Title:

Difference in accumulation pattern of the allergens within the same PR10 family in transgenic rice

Running title: Expression of Mal d 1 in transgenic rice

Authors:

Fumio Takaiwa[1]*, Yuko Ogo [1,2], Yuhya Wakasa[1],

Affiliations

[1] Institute of Agrobiological Sciences, National Agriculture and Food Research Organization Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan

[2]Institute of Crop Science, National Agriculture and Food Research Organization, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan

E-mail address

Fumio Takaiwa: takaiwa@affrc.go.jp, Yuhko Ogo: ogogogo@affrc.go.jp, Yuhya Wakasa: ywakasa@affrc.go.jp

***Corresponding author**:

Fumio Takaiwa

Plant Molecular unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan

TEL: +81-29-838-8373

E-mail**.:** takaiwa@affrc.go.jp

The date of submission June 27, 2018

Number of Figures :9

Words (introduction to acknowledgement) 5956

Figures should be in color only online 9

Numbers of Supplementary Figures: 7

Difference in accumulation pattern of the allergens within the same PR10 family in transgenic rice

Highlight

Specific region of PR10 proteins is mainly implicated in their stability in vegetative tissues when expressed in transgenic rice.

Abstract

Apple food allergen Mal d 1 and birch pollen allergen Bet v 1 belong to the same pathogen related protein 10 (PR10) family. When each of these allergens was expressed as a secretory protein by fusion with the GFP reporter in transgenic rice by ligating an N terminal signal peptide and a C terminal KDEL ER retention signal under the control of the maize ubiquitin constitutive promoter, the GFP:Mald1 highly accumulated in various tissues, whereas the accumulation level of GFP:Betv1 was remarkably reduced in vegetative tissues except for seed. Analysis by RT-PCR exhibited that there was little difference in transcript levels between them, indicating the involvement of post-transcriptional regulation. To investigate the cause of such difference in accumulation levels, deletion analysis of the Mal d 1 and domain swapping between them were carried out in transgenic rice. These results showed that the region between positions 41-90 in the Mal d 1 is predominantly implicated in higher level accumulation in vegetative tissues as well as seed compared with the Bet v 1. It is notable that GFP:Mald1 directed by the ubiquitin promoter is deposited in huge PBs in aleurone layer rather than starchy endosperm.

Key words: Apple allergen, Bet v 1, PR10, protein body, Mal d 1, transgenic rice

Abbreviations: Cys, cysteine; BiP, binding protein; ER, endoplasmic reticulum; ER, elastin-like polypeptide; GFP, green fluorescence protein; OSA, oral allergy syndrome; PB, protein body; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RT-PCR; reverse transcription-polymerase chain reactions; PR10, pathogen related protein 10, PDIL; protein disulfide isomerase

Introduction

Mal d 1 is the major allergen of apple fruit eliciting oral allergy syndrome (OAS) such as itching and swelling of lips, tongue and throat, which belongs to the family of pathogen-related 10 (PR10) proteins within the Bet v 1 superfamily (Mari *et al*. 2005; Fernandes *et al*, 2013). It has been reported that more than 70% of birch pollen-allergenic patients sensitizing to the major birch pollen allergen Bet v 1 display OAS after apple consumption (Geroldinger-Simic *et al*., 2011). Namely, the OAS by apple is provoked by the allergic cross-reaction between the Mal d 1 and Bet v 1 specific IgE, indicating the presence of common sequences (IgE epitopes) and tertiary structure required for its binding to the Bet v 1 specific IgE. (Ebner *et al*., 1991; Geroldinger-Simic *et al*., 2011).

Mal d 1 is composed of 159 amino acids with the estimated molecular mass of 17.5 kDa. Its subcellular localization is estimated to be intracellular and cytoplasmic like the other PR10 proteins, since there is no signal peptide sequence directing to the endomembrane system as a secretory protein (Fernandes *et al.* 2013). Mal d 1 is mainly present in the pulp and peel of apple, but is not only expressed in apple fruit but also in various vegetative tissues and seed (Beuning *et al*., 2004; Marzban *et al.,* 2005). Although the biological function of Mal d 1 remains unknown well, its expression has been studied to respond to fungal or bacterial infection and also to be activated by stress and storage, suggesting that it participates in defense mechanism against pathogens or abiotic stress like other PR10 proteins (Fernandes *et al.* 2013).

Mal d 1 is encoded by a multigene family composed of 31 members, which are divided into four groups based on amino acid sequence similarities, Mal d 1.01, Mald d 1.02, Mal d 1.03 and Mal d 1.04 (Mal d 1 a-d) (Pagliarani *et al*., 2013). Mal d 1.01 and Mal d 1.02 are predominantly expressed in apple fruit (peel and pulp) （Marzban *et al*., 2005）. Amino acid sequence identities between Mal d 1 isoforms and Bet v 1 range from 55 to 68 % (Ma *et al.,* 2006).

Rice seed provides good production platform for high-value recombinant proteins in terms of scalability, safety, stability and cost-effectiveness (Stoger *et al.*, 2005; Takaiwa *et al*., 2015). Especially, when recombinant proteins are produced as secretory protein and then deposited into protein-bodies (PBs) in the endosperm cells, they are highly and stably accumulated (Takaiwa *et al.*, 2017). Moreover, when orally administered, it has been known that recombinant proteins bio-encapsulated in PBs are effectively delivered to small intestine without degradation with digestive enzymes in gastrointestinal tract, providing an ideal delivery system to gut-associated lymphoid tissues (GALT) as oral mucosal vaccines (Hofbauer and Stoger, 2013; Takaiwa *et al.*, 2015).

We previously generated transgenic rice seeds containing versatile hypo-allergenic derivatives of major birch pollen allergen Bet v 1 referred to as tree pollen chimera7 (TPC7) or TPC9, which have been developed as allergy vaccines (tolerogens) against the major allergens of trees belonging to the Fagales order Bet v 1 family for allergen-specific immunotherapy (Wang *et al*., 2013). When these native Bet v 1 and its derivatives (TPC7 and TPC9) were expressed as secretory protein in rice seeds under the control of the endosperm-specific glutelin *GluB-1* promoter by ligating the glutelin GluB-1 signal peptide and the KDEL ER retention signal to their N- and C-termini, they were deposited in remarkably huge spherical ER-derived PBs designated as TPC7 bodies with the size of >10 μm (Wang *et al.*, 2013; Ogo *et al.*, 2014). Furthermore, their accumulation levels reached about 23% of total seed proteins (Ogo *et al.,* 2014). Such ability to form huge PBs

112 is expected to have a potential to produce high amounts of foreign recombinant proteins
113 by fusion to them. Moreover, it is interesting to examine whether properties yielding such
114 huge PB formation and high productivity are retained in vegetative tissues, when expressed
115 in vegetative tissues.

116 In this study, we examined whether Mal d 1, Bet v 1 and TPC7 within the same
117 PR10 family are deposited in huge ER-derived PBs in various tissues of transgenic rice.
118 When expressed under the control of the maize ubiquitin constitutive promoter. it was
119 shown that the Mal d 1 stably and highly accumulates in vegetative tissues as well as seed,
120 whereas the accumulation levels of the Bet v 1 and TPC7 are very low in vegetative
121 tissues except for seed. Analysis by deletion and domain-swapping between the Mal d 1
122 and Bet v 1 demonstrated that this difference is mainly attributed to the region between
123 positions 41-90.

124

125 **Methods**

126 **Construction of expression vectors and generation of transgenic rice**

127 The DNA sequences encoding Mal d 1, Bet v 1 and TPC7 were optimized for translation
128 based on the codons frequently used in rice seed storage protein gens and their DNA
129 sequences were synthesized by GenScript Corporation (NJ, USA). The N terminal signal
130 peptide of the glutelin GluB-1 that is required for translocation into the ER and the KDEL ER
131 retention signals were attached to their N and C termini, respectively. The gene encoding
132 the green fluorescence protein (GFP) reporter was inserted in frame between the GluB-1
133 signal and these PR10 related genes. In order to express specifically in the endosperm or
134 constitutively in vegetative tissues, 2.3kb glutelin *GluB-1* promoter or maize ubiquitin
135 promoter were ligated upstream of these three fusion constructs, which were followed by
136 the 0.65kb of glutelin *GluB-1* terminator (Fig. 1B).

