

# Endosperm tissue is good production platform for artificial recombinant proteins in transgenic rice

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Received 12 June 2006;

revised 8 August 2006;

accepted 10 August 2006.

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**Keywords:** AGPase large subunit promoter, endosperm tissue, 7Crp, protein body, transgenic rice, ubiquitin promoter.

## Summary

Transgenic rice plants expressing 7Crp peptide were generated by *Agrobacterium*-mediated transformation. The 7Crp peptide is the hybrid peptide of seven major human T-cell epitopes derived from Japanese cedar pollen allergens Cry j 1 and Cry j 2. When the 7Crp gene was expressed under the control of the rice AGPase large subunit or maize ubiquitin-1 promoters, it could only be detected in the endosperm of rice seed, although high levels of RNA transcript were observed in the leaf, stem, and seed embryo. It was demonstrated by confocal and electron microscopy analysis that the 7Crp peptide was mainly localized in the endoplasmic reticulum-derived protein bodies, designated protein body I (PB-I). Our results indicate that rice endosperm tissue has advantage over other tissues as a production platform for foreign recombinant proteins.

## Introduction

Transgenic plants have been used as attractive bioreactors for the production of recombinant proteins including pharmaceuticals and industrial enzymes (Hood and Woodard, 2002; Larrick *et al.*, 2002). Transgenic plant systems offer several advantages for the expression of genes and production of proteins in comparison to conventional competing systems such as microbial and mammalian cell culture systems. Recombinant proteins expressed in plants are produced with many benefits, including lower production costs, easy control of production scale, and low risk of contamination by human and animal pathogens (Daniell *et al.*, 2001; Fischer *et al.*, 2004). Furthermore, bioactive peptides or vaccines that accumulate in edible parts of crops do not require any processing or purification, thus allowing direct oral delivery.

The accumulation level of recombinant proteins in plant cells is influenced by several factors, including strength of the promoter, RNA and/or protein stability, subcellular localization, genetic background, and metabolism of the host cell (Delaney, 2002; Stoger *et al.*, 2002; Hood *et al.*, 2003; Tada *et al.*, 2003).

Low level accumulation and inconsistent product quantities have been pointed out as limiting factors for commercialization of transgenic plants as reliable protein production systems. In

order to enhance recombinant protein yield in transgenic crops, general approaches are to increase expression levels by taking advantage of tissue-specific strong promoters, codon-optimization of the target gene, adaptation of the 5' and 3' untranslated sequences involved in mRNA stability, improvement of protein stability, subcellular targeting to a suitable organelle, and choosing hosts fitted to expression of recombinant proteins (Twyman *et al.*, 2003). Seed, however, is an ideal plant production platform, because it is the natural storage organ for protein and starch and it offers several subcellular storage compartments for foreign recombinant proteins (Stoger *et al.*, 2005). Furthermore, when expressed in seed, recombinant proteins are stable for more than a year even if stored at room temperature, which would allow extensive transport and storage options that are not available in other plant organs (Stoger *et al.*, 2002). The seed is also an ideal direct delivery system because it can be used as a food or as a feed supplement.

Choosing the most ideal host plant for expression of recombinant proteins is also an important factor (Delaney, 2002; Stoger *et al.*, 2002). Critical factors are the ease of transformation and yield of recombinant protein per unit biomass the host plant can produce. To date, maize has been utilized as one of the best production platforms for industrial enzymes. The commercialized enzymes avidin and  $\beta$ -glucuronidase

(GUS) have been produced in maize (Hood *et al.*, 1997; Witcher *et al.*, 1998). Rice has also been regarded as an attractive alternative for the production of recombinant proteins based on high grain yield, ease of transformation, capacity for rapid scale up, self-pollinating, and oral delivery (Stoger *et al.*, 2005). Furthermore, rice is the only cereal crop in which the whole complete genome sequence is available.

We have recently generated transgenic rice plants accumulating the 7Crp epitope peptide in the seed by expressing it under the control of the endosperm-specific glutelin *GluB-1* promoter (Takagi *et al.*, 2005a). The 7Crp peptide is an artificial peptide composed of seven dominant human T-cell epitopes of Japanese cedar pollen allergens, Cry j 1 and Cry j 2. It has been demonstrated that this peptide can be used as safe and effective tolerogen for immunotherapy of cedar pollen allergic disease because of lack of its binding to specific immunoglobulin E (Hirahara *et al.*, 2001). We have recently succeeded in generating transgenic rice plants that can accumulate high amounts of this artificial peptide in seeds (~50–60 µg/~20 mg grain) (Takagi *et al.*, 2005a). When these seeds were orally administered to mice, which were then challenged with Cry j 1, T-cell proliferation and immunoglobulin E levels were significantly suppressed compared to mice that were fed non-transgenic rice seeds. These results indicate that oral administration of the transgenic rice grains containing 7Crp peptide can induce immune tolerance (Takagi *et al.*, 2005a). This underlying molecular mechanism of oral immune tolerance using transgenic rice grains containing T-cell epitopes has recently been examined (Takagi *et al.*, 2005b).

