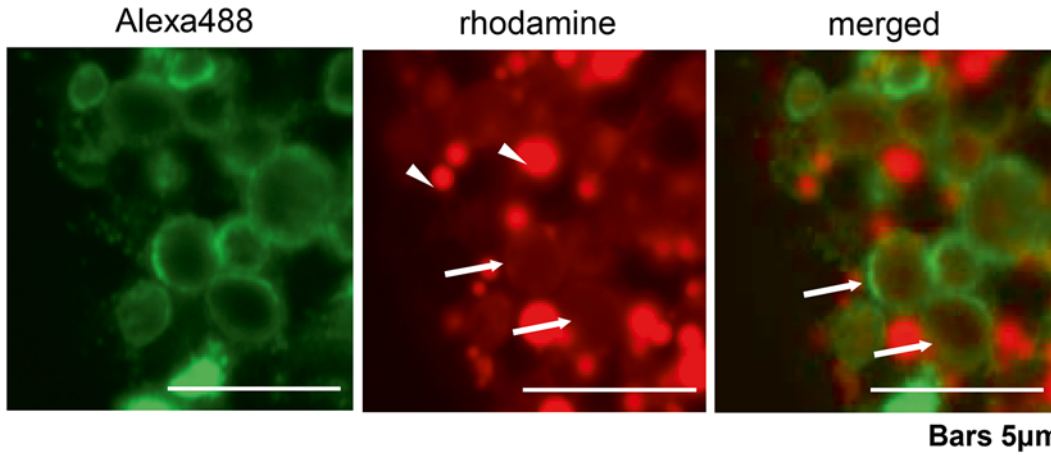

Fig. 11 Over-staining (for more than 1 h) of rhodamine B in transgenic rice seeds. The *left panel* shows the localization of recombinant proteins (*green*, Alexa 488), the *middle panel* shows the localization of PB-I (*arrowhead*) and PB-II (*arrow*), and the *right panel* shows merged images

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