137 These gene expression cassettes inserted into entry vector were transferred into

destination binary vector (p35SHPTAg7-GW) harboring the CaMV35S promoter::hygromycine phosphotransferase gene (*HPT*) as selectable marker gene via the MultiSite Gateway LR Clonase reaction (Invitrogen) (Wakasa *et al.,* 2006). These binary vector plasmids containing various expression constructs were introduced into rice genome via *Agrobacterium*-mediated transformation (Goto *et al.,* 1999).

For each construct, more than 16 independent transgenic lines (16-30 lines) were generated. Transgenic rice plants were selected by hygromycin resistance and grown in the closed control greenhouse (33°C, 16h light/8h dark cycle).

SDS-PAGE and immunoblot analysis

Individual mature grain (four grains per independent transgenic line) and leaves and roots frozen in liquid nitrogen were ground into a fine powder using a Multibeads shocker (Yasui Kikai, Osaka, Japan). Total proteins were extracted from the powder of a mature grain, leaves and roots with 400 µl (grain) or 200 µl (leaves and roots) of urea-SDS buffer (50 mM Tris-HCl, pH 6.8, 8M urea, 4% SDS, 5% 2-mercaptoethanol (2-MER), 20% glycerol) as described previously (Tada *et al*., 2003). After separation of total protein extract (2-5 µl) by electrophoresis on 13% SDS-PAGE, proteins were visualized by Coomassie Brilliant Blue (CBB)-R250 staining or were transferred to PVDF membranes (Millipore, Billerica, MA, USA) for immunodetection with rabbit anti-GFP or appropriate antibodies. Rabbit antibodies against TPC7, rice glutelin A (GluA), and rice chaperones of binding protein 1 (BiP1) and 4&5 (BiP4/5), protein disulfide isomerase-like 1-1 and 2-3 (PDIL1-1 and PDIL2-3), and calnexin (CNX) were prepared as described previously (Yasuda *et al.,* 2009; Oono *et al.,* 2010; Wakasa *et al.,* 2011; Wang *et al.,* 2013). For immunodetection, membranes were incubated with rabbit antibodies, followed by horseradish peroxidase-conjugated anti-rabbit secondary IgG antibodies (dilution 1:5000) (Cell Signaling Technology, Danvers, MA, USA). Bands were visualized using Clarity Western ECL detection kit (Bio-Rad).

Confocal laser scanning microscopy and electron immunomicroscopy

Developing seeds were harvested 15–20 days after flowering (DAF) from various transgenic lines and then cross-sectioned with a DTK-1000 Microslicer (Dosaka EM) to approximately 150 μm in thickness for indirect immune-histochemical analysis (Yasuda *et al*. 2006). Mouse anti-GFP monoclonal antibody, rabbit anti-CNX, anti-PDIL1-1, anti-PDIL2/3, anti-BiP1-1, anti-BIP4/5, anti-TiP3 and anti-RM1 (14kDa prolamin) polyclonal antibodies were used to investigate their intracellular localization by Alexa-488 conjugated anti-mouse IgG antibody and Alexa-546 conjugated anti-rabbit IgG antibody. After immunostaining of samples, rhodamine B was used to stain ER-derived protein bodies (PB-Is). Samples cross-sections were spread on glass slides, sealed with a cover glass, and observed with a confocal laser scanning microscope (FLUOVEIW; OLYMPUS, Tokyo, Japan).

RNA extraction and RT-PCR analysis

Total RNA was extracted from seeds as previously described (Takaiwa *et al.*, 1987). RT-PCR analysis was carried out using ReverTraAce qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) with gene-specific primers for GFP and 17S rRNA.

Extraction of recombinant proteins from mature seeds

Leaves and roots were collected from each transgenic rice plant and frozen instantly in liquid nitrogen and then powdered using a Multibeads shocker. Pulverized seed, leaves and roots were treated with ten volume of various extraction buffer (200 μl) for 20 min by vortex, and then centrifuged at 15000 rpm for 10 min. The supernatant was transferred to new tube and then equal volume of 2x sample buffer （125 mM Tris-HCl (pH6.8), 20% glycerol, 4%SDS） without 2-MER was added. Some aliquot (5ul) was

applied and subjected to electrophoresis on 6 or 8 % SDS-PAGE.

Results

Endosperm-specific expression of Mal d 1 in transgenic rice

When GFP:Mald1 fusion protein were expressed as the secretory protein by ligating an N terminal glutelin signal peptide and a C terminal KDEL ER retention signal under the control of the endosperm-specific 2.3 kb glutelin *GluB-1* promoter, it accumulated at high levels in the endosperm of transgenic rice, which was detected as a visible band with the molecular mass of 50 kDa on CBB staining gel (Fig. S1). However, when the KDEL ER retention signal was removed, its accumulation (GFP:Mald1(-KDEL)) was significantly decreased. Furthermore, when produced in the cytoplasm by the absence of both the signal peptide and KDEL tag, its accumulation level (GFP:Mald1(-sig/-KDEL)) was remarkably reduced to undetectable level (Fig. S1). These results are in remarkable contrast with the expression of the inherent Mal d 1 in apple fruit, since it accumulates at relatively high level (1-20 μg/g FW) irrespective of absence of the signal peptide (Matthes and Schmitz-Eiberger, 2009).

On the other hand, when GFP:Mald1 was expressed as secretory protein in seeds, its effect on expression levels of various chaperones proteins was analyzed by immunoblotting using specific antibodies. In these transgenic seeds BiPs (BiP1 and BiP4/5) and PDIL2-3 were significantly increased compared with those in GFP:Mald1(-sig/-KDEL) construct, indicating that ER stress was induced by production at high level as secretory protein (Fig. S1).

Subcellular localization of GFP:Mald1 with the presence or absence of the KDEL signal was investigated in maturing transgenic seeds by immuno-histological analysis using confocal laser scanning microscopy. As shown in Fig. 2A, the GFP:Mald1 containing the KDEL signal was deposited in a spherical huge ER-derived protein body called as TPC7

body (Wang *et al.,* 2013) with a diameter of 5-10 μm in maturing endosperm cells. By contrast, when the KDEL tag was removed, generation of giant PBs was lost and the GFP:Mald1(-KDEL) was sequestered in small granules in ER lumen (Fig. 2B).

Constitutive expression of Mal d 1, Bet v 1 and TPC7 in transgenic rice

The GFP:Mald1, GFP:Betv1 and GFP:TPC7 containing the GluB-1 signal peptide and KDEL ER retention signal at their N and C termini were expressed under the control of the maize ubiquitin promoter. The GFP:Mald1 accumulated in mature seeds at higher levels than the GFP:Betv1 and GFP:TPC7 (Fig. 3A). Next, individual whole seed expressed from these expression constructs was divided into the embryo and endosperm parts and their accumulation levels were examined by immunoblotting using anti-GFP antibody. As shown in Fig. 3C , accumulation levels of the GFP:Betv1 and GFP:TPC7 in most embryos were under detectable level, whereas the GFP:Mald1 highly accumulated in most embryos. These results are in contrast with that they accumulated at similar expression levels in their endosperm (Fig. 3B). Thus, difference in their accumulation levels in whole grains may be accounted for by their differences in embryos. On the other hand, when the GFP reporter containing the KDEL tag at its C terminus was directly expressed, it accumulated in both embryo and endosperm like the GFP:Mald1 construct (Fig. S2). These findings suggested that fusion to the Bet v 1 or TPC7 may give rise to unstability of their fusion products in embryos.

When accumulation levels of the GFP:Mald1 in mature seeds that were directed by the endosperm-specific glutelin *GluB-1* promoter were compared with those that were driven by the ubiquitin promoter, the endosperm-specific promoter led to higher levels of its accumulation (Fig. 3A). This result may reflect the difference in promoter activities of maturing seeds between them (Qu and Takaiwa, 2004).