In this study, we investigated the tissue and intracellular localization suitable for production of artificial recombinant

7Crp peptide. We reported here that this peptide could be only accumulated in the endosperm tissue of transgenic rice plant irrespective of high amounts of transcripts in vegetative tissues, when it was expressed under the control of the rice AGPase large subunit and maize ubiquitin-1 promoters. These results indicate that endosperm tissue is the best production platform when foreign recombinant protein gene is constitutively expressed in transgenic rice plant.

## Results and discussion

### Production and characterization of transgenic rice plants

The glutelin GluB-1 signal peptide required for endoplasmic reticulum (ER) targeting and the KDEL (Lys-Asp-Glu-Leu) ER retrieval signal were fused to the 7Crp gene at the N and C termini, respectively. We have recently shown that the signal peptide was inevitable for accumulation of the 7Crp peptide, and attachment of the KDEL ER retention signal enhanced the accumulation levels (Takagi *et al.*, 2005a).

Two constructs were used to transform rice plants (Figure 1). The difference in the two constructs is the promoter used for expression of 7Crp gene. These promoters essentially direct constitutive expression, although their expression levels are different among tissues. The numbers of generated independent transgenic rice lines were 38 and 15 T<sub>0</sub> lines for the pAGPasePsig/7CrpKDEL/Nos3' and pUbiPsig/7CrpKDEL/Nos3' constructs, respectively. Integration of the 7Crp gene into the nuclear genome in these transgenic rice plants was verified by polymerase chain reaction (PCR) using 7Crp gene-specific primers (data not shown).

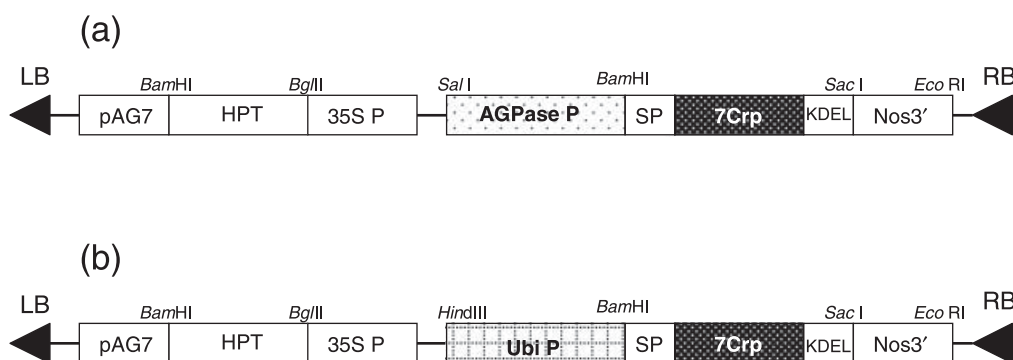


**Figure 1** Plant transformation vectors for the expression of the 7Crp peptide consisting of major human T-cell epitope peptides derived from Cryj1 and Cryj2 in rice. The 7Crp gene was fused upstream to a rice glutelin GluB-1 signal peptide, driven by the rice 1.9 kb AGPase large subunit promoter (a) or the maize ubiquitin-1 promoter with its non-translated first exon and intron (b), and fused downstream to 0.3 kb the nopal synthase terminator (Nos3'). The GluB-1 signal peptide (SP) and KDEL ER retention signal were attached to the N and C termini of the 7Crp peptide. 35S P, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase gene; pAG7, gene 7 poly(A) signal.

### Expression of 7Crp in transgenic rice plants

When accumulation levels of 7Crp peptide were examined by Western blot analysis, it appeared to be below detectable level in vegetative tissues such as leaf and stem. The 7Crp peptide was only detected in seeds (Figure 2). When we compared the accumulation levels in mature dry grains after expression with the rice AGPase and the maize ubiquitin-1 constructs, we observed that the former construct accumulated much higher levels of 7Crp peptide in grains than the latter. As shown in Figure 3, the concentration of 7Crp peptide in each mature dry grain was examined and presented as dots for the individual transgenic line. Four grains were randomly selected from independent transgenic lines, and the concentration of 7Crp peptide in each grain was analysed (each value was represented as a dot). The highest accumulation line for the AGPase construct was about 10  $\mu\text{g}/\text{grain}$ , whereas grains expressing 7Crp under control of the maize ubiquitin-1 promoter contained only about 0.2  $\mu\text{g}/\text{grain}$ . As shown in Figure 2, total seed proteins extracted from the highest ADPase and ubiquitin-1 expression lines were subjected to the SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The 7Crp peptide was not detectable as a visible band on the Coomassie Brilliant Blue stained gel. This contrasts with the GluB-1 construct in

which the 7Crp peptide was visible and the 57 kDa glutelin precursor had accumulated abnormally due to expression more than five times greater (50–60  $\mu\text{g}/\text{grain}$ ) than in the AGPase or ubiquitin-1 constructs (Takagi *et al.*, 2005a). High accumulation of the 57 kDa glutelin precursor may be due to retarded trafficking from the ER to protein bodies under ER stress. This may be a result in a disturbance of post-translational processing from preglutelin into mature acidic and basic subunits in the protein bodies. Furthermore, there may be competition for the glutelin signal peptide required for entry into the ER lumen, and several chaperones required for proper unfolding between the artificial 7Crp peptide and native glutelins.

In order to examine the characteristics of promoters used for expression of the 7Crp gene, the GUS reporter gene linked to the same rice AGPase large subunit promoter (1.9 kb) and the maize ubiquitin-1 promoter (2.0 kb) was stably introduced into rice genome, the former AGPase promoter directed higher expression in stems and seeds (seed,  $10.2 \pm 4.7$ ; leaf,  $12.5 \pm 5.0$ ; stem,  $40.2 \pm 28.8$  pmol 4 MU/ $\mu\text{g}$  protein/mi; average values from 18 independent transgenic lines), whereas the latter led to higher expression in leaves and stems besides seed (seed,  $7.4 \pm 4.2$ ; leaf,  $31.9 \pm 5.5$ ; stem,  $149 \pm 36.8$  pmol 4 MU/ $\mu\text{g}$  protein/min; average values from 12 independent transgenic lines).

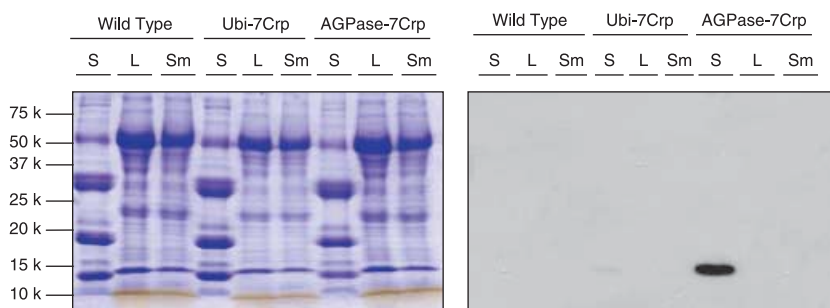


**Figure 2** Western blot analysis of the 7Crp peptide expression in transgenic rice plants. Total protein extracts (about 20  $\mu\text{g}$ ) from transgenic rice leaf, stem and maturing seed (15 DAF) were separated on SDS-PAGE, blotted on to PVDF membrane and probed with an anti-7Crp antibody. Numbers on the left indicate the position of protein size markers. S, seed; Sm, stem; L, leaf.

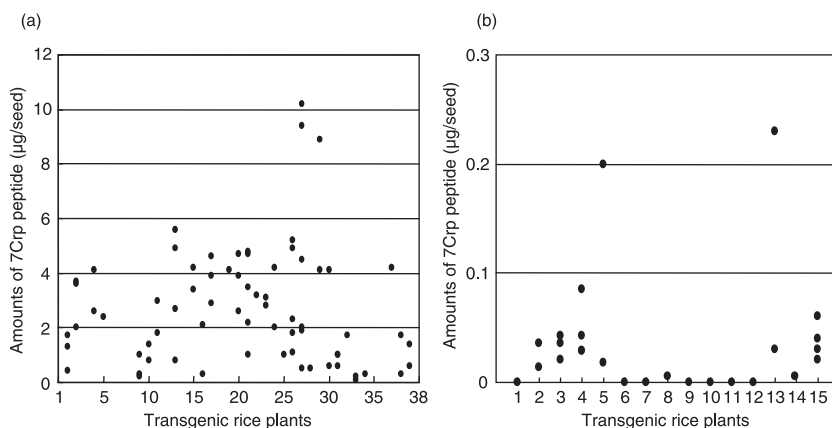


**Figure 3** Accumulation levels of 7Crp peptide in mature seeds of transgenic rice plants transformed with the AGPasePsig/7CrpKDEL/Nos3' and UbiPsig/7CrpKDEL/Nos3' expression vectors. (a) AGPasePsig/7CrpKDEL/Nos3', (b) UbiPsig/7CrpKDEL/Nos3' constructs.

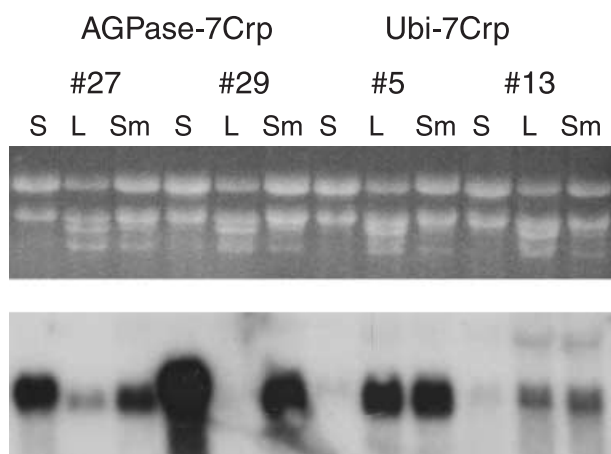


**Figure 4** Northern blot analysis of 7Crp transcript in various tissues of transgenic rice plants. Total RNA was extracted from maturing seeds (S), stem (Sm) and leaf (L). Ten micrograms of RNA was separated on a agarose/formaldehyde gels, blotted on to nylon membrane and hybridized with labelled DNA probes for 7Crp. The 7Crp mRNA was detected in representative transgenic rice plants carrying the AGPasePsig/7CrpKDEL/Nos3' (AGPase-7Crp) and UbiPsig/7CrpKDEL/Nos3' (Ubi-7Crp) constructs.