Next, accumulation levels of the GFP:Mald1, GFP:Betv1 and GFP:TPC7 in leaves

and roots were examined by immunoblotting using anti-GFP antibody. It should be noted that these GFP fusion products with the size of 50kDa is processed (degraded) into 37-39 kDa in vegetative tissues, when analyzed by immunoblotting using anti-GFP antibody. As shown in Fig. 4A, GFP:Mald1 highly accumulated in leaves and roots, whereas the accumulation levels of GFP:Betv1 and GFP:TPC7 were very low. Especially, their accumulation levels in roots were much lower than those of GFP:Mald1. These results suggested that the GFP:Betv1 and GFP:TPC7 products are unstable in vegetative tissues in a similar manner to their expression in their embryos. Therefore, the Mal d 1 may act as stabilizing sequence by protecting fusion proteins from degradation or the Bet v 1 and TPC7 may cause unstability in vegetative tissues.

When the transcript (mRNA) levels in leaf or roots between the Mal d 1 and Bet v 1 were examined by RT-PCR, there was little difference in their transcript levels between them, indicating that their accumulation levels are determined by post-transcriptional level (Fig. 4B).

Subcellular localization of GFP:Mald1

When the GFP:Mald1 was expressed under the control of the maize ubiquitin promoter, it was detected in the whole grain under blue UV fluorescence (Fig. 2C). When this transgenic rice seed was vertically sectioned and observed using the confocal laser scanning microscopy, high fluorescence signal of GFP was detected in aleurone layer and embryo (Fig. 2D). Then, the subcellular localization of GFP:Mald1 in various parts of transgenic rice seed was examined in detail by fluorescence of GFP reporter (Fig. 2E-H) or immuno-histochemical analysis by anti-GFP antibody (Fig. S3) using the confocal laser scanning microscope. In the aleurone cells, the GFP:Mald1 was shown to be predominantly localized in huge spherical protein bodies (PBs) with a dimeter of >5 μm (Fig. 2E-F), whereas in the starchy endosperm it was localized in PB-Is containing

prolamins and small granules with a size of ≦1 μm (Fig. 2F, Fig. S3L). In the embryo of maturing seed, it was predominantly localized in ER lumen (Fig. 2H, Fig. S3H,K). To investigate the participation of chaperon proteins in huge PB formation, intracellular localization of several chaperon proteins was analyzed. As shown in Fig. S3 (A,B,D,I) , BIP1, BiP4/5, PDIL1-1 and PDIL2-3 were co-localized in huge peripheral PBs in the aleurone cells, whereas they are also localized in ER lumen and PB-Is in the starchy endosperm. When antibodies against CNX (Fig. S3F), OsTiP3 (Fig. S3G) and Cys-rich 14kDa prolamin (RM1) (Fig.3SL) were used as markers for subcellular compartments of ER, protein storage vacuole (PB-II) and ER-derived PBs (PB-Is), respectively, they were not observed to be over-lapped with huge PBs. These results indicate that huge PBs accumulating GFP:Mald1 are formed by aid of BiPs and PDILs chaperone proteins.

Next, when intracellular localization of the GFP:Mald1 was examined in root, it was shown to be mainly localized as small granules in ER lumen (Fig. S4), since CNX, PDIL1-1, BIP1 were co-localized with the GFP:Mald1 at the peripheral sites of individual vegetative cell. Huge PBs could not be observed in root tissues. Strong fluorescence of GFP was observed at rooting site.

Identification of regions of Mal d 1 responsible for stable accumulation in vegetative tissues

To investigate which domain(s) in the Mal d 1 molecule is involved in high and stable accumulation in vegetative tissues, the Mal d 1 was progressively deleted from the N or C termini and their resultant regions were fused to the GFP reporter as shown in Fig. 5A. They were expressed under the control of the maize ubiquitin constitutive promoter.

When these fusion product levels were examined in vegetative tissues (leaf and roots) by immunoblotting using anti-GFP antibody, the regions of the Mal d 1 between positions 1-120, positions 41-159 and positions 41-120 conferred higher level of accumulation than the

294 regions between positions 81-120 and positions 81-159 (Fig. 5B). This result indicates
295 that the region between positions 41-120 is critical to confer high level of accumulation in
296 vegetative tissues. Then, to characterize the minimum region required for high expression in
297 vegetative tissues, accumulation levels from the regions between positions 41-107,
298 41-90 and 41-70 were examined. As shown in Fig 5C, the region between positions 41-90
299 led to the highest accumulation level. Notably, further deletion of 20 amino acids (region
300 between positions 41-70) or addition of 17 amino acids (region between positions 41-107)
301 resulted in reduction of accumulation levels. These results indicate that the minimum region
302 implicated in high accumulation in vegetative tissue is localized between positions 41-90.
303 This region contains 'P-loop' (phosphate-binding loop) motif, (GxGGxGxxK) rich in glycine
304 residue between positions 48-54 that is conserved in most of PR10 proteins (Fig. 1).
305 In order to examine its function, this sequence (GNGGPGTIK) was substituted to
306 ANAAPATIK. However, mutation of this sequence did not have any effect on
307 accumulation levels of the linked GFP in most of leaves as well as seeds (Fig. S5). This
308 result indicated that P-loop motif is not responsible for enhancing the accumulation level of
309 the Mal d 1 in transgenic rice.

310 Next, it was examined how above deletions of the Mal d 1 have an effect on
311 accumulation levels of the linked GFP in transgenic rice seeds (Fig. S6). Progressive
312 deletion from the N terminus reduced the linked GFP accumulation levels (Fig. S6A). The
313 region between positions 41-90 gave rise to higher level accumulation than the regions
314 between positions 41-159, positions 41-120 and positions 41-70 (Fig. S6B). Furthermore,
315 the region between positions 41-120 led to higher levels of accumulation than the
316 regions between positions 81-120 and positions 1-120 (Fig. S6C). These results are
317 fundamentally similar to those observed in vegetative tissues. Taken together, the region
318 between positions 41-90 is involved in stable and high accumulation in seed as well as
319 vegetative tissues.

Domain swapping between the Mal d 1 and Bet v 1

To identify the domain that is implicated in high accumulation in vegetative tissues of transgenic rice harboring GFP:Mald1, domain swapping was carried out between the Mal d 1 and Bet v 1 as shown in Fig. 6A. When the C terminal regions of the Bet v 1 between positions 90-159 and positions 127-159 were exchanged with the corresponding regions of the Mal d 1, accumulation levels of these chimeric products (Bet:Mal90, Bet:Mal127) were very low in leaves like the native Bet v 1. That is, accumulation levels of these chimeric products were not significantly altered by fusion with the C terminal region of Mal d, indicating that the C terminal half region of the Mal d 1 has little enhancing effect. On the other hand, when the N terminal half region of Bet v 1 between positions 1and 90 was exchanged with the corresponding region of the Mal d 1, much higher level of this chimeric product (Mal:Bet90) than the native Bet v 1 was observed in leaves. These findings indicated that the N terminal half region of the Mal d 1 is responsible for high accumulation of the Mal d 1 in vegetative tissues. Therefore, in order to ascertain that the region between positions 41-90 is critical for high accumulation in vegetative tissue, the corresponding region was mutually exchanged between the Mal d 1 and Bet v 1. The Bet:Mal:Bet chimeric product, in which the region between positions 41-90 was substituted with the corresponding region of the Mal d 1, gave rise to high levels of accumulation in leaves of most of transgenic lines, whereas the Mal:Bet:Mal chimeric product containing the Bet v 1 sequence between positions 41-90 resulted in lower levels of accumulation in most of transgenic rice leaves. These results indicated that the region between positions 41-90 is mainly responsible for high accumulation in vegetative tissues of Mal d 1.