In order to ascertain whether the 7Crp gene was highly expressed in the vegetative tissues of transgenic rice plants, we selected two representative lines with high expression of the construct (#27 and #29 lines for the AGPase construct, #5 and #13 lines for the ubiquitin-1 construct). The total RNAs were isolated from maturing seed, leaf and stem and were subjected to Northern blot analysis (Figure 4). High 7Crp transcript levels were observed in both leaf and stem for the ubiquitin-1 construct and in both stem and seed for the AGPase construct, respectively (Figure 4). These results were in remarkable contrast with the results of our Western blots. The 7Crp peptide was not detected in the vegetative tissues exhibiting high GUS and RNA transcript activities. There has never been such a dramatic tissue-specific difference reported. It is not yet known if there is any translation at all in vegetative tissues. However, the 7Crp peptide can be produced in maturing seed of the same transgenic lines used for analysis, suggesting that it is translated in vegetative tissue but is very unstable. This instability may be related to the artificial nature of the 7Crp peptide, which may target it for digestion as an abnormal protein in vegetative tissues. Considering that the 7Crp peptide is designed to be transported to the ER by means of a KDEL signal sequence fused to its C terminus, further work will need to be done to locate the site of 7Crp peptide degradation in vegetative tissue cells.

We then further examined the ability of the 7Crp peptide to accumulate in vegetative tissues of other plants. In this experiment, the tomato ribulose biphosphate carboxylase-

oxygenase (*rbc*) 3A-small subunit promoter with (1812 bp) or without (1630 bp) its transit signal peptide required for targeting to plastids was fused to the 7Crp gene. We introduced these chimeric genes into the tobacco nuclear genome by *Agrobacterium*-mediated transformation (data not shown). We selected high expression lines of 7Crp transcripts in leaves by Northern blot analysis and their crude protein extracts were subjected to Western blot analysis. Despite high levels of mRNA transcripts, the 7Crp peptide could not be detected by Western blot analysis (data not shown). Taken together, these results suggest that artificial peptides such as the 7Crp are unstable in vegetative cells, even if the peptide was targeted and expressed in plastid or in the cytoplasm.

However, our observations contradicted the work showing that the foreign scFv antibody gene, driven by the maize ubiquitin-1 promoter, can be accumulated in transgenic rice leaves and grain at similar levels (Stoger *et al.*, 2000; Torres *et al.*, 2001). Furthermore, proteins such as antibodies, vaccines and other biopharmaceuticals that are produced in stable transgenic plants under the control of constitutive promoters (e.g. CaMV 35S and the ubiquitin-1 promoters) accumulated in leaves, roots and tubers despite the low level expression (less than 1%) of total soluble proteins (Daniell *et al.*, 2001). One possible reason is that the use of the glutelin signal peptide has an effect on tissue-specific accumulation. However, this explanation may not be applicable, since the 7Crp peptide was not detectable in tobacco leaves, even if 7Crp gene was highly expressed in leaves under the control of the tomato *rbc*3A small subunit promoter containing the transit peptide in place of the glutelin signal peptide. Taken together, this discrepancy may be due to protein properties used for expression and/or the scFv may be folded in the ER, resulting in stable accumulation.

#### Localization of the 7Crp peptide in whole seed

In order to examine where the 7Crp peptide is accumulated in rice seed, the seed was separated into embryo and endosperm and then subjected to Western blot analysis. As shown in Figure 5c, the 7Crp peptide could not be detected in embryo cells, but could be detected in the endosperm. In order to examine the expression sites of the 7Crp peptide in seed, *in situ* histochemical Western hybridization (Qu *et al.*, 2003) was carried out with immature seeds using anti-7Crp peptide antibody. As shown in Figure 5a, 7Crp peptide (visible as purple staining) was mainly accumulated in endosperm, whereas its accumulation level in embryo was very weak. This accumulation pattern of the 7Crp peptide was almost identical when analysing seeds that were transfected