Aggregation property of Mal d 1

When the Mal d 1 containing KDEL ER retention tag was expressed under the control of the endosperm-specific glutelin *GluB-1* promoter, it was shown to be accumulated as an

346 oligomeric form in a similar manner to the TPC7 and Bet v 1 that are deposited into huge
347 PBs in endosperm (Fig. 7).
348 Next, to examine whether there is a difference in oligomeric formation among various
349 tissues, the oligomeric formation of the GFP:Mald1 directed by the glutelin
350 endosperm-specific or ubiquitin constitutive promoter was compared by separation on 8%
351 non-reduced native PAGE. As shown in Fig.7A, dimer of GFP:Mald1 fusion protein was
352 observed in the extracts from these transgenic rice seed and leaf. Furthermore, higher
353 aggregates such as trimer and tetramer were also observed by separation on 6%
354 non-reducing native PAGE (Fig. 7B). Such aggregates are suggested to be formed by
355 self-aggregation via disulfide bond, because there is only one free cysteine (Cys) residue
356 in the Mad d 1 like the Bet v 1 and TPC7. Therefore, the Cys residue in the Mal d 1 was
357 substituted to the Ala residue to eliminate the Cys residue, which was then produced as a
358 fusion protein with GFP in transgenic rice. When this GFP:Mal $_{C\text{-}A}$ was extracted from
359 seed and leaf and then analyzed by native PAGE, dimeric form of the modified Mal d 1 was
360 observed irrespective of the absence of Cys residue (Fig. 7A line2 and 5). However, the
361 migration of this aggregate was slightly different from that of the inherent one.
362 To examine whether this difference is attributed to the aggregate form, these GFP fusion
363 proteins were extracted from transgenic seeds with different concentration of SDS (0.2%,
364 0.5% and 1%(w/v)). The GFP:Mald1 could be extracted with all the concentrations of
365 SDS used here, whereas the GFP:Madl$_{C\text{-}A}$ could not be extracted with 0.2% or 0.5% SDS
366 buffer (Fig. 7D). This finding suggested that Cys free modified GFP:Mald1 is aggregated in
367 different manner from the native one.
368
369 **Extraction of GFP:Mald1 from transgenic rice plants**
370 GFP:Mald1 was extracted from nature seed and leaf of transgenic rice plants with various
371 extraction buffers. Different concentration of SDS (0.2% and 1%) and 1% Tween20 and

1%Triton-X100 were used for solubilizing membrane proteins. When expressed in the endosperm under the control of the glutelin promoter, higher concentration of SDS (1%) was required for its extraction from mature seed. However, this extraction efficiency was much lower than the SDS-urea buffer. On the other hand, when expressed under the control of the ubiquitin promoter, it could be easily extracted from seed and leaf with all types of buffer used here. It is notable that extraction efficiency was almost same to the SDS-urea buffer. These results may be related to that the GFP:Mald1 directed by the maize ubiquitin promoter is localized in aleurone layer of seed and in ER lumen of leaf.

Next, to examine the cause of low extraction efficiency of GFP:Mald1 from transgenic rice seed that was directed by the endosperm-specific glutelin promoter, mature seed was treated with various extraction buffers containing reducing agent (10mM DTT) and various detergents. As shown in Fig. 8B, extraction efficiency of the GFP:Mald1 was improved by the presence of reducing agent (DTT). Furthermore, there is little difference in extraction efficacy by the presence or absence of KDEL ER retention sequence. These results suggest that the Mal d 1 interacts with Cys-rich prolamins via disulfide bond in starchy endosperm.

Discussion

TPC7 and TPC 9 are derivatives of major birch pollen allergen Bet v 1, which have been developed through *in vitro* random recombination by means of DNA shuffling as versatile recombinant hypoallergenic allergen against multiple Fagales pollen allergens (Wallner *et al*. 2007). Since the TPC7 and TPC9 exhibit lower allergenicity and higher immunogenicity than the native Bet v 1, they are expected to act as the ideal tolerogens for allergen-specific immunotherapy against the major pollen allergens of trees belonging to the Fagales order Bet v 1 family. Therefore, to create rice seed-based oral allergy vaccine against the pollen allergy of Fagales order, we have generated transgenic rice

accumulating the Bet v 1 and its derivatives, TPC7 and TCP9. We previously reported that high amounts of TPC7 (540 ug/seed) and native Bet v 1 were deposited in spherical huge protein bodies in endosperm cells of transgenic rice seeds, when expressed as secretory protein under the control of glutelin endosperm-specific promoter (Wang *et al*. 2013; Ogo *et al.* 2014).

It has been reported that more than 70% of birch pollen allergy patients develop an IgE-mediated hypersensitivity reaction termed as OAS after apple consumption (Geroldinger-Simic *et al*., 2011). This is mainly attributed to the immunological cross-reaction between the Bet v 1 specific IgE and apple major allergen Mal d 1, because the Bet v 1 and Mal d 1 share high homology in the primary sequence. Pollen allergen Bet v 1 is specifically expressed in pollen, while food allergen Mal d 1 is expressed in vegetative tissues. Therefore, it is important to examine how these allergens are deposited in various tissues of transgenic rice, when these allergens are produced in transgenic rice and then used as rice-based allergy vaccines against these pollen and food allergy diseases.

As shown in Fig. 3, when the GFP:Betv1, GFP:TPC7 and GFP:Mald1 were expressed as secretory protein by ligating the N terminal signal peptide and C terminal KDEL tag under the control of the maize ubiquitin promoter, accumulation levels of GFP:Betv1 and GFP:TPC7 were very low in various vegetative tissues such as leaf, root and seed embryo except for seed (endosperm). In contrast, the GFP:Mald1 highly accumulated not only in seed, but also in leaf and root. When intracellular localization of the GFP:Mald1 was examined in maturing transgenic seeds, it is mainly deposited in huge PBs in aleurone cells and in ER-derived PBs (PB-Is) containing prolamin storage proteins in starchy endosperm cells. This accumulation pattern was remarkably different from that directed by rice endosperm-specific glutelin promoter, in which the GFP:Mald1 is predominantly deposited in huge PBs in the starchy endosperm cells. On the other hand, huge PBs were

not observed in vegetative tissues, although high amounts of GFP:Mald1 accumulate in vegetative tissues. Then, taking these results into consideration, we examined why the GFP:Mald1 can be deposited into huge bodies in aleurone cells, but not in endosperm and vegetative tissues under the control of the maize ubiquitin promoter.

It has been suggested that huge PB formation is associated with the aggregate property of the recombinant proteins (Ogo *et al.*, 2014). Furthermore, expression levels may be also important for the formation of huge PBs, since maize ubiquitin promoter directs stronger expression in aleurone layer than in starchy endosperm of maturing seed, when examined by expression of the GUS reporter gene driven by the same maize ubiquitin promoter (Takaiwa *et al.*, 2007). Requirement for high accumulation of recombinant proteins above a threshold level has been pointed out for the formation of PB in elastin-like polypeptide (ELPs) and hydrophobin fusion proteins (Gutierrez *et al.,* 2013).

PB formation is suggested to be efficiently initiated by ER retention via the C terminal KDEL signal of GFP:Mald1. Actually, localization of GFP:Mald1 in ER lumen is observed in various tissues including aleurone layer and seed embryo, since small granules with green fluorescence are observed along ER membrane network all over the whole cell, which are connected as ER network. That is, the GFP:Mald1 is synthesized on rough ER, and subsequently transferred into the ER lumen. The size of PB gradually increases through binding of small aggregates, resulting in generation of huge spherical PB with a maximum size of >10 um. As shown in Fig. 9, a single or a few huge spherical PBs are finally formed by gathering several small size GFP:Mald1 aggregates in one cell. Aggregates are observed to be concentrated into a single huge PB through ER network connection. Finally, a single huge spherical PB is formed in many aleurone cells. Growth in PB size is accompanied by an increase in accumulation levels. Furthermore, PB formation is known to be mediated by aid of some chaperon proteins such as BiPs and PDILs that are implicated in folding and assembly. When subcellular localization of some

BiPs and PDILs were examined by immuno-histochemical analysis, these chaperons are localized within huge PBs containing GFP:Mald1 as shown in Fig. S5, indicating that huge PBs in aleurone cells are formed in a similar manner as huge PBs observed in the starchy endosperm (Ogo *et al.* 2014). Aleurone cells are rich in protein storage vacuoles (PSVs) referred as aleurone bodies and lipid bodies. Some storage proteins such as rice embryo globulin-2 proteins (REG2) and phytic acid are accumulated inside aleurone bodies. Antibodies against OsTiP3 and CNX as PSV and ER marker are not localized to huge PBs in the aleurone cells. Huge PBs are suggested to be independently formed by self-aggregation of Mal d 1. Generation mechanism of this huge PB is fundamentally same to that of Zera, ELPs and hydrophobin-I in plant cells (Conley et al, 2011; Saberianfar and Menassa, 2017).