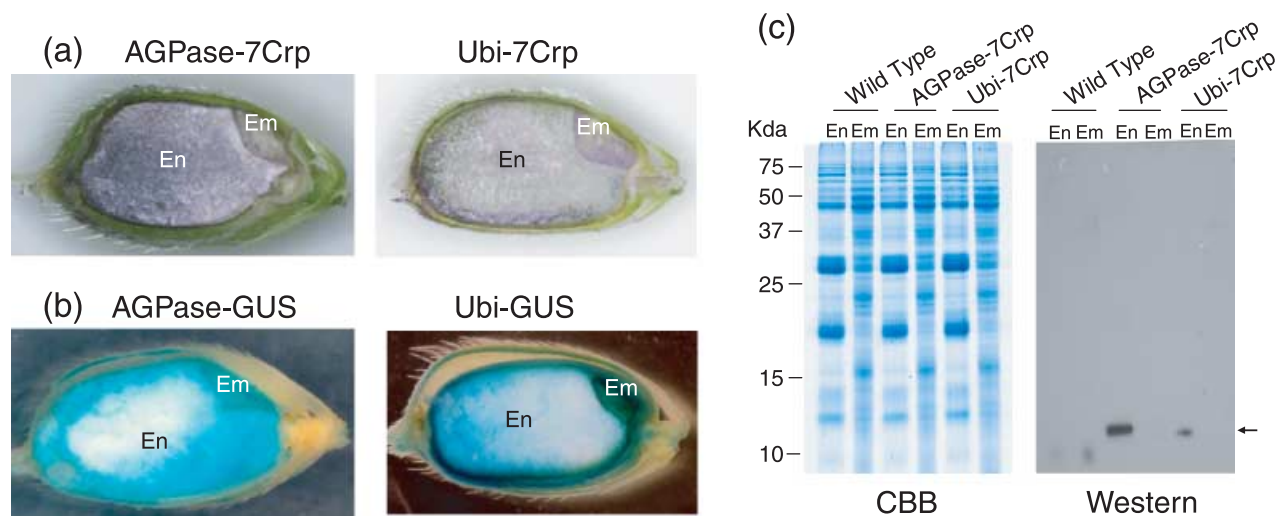


**Figure 5** Characteristic accumulation of the 7Crp peptide in the endosperm tissue of transgenic rice seeds. (a) *In situ* immunolocalization of 7Crp peptide in seed. Maturing rice seeds from transformants were cut in half at the vertical section, and then analysed by *in situ* hybridization using anti-7Crp antiserum. For visualization of signals derived from 7Crp, anti-rabbit IgG antibody labelled with alkaline phosphatase was used as a secondary antibody. En, endosperm; Em, embryo. (b) Specific expression of the GUS reporter gene in developing seed, which was under the control of the same rice AGPase large subunit and maize ubiquitin-1 promoters used for expression of the 7Crp gene. (c) Accumulation of 7Crp in the endosperm and detached embryo of the representative transgenic rice seed containing AGPasePsig/7CrpKDEL/Nos3' (AGPase-7Crp) and UbiPsig/7CrpKDEL/Nos3' (Ubi-7Crp) constructs.

with the different constructs. However, it was in remarkable contrast with the results of GUS activities directed by the AGPase and maize ubiquitin-1 promoters, which exhibited higher expression levels in embryo cells than in endosperm (Figure 5b). These results confirm that the 7Crp peptide is unstable in any other tissues than the endosperm in seed, suggesting that the artificial 7Crp peptide is degraded in other tissues.

However, high accumulation of foreign gene product in the endosperm such as what we observed in transgenic rice seeds was not applicable to other seeds. When sweet protein brazzein (Lamphear *et al.*, 2005) and fungal laccase genes (Hood *et al.*, 2003) were expressed in stable transgenic maize under the control of seed-specific promoters (embryo- or endosperm-specific promoters), the proteins were more highly accumulated in the embryo part of the seed. It has been demonstrated that the accumulation level was about 100-fold higher when driving expression with an embryo-specific promoter compared to an endosperm-specific promoter. Furthermore, it is interesting to note that recombinant egg white avidin and GUS were also located primarily in the embryo of the transgenic maize seed, when these genes were expressed under the control of maize ubiquitin-1 promoter. It should be noted that the endosperm, representing about 80% of the total kernel weight, contributed only 22% and 7% of the avidin and GUS activities found in whole kernels, respectively (Hood *et al.*, 1997; Witcher *et al.*, 1998).

It was demonstrated that the avidin protein was primarily localized in the intercellular spaces of the cell wall (apoplasts), whereas GUS was associated with a series of small spherical organelles throughout the cytoplasm (Hood *et al.*, 1997; Witcher *et al.*, 1998). The accumulation of the brazzein protein was enhanced by fusing to the secretion signal peptide of  $\alpha$ -amylase, confirming the validity of this intracellular target site. In summary, the difference in suitable accumulation sites between maize and rice may be responsible for properties of seeds as an expression platform or expressed recombinant protein properties.

#### Intracellular localization of the 7Crp peptide

*In situ* localization experiments were performed on thin sections of endosperm and embryos using anti-7Crp primary antibodies and fluorescently labelled secondary antibodies with Alexa-Fluor 488 (Figure 6a–f). It was shown that green fluorescence was mainly restricted to ER-derived protein bodies named PB-I or type I protein body, because prolamin PBs are visualized by staining with rhodamine B, and the fluorescence of Alexa-Fluor 488 colocalized mainly with the red fluorescence of rhodamine B. Weak signals that are independent of prolamin are also sometimes observed. Those signals may be localized in the ER because 7Crp peptide can be translated and deposited there. These results indicate that the 7Crp peptide is mainly transported into PB-I.