Bet v 1 and Mal d 1 belong to the same PR10 group and share about 55-68% amino acid sequence homology to each other. However, it is notable that their accumulation levels in various tissues are quietly different even when expressed under the control of the same constitutive ubiquitin promoter. The GFP:Mald1 highly accumulates in various vegetative tissues and seed, whereas high accumulation of the GFP:Betv1 is limited to the endosperm and its accumulation level in the vegetative tissues is relatively low. It is notable that transcript levels between the GFP:Mald1 and GFP:Betv1 in leaves and roots are almost same, when examined by RT-PCR using GFP primers. Post-translational regulation is involved in such difference in accumulation levels. Non-correlationship between transcript levels and accumulation products have been reported for several seed storage proteins in maize aleurone cells (Reyes *et al.,* 2011). By contrast, foreign recombinant protein such as 7Crp peptide selectively accumulated in endosperm rather than other tissues (embryo and vegetative tissues) when expressed under the constitutive promoter (Takaiwa *et al.,* 2007). Difference in intracellular localization or post-translational modification such as glycosylation may be also implicated in such post-translational regulation.

476 In this study, we first demonstrated by deletion analysis of the Mal d 1 that the region
477 between positions 41-90 is mainly responsible for high and stable accumulation in
478 vegetative tissues. Participation of this region in high accumulation in vegetative tissues
479 was further confirmed by domain swapping between the Mal d 1 and Bet v 1. As shown
480 in Fig. 6, the exchange of this Mal d 1 region between positions 41-90 with the
481 corresponding region of Bet v 1 (Bet:Mal:Bet) resulted in higher accumulation of the linked
482 GFP in vegetative tissue than the native Bet v 1. This region includes very conserved P
483 element motif and N-glycosylation site of Bet v 1 and TPC7. Moreover, we previously
484 reported by domain swapping between TPC7 and Bet v 1 that the sequence between
485 positions 32-160 is important for the formation of huge and high number of PBs (Ogo *et al.,*
486 2014). This result also supports the contribution of C terminal half region to the huge PB
487 formation. Furthermore, this region has been suggested to be involved in immunological
488 cross-reactivity between Mal d 1 and Bet v 1, since this region was identified as
489 conformational B-cell epitope (discontinuous epitopes) by antibody binding (Mirza et al.,
490 2000). This epitope is formed by the segment between Glu42 and Thr52 along with Arg70,
491 Asp72, His76, Ile86 and Lyn97 of Bet v 1. The tertiary structure of this region exhibits
492 antiparallel β-sheet structure (β-2, β-3, β-4 and β-5), which covers a large proportion of
493 the inner cavity surface (Ahammer *et al*., 2017). Mutagenesis of conserved amino acid
494 within this region (E45S) also reduced IgE binding (Spangfort *et al.,* 2003). These
495 evidences also suggest that the region between positions 41-90 is structurally important and
496 is localized on the protein surface of the Mal d 1..
497 When the GFP:Mald1 was specifically expressed under the control of the
498 endosperm-specific promoter, it was difficult to extract it from mature seed. This is due to
499 that it is deposited into huge ER-derived PBs by self-aggregation or PB-Is by interaction
500 with Cys-rich prolamins via disulfide bonds (Takaiwa *et al*, 2009). Higher concentration of
501 detergent (SDS) and reducing agent (DTT) are required for efficient extraction of Mal d 1

502 from transgenic rice seed. By contrast, when expressed under the control of the constitutive
503 promoter, the GFP:Mald1 can be easily extracted from various tissues. Especially, it
504 can be extracted from transgenic mature seeds even with saline solution. This can be
505 explained by the observation that the GFP:Mald1 is localized in ER lumen of vegetative
506 tissue or accumulated mainly in huge PBs of aleurone cells.

507 Bet v 1 has been reported to generally form a mixture of monomers, dimers and higher
508 order oligomers (Scholl *et al.,* 2005). Dimerization was also reported for Mal d 1 (Ma *et al.,*
509 2006). When GFP:Mald1 was produced in transgenic rice, it displays multimeric formation in
510 seed and vegetative tissues (Fig. 8). The Mal d 1 has one cysteine residue like the Bet v
511 1. This cysteine residue is suggested to be implicated in disulfide bond formation for
512 self-aggregation or interaction with the Cys-rich prolamins in transgenic rice seeds.
513 However, even though this cysteine residue was substituted to Ala by mutagenesis,
514 oligomeric (dimeric) formation was retained. Notably, this mutagenesis resulted in the
515 alternation in physicochemical property of the accumulated modified Mal d 1, since it
516 became difficult to extract the mutagenized Mal d 1 from vegetative tissues. The
517 mutagenized Mal d 1 lacking Cys residue suggested to be falsely folded, leading to
518 change in physicochemical property of Mal d 1. Therefore, cysteine residue in Mal d 1
519 molecules may be critical for the correct folding or oligomeric formation via disulfide bond.

520 It has been demonstrated in this study that localization and generation of such huge
521 PBs in transgenic rice are dependent on the tissue specificity and strength of the
522 promoter used for expression. When directed by the maize ubiquitin constitutive promoter,
523 huge PBs are observed to be formed mainly in aleurone cells, whereas expression by the
524 endosperm-specific glutelin promoter gives rise to their production in starchy endosperm.
525 Higher expression above a threshold level is essentially required for huge PB formation.
526 However, although many various foreign recombinant proteins have been produced in our
527 laboratory, such huge PBs could not be formed except for the PR10 proteins such as Bet v 1

and Mal d 1 even when the same strong endosperm-specific promoter has been utilized, suggesting that the specific physicochemical property such aggregation property may be associated with the huge PB formation. Their tertiary structure leading to high aggregates may be responsible for the huge PB formation by helping to increase the accumulation levels. When the GFP was produced by fusion with Mal d 1, it was highly and stably accumulated not only in seed, but also in leaf and root (Fig. S7). Notably, accumulation level of GFP has been significantly increased by fusion with Mal d 1. Furthermore, GFP:Mald1 fusion protein can be more easily extracted from various tissues of transgenic rice than that specifically expressed in transgenic rice seeds. Therefore, Mal d 1 may be utilized as fusion partner for the production of high-value recombinant proteins in plant like Zera and ELPs. Further works will be required whether production of foreign recombinant proteins can be enhanced by fusion with Mal d 1.

Acknowledgments

We thank Ms. K. Miyashita, Y. Ikemoto and Y. Yajima for technical assistance and Dr. Kenjirou Ozawa for encourage of this research.

References

Agarwall P, Agarwal PK. 2014. Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. Molecular Biology Reports **41**, 599-611.

Ahammer L, Grutsch S, Kamenik AS, Liedl KR, Tollinger M. 2017. Structure of the Major Apple Allergen Mal d 1. Journal of Agricultural and Food Chemistry **65**, 1606-1612.

Beuning L, Bowen J, Persson H, Barraclough D, Bulley S, Macrae E. 2004. Characterisation of Mal d 1-related genes in Malus. Plant Molecular Biology **55**, 369-388.

Conley AJ, Joensuu JJ, Richman A, Menassa R. 2011. Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. Plant Biotechnology Journal **9**, 419-433

Ebner C, Birkner T, Valenta R, Rumpold H, Breitenbach M, Scheiner O, Kraft D. 1991. Common epitopes of birch pollen and apples – studies by western and northern blot. Journal Allergy and Clinical Immunology **88**, 588-594.

559 **Fernandes H, Michalska K, Sikorski M, Jaskolski M**. 2013. Structural and functional
560 aspects of PR-10 proteins FEBS Journal **280**, 1169-1199.
561 **Fritsch R, Bohle B, Vollmann U, Wiedermann U, Jahn-Schmid B, Krebitz M,**
562 **Breiteneder H, Kraft D, Ebner C.** 1998. Bet v 1, the major birch pollen allergen,
563 and Mal d 1, the major apple allergen, cross-react at the level of allergen-specific T
564 helper cells. Journal Allergy and Clinical Immunology **102**, 679–686.
565 **Geroldinger-Simic M, Zelniker T, Aberer W.** ***et al.*** 2011. Birch pollen–related food
566 allergy: Clinical aspects and the role of allergen-specific IgE and IgG4 antibodies.
567 Journal of Allergy and Clinical Immunology **127,** 616–622.
568 **Goto F, Yoshihara T, Sugimoto N, Toki S, Takaiwa F.** 1999. Iron fortification of rice seed
569 by the soybean ferritin gene. Nature Biotechnology **17**, 282-286.
570 **Gutiérrez SP, Saberianfar R, Kohalmi SE, Menassa R.** 2013. Protein body formation in
571 stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion
572 proteins. BMC Biotechnology **13,** 40.
573 **Hofbauer A, Stoger E.** 2013. Subcellular accumulation and modification of pharmaceutical
574 proteins in different plant tissues. Current Pharmaceutical Design **19**, 5495-5502.
575 **Holm J, Ferreras M, Ipsen H, Wurtzen PA, Gajhede M, Larsen JN, Lund K**.
576 2011.Spangfort, M. D. Epitope grafting, re-creating a conformational Bet v 1 antibody
577 epitope on the surface of the homologous apple allergen Mal d 1. Journal of Biological
578 Chemistry **286**, 17569−17578.
579 **Liu J, Ekramoddoullah AKM.** 2006. The family 10 of plant pathogenesis-related proteins:
580 Their structure, regulation, and function in response to biotic and abiotic stresses. Physiol
581 Molecular Plant Pathology **68**, 3-13.
582 **Ma Y, Gadermaier G, Bohle B, Bolhaar S, Knulst A, Markovic-Housley Z, Breiteneder H,**
583 **Briza P, Hoffmann-Sommergruber K, Ferreira F.** 2006. Mutational analysis of amino
584 acid positions crucial for IgE-binding epitopes of the major apple (Malus domestica)
585 allergen, Mal d 1. International Achieves of Allergy and Immunology **139**, 53–62.
586 **Mari A, Ballmer-Weber BK, Vieths S.** 2005. The oral allergy syndrome: improved
587 diagnostic and treatment methods. Current Opinion in Allergy and Clinical Immunology. **5**,
588 267–273.
589 **Marzban G, Puehringer H, Dey R,** ***et al.*** 2005. Localisation and distribution of the major
590 allergens in apple fruits. Plant Science **169**, 387−394.
591 **Mirza O, Henriksen A, Ipsen H, Larsen JN, Wissenbach M, Spangfort MD, Gajhede M.**
592 2000. Dominant epitopes and allergic cross-reactivity: complex formation between a Fab
593 fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen
594 Bet v 1. Journal of Immunology **165**, 331-338.
595 **Ogo Y, Takahashi H, Wang S, Takaiwa F.** 2014,Generation mechanism of novel, huge
596 protein bodies containing wild type or hypoallergenic derivatives of birch pollen allergen
597 Bet v 1 in rice endosperm. Plant Molecular Biology **86**, 111-123.
598 **Oono Y, Wakasa Y, Hirose S, Yang L, Sakuta C, Takaiwa F**. (2010) Analysis of ER stress

599 in developing rice endosperm accumulating β-amyoid peptide. Plant Biotechnology
600 Journal **8**, 691-718.
601 **Pagliarani G, Paris R, Arens P, Tartarini S, Ricci G, Smulders MM, van de Weg WE.**
602 2013. A qRT-PCR assay for the expression of all Mal d 1 isoallergen genes.
603 BMC Plant Biology **23**, 13:51.
604 **Rouvinen J, Jänis J, Laukkanen ML, Jylhä S, Niemi M, Päivinen T, Mäkinen-Kiljunen S,**
605 **Haahtela T, Söderlund H, Takkinen K.** 2010. Transient dimers of allergens. PLoS
606 One **5**, e9037.
607 **Puehringer H, Moll D, Hoffmann-Sommergruber K, Watillon B, Katinger H, Machado**
608 **MLD.** 2000.The promoter of an apple Ypr10 gene, encoding the major allergen Mal d 1,
609 is stress- and pathogen-inducible. Plant Science **152**, 35−50.
610 **Qu LQ, Takaiwa F.** 2004. Evaluation of tissue specificity and expression strength of rice
611 seed component gene promoters in transgenic rice. Plant Biotechnology Journal **2,**
612 113-125.
613 **Reyes FC, Chung T, Holding D, Jung R, Vierstra R, Otegui MS.** 2011. Delivery of
614 prolamins to the protein storage vacuole in maize aleurone cells. Plant Cell. **23,** 769-784.
615 **Saraste M, Sibbald PR, Wittinghofer A.** 1990. The P-loop- a common motif in ATP-and
616 GTP-binding proteins. Trends in Biochemical Sciences **15,** 430-434.
617 **Saberianfar R, Menassa R.** 2017. Protein bodies: how the ER deals with high accumulation
618 of recombinant proteins. Plant Biotechnology Journal **15,** 671-673.
619 **Scholl I, Kalkura N, Shedziankova. *et al.*** 2005. Dimerization of the major birch pollen
620 allergen Bet v 1 is important for its in vivo IgE-cross-linking potential in mice. Journal of
621 Immunology **175,** 6645-6650.
622 **Spangfort MD, Mirza O, Ipsen H, Van Neerven RJ, Gajhede M, Larsen JN.** 2003.
623 Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen,
624 characterized by X-ray crystallography and site-directed mutagenesis. Journal of
625 Immunology **171,** 3084-3090.
626 **Stoger E, Ma JK, Fischer R, Christou P.** 2005. Sowing the seeds of success:
627 pharmaceutical proteins from plants. Current Opinion in Biotechnology **16,** 167-173.
628 **Tada Y, Utsumi S, Takaiwa F.** 2003. Foreign gene products can be enhanced by
629 introduction into storage protein mutants. Plant Biotechnology Journal **1,** 411-422.
630 **Takaiwa F, Kikuchi S, Oono K.** 1987. A rice glutelin gene family-A major type of glutelin
631 mRNAs can be divided into two classes. Molecular General Genetics **208,** 15-22.
632 **Takaiwa F, Takagi H, Hirose S, Wakasa Y.** 2007. Endosperm tissue is good production
633 platform for artificial recombinant proteins in transgenic rice. Plant Biotechnology
634 Journal **5**, 84-92.
635 **Takaiwa F, Hirose S, Takagi H, Yang L, Wakasa Y**. 2009. Deposition of a recombinant
636 peptide in ER-derived protein bodies by retention with cysteine-rich prolamins in
637 transgenic rice seed. Planta **229,** 1147–1158.
638 **Takaiwa F.** 2013. Update on the use of transgenic rice seeds in oral immunotherapy.

639 Immunotherapy **5,** 301-312.
640 **Takaiwa F, Wakasa Y, Takagi H, Hiroi T.** 2015. Rice seed for delivery of vaccines to gut
641 mucosal immune tissues. Plant Biotechnology Journal **13,** 1041-1055.
642 **Takaiwa F, Yang L, Maruyama N, Wakasa Y, Ozawa K.** 2016. Deposition mode of
643 transforming growth factor-β expressed in transgenic rice seed. Plant Cell Reports **35,**
644 2461-2473.
645 **Takaiwa F, Wakasa Y, Hayashi S, Kawakatsu T.** 2017. An overview on the strategies to
646 exploit rice endosperm as production platform for biopharmaceuticals. Plant Science **263,**
647 201-209.
648 **Wakasa Y, Yasuda H, Takaiwa F.** 2006. High accumulation of bioactive peptide in
649 transgenic rice seeds by expression of introduced multiple genes. Plant
650 Biotechnology Journal **4,** 499–510.
651 **Wakasa Y, Hayashi S, Takaiwa F**. (2012) Expression of OsBiP4 and OsBiP5 is highly
652 correlated with the endoplasmic reticulum stress response in rice. *Planta* **236**, 1519-1527
653 **Wallner M, Stöcklinger A, Thalhamer T, *et al.*** 2007. Allergy multivaccines created by
654 DNA shuffling of tree pollen allergens. Journal of Allergy and Clinical Immunology **120,**
655 374-380.
656 **Wang S, Takahashi H, Kajiura H, Kawakatsu T, Fujiyama K, Takaiwa F.** 2013. Transgenic
657 rice seeds accumulating recombinant hypoallergenic birch pollen allergen Bet v 1
658 generate giant protein bodies. *Plant and Cell Physiology* **54,** 917-933.
659 **Yasuda H, Hayashi Y, Jomori T, Takaiwa F.** 2006. The correlation between expression and
660 localization of a foreign gene product in rice endosperm. *Plant and Cell Physiology* **47**,
661 756-763
662 **Yasuda H, Hirose S, Kawakatsu T, Wakasa Y, Takiwa F.** 2009. Overexpression of BiP has
663 inhibitory effects on the accumulation of seed storage proteins in endosperm cells of rice.
664 *Plant and Cell Physiology* **50,** 1532-1543.
665
666
667
668
669
670
671
672
673
674