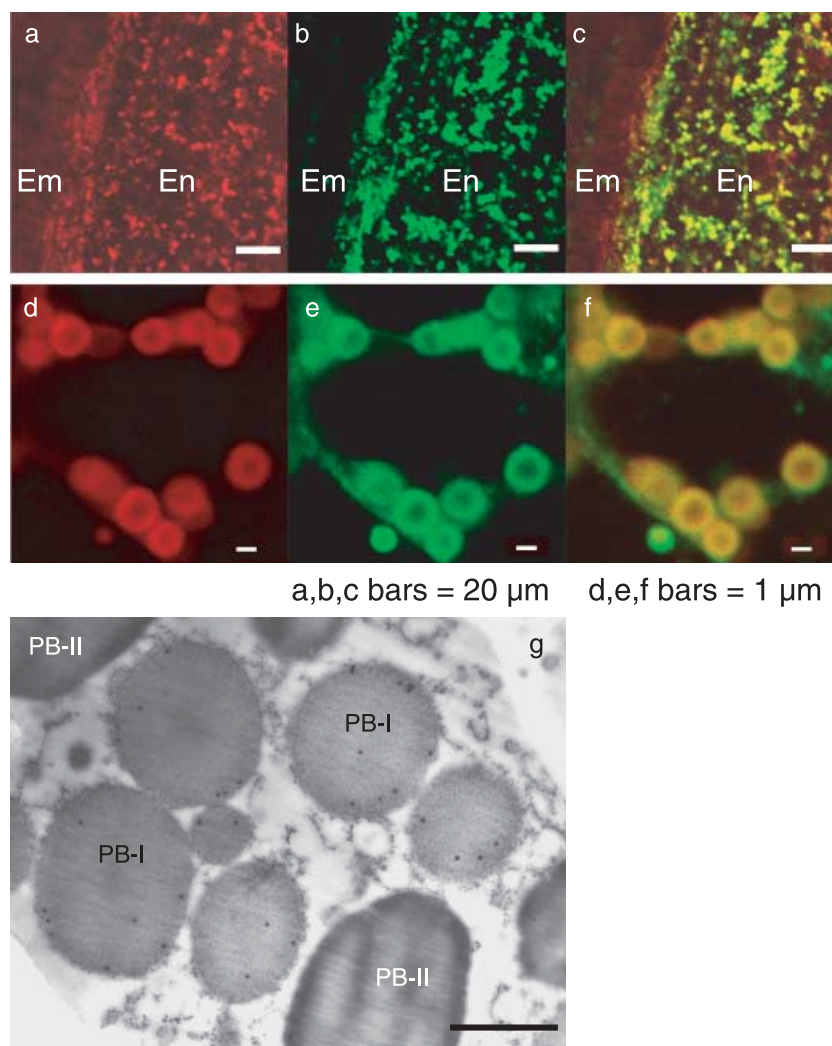


**Figure 6** Intracellular localization of 7Crp peptide in the immature seeds of AGPase Psig/7CrpKDEL/Nos 3' transgenic rice. (a–f) 7Crp peptide localization observed by a confocal laser scanning microscopy. (a and d) Seed sections were stained with rhodamine B (red fluorescence), which specifically binds prolamins in PB-I. (b and e) 7Crp peptide were detected by anti-7Crp antibody and the second antibody with green fluorescence. (c and f) Two images (a, d) and (b, e) were merged. (a–c) An area of the seed section contains endosperm (En) and embryo (Em). Note that prolamins and 7Crp were localized on the same protein bodies in the endosperm cells and not in the embryo cells. Bars = 20  $\mu$ m. (d–f) High magnification view of prolamins and 7Crp peptide in the endosperm cell. Prolamins and 7Crp peptide are located in the same bodies. Bars = 1  $\mu$ m. (g) 7Crp peptide localization detected by immunoelectron microscopy. Twenty-five-nanometre (25 nm) gold particles are localized on the peripheral region of PB-I. Bar = 1  $\mu$ m.

In order to examine further the intracellular distribution of the 7Crp peptide in transgenic rice seed, the 7Crp peptide was localized within the cellular ultrastructure and detected with specific primary antisera and secondary antibodies conjugated to 25-nm gold particles. The experiments were performed with ultrathin sections of immature seeds (15 DAF) from transgenic rice plants harbouring the AGPase construct.

In the rice endosperm tissue, there are two types of storage protein bodies in the same cell: the ER-derived protein body and the protein storage vacuole (PSV) (Tanaka *et al.*, 1980; Takaiwa *et al.*, 1999). Prolamin is localized in the round-shaped former storage protein bodies (PB-I), which are formed within the ER, whereas major storage proteins such as glutelin and the 26 kDa globulin are localized with high density within the irregular-shaped latter protein body (PB-II).