675 **Legends of figures**

676 **Figure 1. Gene constructs used for expression of Mal d 1, Bet v 1 and TPC7 in**
677 **transgenic rice. (A)** Alignment of Mal d 1, Bet v 1 and TPC7 amino acid sequences.
678 Cysteine residue and glycosylation sites are indicated by red and green colors. Red square
679 represents P loop motif. **(B)** Schematic representation of the gene constructs used for
680 expression of GFP, GFP:Mald1, GFP:Betv1 and GFP:TPC7 in transgenic rice. Mal d 1,
681 Bet v 1 and TPC7 were expressed as fusion proteins with GFP under the control of the 2.3
682 kb glutelin *GluB-1* promoter and maize ubiquitin promoter. SP and *GluB-1* T represent the
683 glutelin B1 signal peptide sequence and 0.65 kb 3' untranslated region of *GluB-1*,
684 respectively. KDEL, endoplasmic reticulum retention signal; HPT, hygromysin
685 phosphotransferase gene; CaMV35S P, cauliflower mosaic virus 35S promoter; UbiP,
686 maize ubiquitin gene promoter; RB, right border; LB, left border
687
688 **Figure 2. Expression pattern of GFP:Mald1 in transgenic rice seed. (A and B)**
689 Intracellular localization of GFP:Mald1 in transgenic rice seed harboring the GluP::GFP:Mal
690 or GluP::GFP:Mal(-KDEL). **(C-H)** Expression pattern of GFP:Mald1 of transgenic rice seeds
691 harboring UbiP::GFP:Mal and its intracellular localization in aleurone layers, starchy
692 endosperm and embryo. ALU, aleurone layer; END, endosperm; EMB, embryo; p,
693 pericalp thin Bars= 5 μm. thick Bars=100 μm
694
695 **Figure 3. Immunoblot analysis of GFP:Mald1, GFP:Betv1 and GFP:TPC7 expressed**
696 **in transgenic rice seeds under the control of the maize ubiquitin promoter.**
697 GFP:Mald1 was also expressed under the control of the glutelin *GluB-1* promoter for
698 comparison. Total proteins were extracted from the whole seed **(A)**, endosperm **(B)** and
699 embryo **(C)** with SDS-urea buffer, and subsequently separated by 13% SDS-PAGE.
700 Accumulation levels were examined by immunoblotting with anti-GFP antibody. GFP fusion

products with the predicted size of 50 kDa are indicated by arrow head. Degradation products of about 37 kDa and 20 kDa are detected in high expression lines.

Figure 4. Expression of GFP:Mald1, GFP:Betv1 and GFP:TPC7 in leaf and roots of transgenic rice lines. (A) Immunoblot analysis of GFP:Mald1, GFP:Betv1 and GFP:TPC7 expressed in leaf and roots of transgenic rice lines under the control of the maize ubiquitin promoter. Processed 37-38 kDa fragments are indicated by arrow head. **(B)** RT-PCR of GFP:Mald1 and GFP:Betv1 transcripts in leaf and roots. Total RNAs were extracted from leaf and roots. The 17S rRNAs were used for normalization of starting RNA concentration in RT-PCR analysis.

Figure 5. Deletion analysis of Mal d 1 molecule. (A) Schematic representation of the gene constructs used for deletion analysis of Mal d 1. α, α helical structure; β, β sheet structure **(B-C)** Effect of deleted Mal d 1 on accumulation levels of linked GFP in leaf and roots of transgenic rice. Total proteins extracted from leaves and roots of 4-5 representative independent transgenic lines for each expression construct were subjected to immunoblot analysis using anti-GFP antibody.

Figure 6. Domain swapping between the Mal d 1 and Bet v 1. (A) Schematic representation of gene constructs used for domain swapping. **(B-C)** Immunoblot analysis of various fusion products extracted from mature seeds and leaves of transgenic rice plants harboring various expression constructs using anti-GFP antibody.

Figure 7. Comparison of aggregation property between GFP:Mald1 and GFP:Mald1$_{C\text{-}A}$. (A) Immunoblot analysis of GFP:Mald1 and GFP:Mald1$_{C\text{-}A}$ expressed in transgenic rice seeds and leaves. They were extracted with 50mM Tris-HCl (pH7.5) and 1%SDS without

727 reducing agent and then separated by 8% SDS-PAGE. 1. Seed extract from transgenic
728 rice harboring GluP::GFP:Mal 2. Seed extract from transgenic rice harboring
729 UbiP::GFP/Ma$_{lC-A}$ 3. Leaf extract from transgenic rice harboring UbiP::GFP:Mal, 4. Leaf
730 extract from transgenic rice harboring UbiP::GFP:Mald1$_{C-A}$. 5 and 6. Seed extracts of
731 GluP::GFP:Mal and UbiP::GFP:Mal were treated with reducing agent before loading to
732 SDS-PAGE. Aggregates were detected by immunoblotting using anti-GFP antibody **(B)**
733 Separation of seed extracts of transgenic rice harboring GluP::GFP:Mal (1) and
734 UbiP::GFP:Mal (2) by 6% SDS-PAGE. **(C)** Comparison of accumulation levels in
735 transgenic rice seeds between GFP:Mald1 and GFP:Mald1$_{C-A}$. **(D)** Difference in
736 extraction efficiency from mature seeds and leaves of transgenic rice harboring
737 UbiP::GFP:Mal and UbiP::GFP:Mal$_{C-A}$ that were treated with 0.2%SDS (1), 0.5%SDS (2)
738 and 1%SDS (3) in 50mM Tris-HCl (pH7.5). EXT, extracted; PPT, not extracted Arrow
739 head represents the expressed GFP:Mald1 and GFP:Mald1$_{C-A.}$
740
741 **Figure 8. Extraction of GFP:Mald1 from seed and leaves of transgenic rice. (A)** Seed
742 powder and frozen leaf powder of transgenic rice harboring GluP::GFP:Mal or
743 UbiP::GFP:Mal construct were treated with saline buffer (1) and saline buffer containing
744 0.2% SDS (2) 1% SDS (3) 1% TritonX-100 (4) and 1%Tween-20 (5) and urea-SDS
745 buffer (6) **(B)** Seed powder of transgenic rice harboring GluP::GFP:Mal or
746 GluP::GFP:Mal-KDEL) was treated with saline solution containing 1%CTAB (1), 10mM DTT
747 (2), 1% SDS (3), 0.2% SDS and 10mM DTT (4), 0.5% SDS and 10mM DTT (5), 1% SDS
748 and 10mM DTT (6), 6M urea and 10mM DTT (7) and urea-SDS buffer (8). Extraction
749 efficiency by these treatments was examined by immunoblotting using anti-GFP and
750 anti-GluA antibodies.
751
752 **Figure 9. Subcellular localization of GFP:Mald1 in aleurone cells.** Green

753 fluorescence by GFP represents the localization of GFP/Mald1. Red represents the
754 rhodamine B stained particles. **(A)** Ventral site of sectioned seed **(B)** Dorsal site of
755 sectioned seed **(C-F)** Huge PB formation process of GFP:Mald1 in alurone layer. ALU,
756 aleurone layer; END, endosperm; EMB, embryo; OV, ovular vascular trace. Thick bars=
757 200 µm Thin bars= 5 µm
758
759
760 **Supplementary Figures**
761
762 **Figure S1. Immunoblot analysis of GFP:Mald1 fusion proteins expressed in**
763 **transgenic rice seeds under the control of the endosperm-specific glutelin 2.3 kb**
764 ***GluB-1* promoter.** Total proteins were extracted from mature seeds with SDS-urea
765 buffer, and then separated by 13% SDS-PAGE. Proteins were visualized with CBB
766 staining and accumulation level of GFP:Mald1 was analyzed by immunoblotting using
767 anti-GFP or anti-TPC7 antibody. Effect on expression of chaperon proteins by accumulation
768 of GFP:Mald1 were examined by immonoblotting using several anti-chaperone proteins.
769
770 **Figure S2. Immunoblot analysis and SDS-PAGE of GFP containing KDEL tag**
771 **expressed as secretory protein in transgenic rice seed under the control of the**
772 **ubiquitin promoter.** GFP accumulates in both embryo and endosperm of mature seed.
773
774 **Figure S3. Subcellular localization of GFP:Mald1 in aleurone and starchy**
775 **endosperm cells in maturing seeds at 15-18 DAFs.** Green signal indicates GFP
776 fluorescence or immunofluorescence of GFP immune-stained with anti-GFP antibody.
777 Red signal indicates immunofluorescences of OSBiP-1, OSBiP4/5, OSPDIL1-1,
778 OSPDIL2-3, CNX, OsTiP3 and RM1 (Cys-rich 14kDa prolamin) immunostained with their