When immature endosperm of the transgenic rice plants containing the AGPasePsig/7CrpKDEL/Nos3' construct was

observed by immuno-electron microscopy in order to identify localization of the 7Crp peptide, gold particles were predominantly localized at the periphery of the PB-I (Figure 6g). It is notable that gold particles for the 7Crp antibody were not detected at any organelle in the endosperm cell of non-transgenic rice (data not shown). The intracellular localization pattern of the 7Crp peptide is slightly different from that observed in the transgenic rice seed containing the GluB-1Psig/7CrpKDEL/GluB3' construct. In the case of the GluB-1 promoter construct, the 7Crp peptide was predominantly deposited in PB-I and PB-II in the developing endosperm (Takagi *et al.*, 2005a). One explanation for this discrepancy is that it may be due to the difference in the 3' flanking sequences of the constructs. It has been reported that the 3' untranslated region (3'UTR) of glutelin mRNA has *cis* elements that target mRNAs to the cisternal ER (Hamada *et al.*, 2003). Thus, it was expected that the glutelin GluB-1 3'UTR may actively participate in transportation of the 7Crp mRNA to the

cisternal ER, resulting in trafficking of 7Crp to the PB-II. It is also possible that ER resident proteins might be incorporated into PB-I or remain in the ER lumen because of the retrieval to the ER lumen by the KDEL retrieval signal. Alternatively, the 7Crp peptide may have been expressed much more highly by the GluB-1 promoter than by the AGPase promoter, thus contributing to the observed differences in subcellular depositions due to saturation of the KDEL receptor. In addition, the different temporal expression patterns conferred by the GluB-1 and AGPase promoters could also account for some of the difference (Qu and Takaiwa, 2004).

When the scFv gene containing the N-terminal murine immunoglobulin G heavy-chain signal peptide and the C-terminal KDEL ER retention signal was expressed in rice endosperm tissue under the control of maize ubiquitin-1 promoter, the recombinant scFv antibody was accumulated in the PB-I and PB-II (Torres *et al.*, 2001). This intracellular deposition pattern was very similar to that observed in the transgenic rice harbouring the GluB-1 Psig/7CrpKDEL/Nos3' construct. It is notable that the 3' Nos terminator and N-terminal murine immunoglobulin heavy-chain signal peptide were used for expression of the scFv antibody. These results suggest that targeting the scFv antibody to the protein bodies may be dependent on the presence of the KDEL signal attached to the C termini of the scFv antibody. Torres *et al.* (2001) proposed that accumulation of the scFv in PSVs or the vacuole may attribute to the inefficient retention and leakage from the ER through the Golgi network irrespective of the KDEL retrieval signal. It has been demonstrated that non-assembled immunoglobulin accumulated in PB (PB-I) and PSVs (PB-II), whereas the assembled antibody was accumulated predominantly within the PSVs, thus indicating that their intracellular tracking is influenced by its state of assembly (Nicholson *et al.*, 2005). It is interesting to note that targeting to protein bodies of antibodies is determined by their protein properties, because neither the KDEL signal nor the 3' or 5' UTR sequences of storage proteins was utilized for targeting to a specific organelle. It is notable that the ER signal peptide of immunoglobulin was only necessary for trafficking to ER-derived PB and PSVs. Taken together, the intracellular destination of proteins may be influenced by specific sequences, such as the KDEL ER retention signal or N- or C-terminal propeptides involved in vacuolar targeting, or protein conformation within mature structure and oligomeric state as reported by Ellgaard and Helenius (2003).

In summary, although targeting of recombinant proteins to the intracellular locations are divergent dependent on expressed proteins, it was clearly shown here that rice endosperm tissue can be used as an ideal bioreactor for

production of recombinant proteins. Moreover, rice endosperm tissue occupies more than 90% of the total seed weight, and represents a stable storage organ that enables the plant to accumulate high amounts of recombinant products.

## Experimental procedures

### Plasmid construction

The region coding for the GluB-1 signal peptide and 7Crp peptide containing the KDEL ER retention signal was amplified from the pGluB-1pro sig/7CrpKDEL plasmid DNA using primers containing *Bam*HI and *Sac*I sites (Takagi *et al.*, 2005a). After digestion of this amplified PCR fragment with these restriction enzymes, it was fused to the 2.0 kb rice AGPase large subunit promoter (Qu and Takaiwa, 2004) and the 2.0 kb maize ubiquitin-1 promoter containing 899 bp of promoter sequence, 83 bp of 5' untranslated exon and 1010 bp of the first intron sequence, respectively (Christensen and Quail, 1996). The promoter cassettes were cloned between the *Hind*III and *Bam*HI sites of pUC19. These chimeric genes (AGPasePsig/7CrpKDEL/Nos3' and UbiPsig/7CrpKDEL/Nos3') were further inserted into the appropriate sites of the pGPTV35SHPT binary vector, and then transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. The 7Crp gene was introduced into the rice genome by *Agrobacterium*-mediated transformation and the transgenic rice plants were selected by hygromycin resistance as described previously (Goto *et al.*, 1999).

### RNA extraction and Northern blot analysis

Total RNA was isolated from leaf, stem and maturing seeds (15 days after flowering) of T3 homozygous transgenic rice plants as described previously (Takaiwa *et al.*, 1987). Total RNA was extracted from frozen tissues with equal volume of the RNA extraction buffer [0.1 M Tris-HCl (pH 9.0), 50 mM NaCl, 1 mM EDTA, 0.5% SDS] and chloroform-phenol (1 : 1, v/v), and then precipitated with the final concentration of 2 M LiCl.

Resultant high molecular weight RNAs were subjected to electrophoresis on a 1.2% (w/v) formaldehyde-agarose gel and subsequently transferred to a Hybond N+ nylon membrane (Amersham). The 7Crp coding region amplified by PCR was labelled by a random priming reaction with <sup>32</sup>P dCTP and used as a probe. The blot was hybridized with the labelled 7Crp in Rapid-Hyb buffer (Amersham) at 65 °C for 2 h, and washed twice at room temperature in 2× SSC, 0.1% SDS, four times at 55 °C in 0.1× SSC, 0.1% SDS.

### Protein extraction and Western blot analysis

Transgenic rice tissues (seed, leaf, stem, endosperm and embryos) were ground to fine powder using Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Proteins were extracted from these tissues with protein extraction buffer (20 mM Tris-HCl (pH 6.8), 8 M urea, 5% 2-mercaptoethanol, 20% glycerol, and 4% SDS by shaking vigorously for 1 h at room temperature. Extracts were centrifuged and the supernatant fractions were used for analysis.

Proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore) for Western blot analysis.

The blots were reacted with an anti-7Crp antibody followed by anti-rabbit peroxidase-conjugated secondary antibody and the signal was detected using ECL Kit (Amersham) as described previously (Takagi *et al.*, 2005a).

The amount of 7Crp peptide in seed protein extracts of independent transgenic plants was estimated on the basis of the intensity of signals detected by Western blot analysis using purified 7Crp-6 × His fusion protein as a calibration control as described previously (Takagi *et al.*, 2005a).

### Immunocytochemical observations by confocal laser scanning microscopy

Transverse sections of immature seeds (15–20 DAF) approximately 200 µm thick were prepared using a microslicer DTK-1000 (Dosaka EM, Kyoto, Japan). The sections were fixed for 1 h in 3.7% formaldehyde in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>HPO<sub>4</sub>) and were washed three times in PBS at intervals of 10 min. The samples were incubated with cell wall digestion solution containing 1% cellulase, 0.1% pectolyase Y-23 in PBS at 37 °C for 3 min, and then were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. The sections were blocked with PBS/bovine serum albumin (BSA) (1% BSA and 0.01% Triton X-100 in PBS) at room temperature for 1 h to prevent nonspecific binding of antibody. Then they were reacted with anti-7Crp monoclonal antibody from mouse (provided by Dr Saito, Jikei University School of Medicine) diluted 1 : 500 in PBS/BSA overnight at room temperature. After washing with PBS three times for at least 10 min each, the sections were reacted with Alexa-Fluor 488 conjugated with goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR) diluted 1 : 300 in PBS/BSA for 2 h at room temperature. To stain PB-I, the sections were incubated with 1 M rhodamine B in PBS for 30 min and washed three times in PBS. The samples were mounted on a glass slide with a drop of 'component A' of Slow Fade Antifade kit (Molecular Probes) and observed by a confocal laser scanning microscopy (MicroRadiance 2000; Bio-Rad, Richmond, CA).

### Immunocytochemical observations by TEM

The immature seeds (15–20 DAF) were cut into small cubes approximately 1 mm<sup>3</sup> and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde buffered at pH 7.2 with 20 mM PIPES overnight at 4 °C. After washing three times for at least 40 min each in PIPES buffer, the samples were dehydrated in a series of ethanol concentrations and were embedded in LR White resin (London Resin, Berkshire, UK). Ultrathin sections were cut with a glass knife using an ultramicrotome (MT2-B, Sorvall, Newtown, CT) and mounted on copper grids. The grids were floated on a drop of PBS/BSA containing 3% BSA for 1 h and were incubated for 1 h with anti-7Crp monoclonal antibody diluted 1 : 10 with PBS containing 1% BSA. Nonspecifically bound antibodies were removed by washing the grids floated on a drop of PBS containing 0.1% BSA, 0.5 M NaCl and 0.05% Tween 20 three times for 5 min each. The samples were reacted with gold-labelled goat anti-mouse IgG antibody; 25 nm (Aurion, Wageningen, the Netherlands) diluted 1 : 200 in PBS containing 1% BSA for 1 h. Then the grids were washed three times with PBS containing 0.1% BSA, 0.5 M NaCl and 0.05% Tween 20. The sections

were fixed with 1% glutaraldehyde in PBS followed by washing with distilled water twice, stained with uranyl acetate for 2 h and lead citrate for 7 min. The samples were examined by using a transmission electron microscope (H-71000, Hitachi, Tokyo, Japan) running at 75 kV.

### Acknowledgements

This work was supported by research grants (Research for the Utilization and Industrialization of Agricultural Biotechnology; Functional analysis of genes relevant to agriculturally important traits in rice genome) from the Ministry of Agriculture, Forestry and Fisheries of Japan to F. Takaiwa.

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