779 specific anti-rabbit antibodies. Signals were detected using second antibody with the
780 Alexa-488-conjugated goat anti-mouse IgG (green) and Alexa-564-conjuated goat
781 anti-rabbit IgG (red). The images correspond to the merge channels resulting from the
782 combination of anti-mouse IgG recognizing GFP (green) and anti-rabbit IgG recognizing
783 chaperons (red). Co-localization is represented by yellow or orange color. Scale bars are
784 5 μm. ALU, alurone layer; END, endosperm; EMB, embryo
785
786 **Figure S4. Intracellular localization of GFP/Mald1 and various chaperone proteins in**
787 **root tissue.** Transverse section of transgenic rice root. The images correspond to the
788 merged channel resulting from the combination of GFP of GFP:Mald1 (shown in green) and
789 chaperon proteins (shown in red). Colocalization is shown in orange. Bars 5 μm
790
791 **Figure S5. Effect of mutation of Mal d 1 P-loop motif on accumulation level in**
792 **leaf and seed.** P-loop motif of Mal d 1 was mutagenized and this modified Mal d 1 was
793 expressed as fusion protein with GFP in leaves and seeds of several independent
794 transgenic rice lines (GFP:Mal(P)). Accumulation levels in these leaves and mature seeds
795 were compared with those of GFP:Mald1 as control.
796
797 **Figure S6. Effect of deleted Mal d 1 on accumulation levels of linked GFP in seeds of**
798 **transgenic rice.** Total proteins extracted from mature seeds of 4-5 representative
799 independent transgenic lines for each expression construct were subjected to immunoblot
800 analysis using anti-GFP antibody
801
802 **Figure S7. Comparison of accumulation levels of GFP, GFP:Betv1 and GFP:Mald1 in**
803 **leaf and seed.** GFP, GFP:Betv1 and GFP:Mald1 were expressed under the control of the
804 maize ubiquitin promoter.

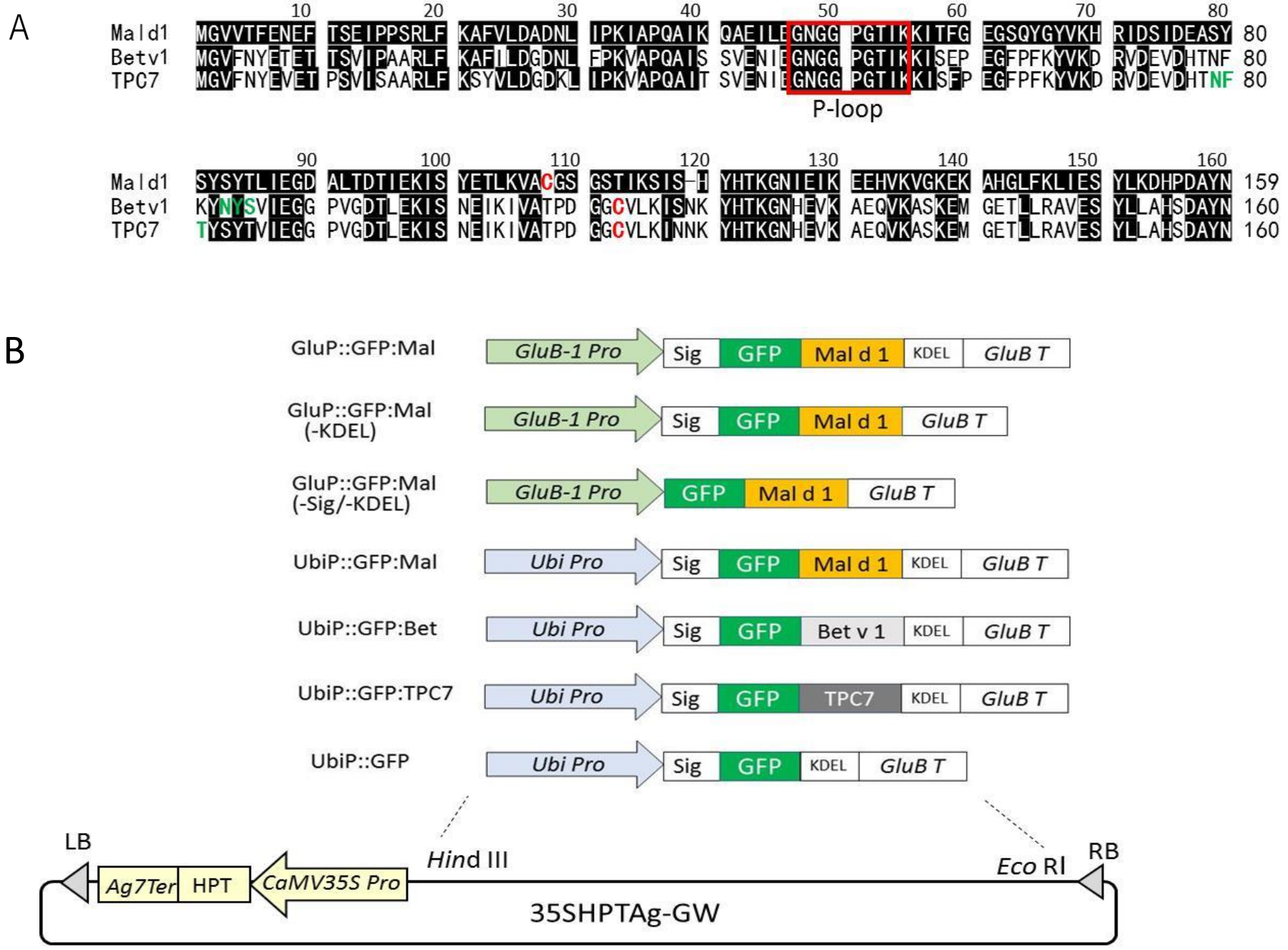

A
Mald1 MGVVTFENEF TSEIPPSRLF KAFVLDADNL IPKIAPQAIK QAEILEGNGG PGTIKKITFG EGSQYGYVKH RIDSIDEASY 80
Betv1 MGVFNYETET TSVIPAARLF KAFILDGDNL FPKVAPQAIS SVENIEGNGG PGTIKKISEP EGFPFKYVKD RVDEVDHTNF 80
TPC7 MGVFNYEVET PSVISAARLF KSYVLDGDKL IPKVAPQAIT SVENIEGNGG PGTIKKISFP EGFPFKYVKD RVDEVDHTNF 80
P-loop
Mald1 SYSYTLIEGD ALTDTIEKIS YETLKVACGS GSTIKSIS-H YHTKGNIEIK EEHVKVGKEK AHGLFKLIES YLKDHPDAYN 159
Betv1 KYNYSVIEGG PVGDTLEKIS NEIKIVATPD GGCVLKISNK YHTKGNHEVK AEQVKASKEM GETLLRAVES YLLAHSDAYN 160
TPC7 TYSYTVIEGG PVGDTLEKIS NEIKIVATPD GGCVLKINNK YHTKGNHEVK AEQVKASKEM GETLLRAVES YLLAHSDAYN 160
B
GluP::GFP:Mal
GluB-1 Pro
Sig
GFP
Mal d 1
KDEL
GluB T
GluP::GFP:Mal (-KDEL)
GluP::GFP:Mal (-Sig/-KDEL)
UbiP::GFP:Mal
Ubi Pro
UbiP::GFP:Bet
Bet v 1
UbiP::GFP:TPC7
TPC7
UbiP::GFP
LB
Ag7Ter
HPT
CaMV35S Pro
Hind III
Eco RI
RB
35SHPTAg-GW

Fig. 1

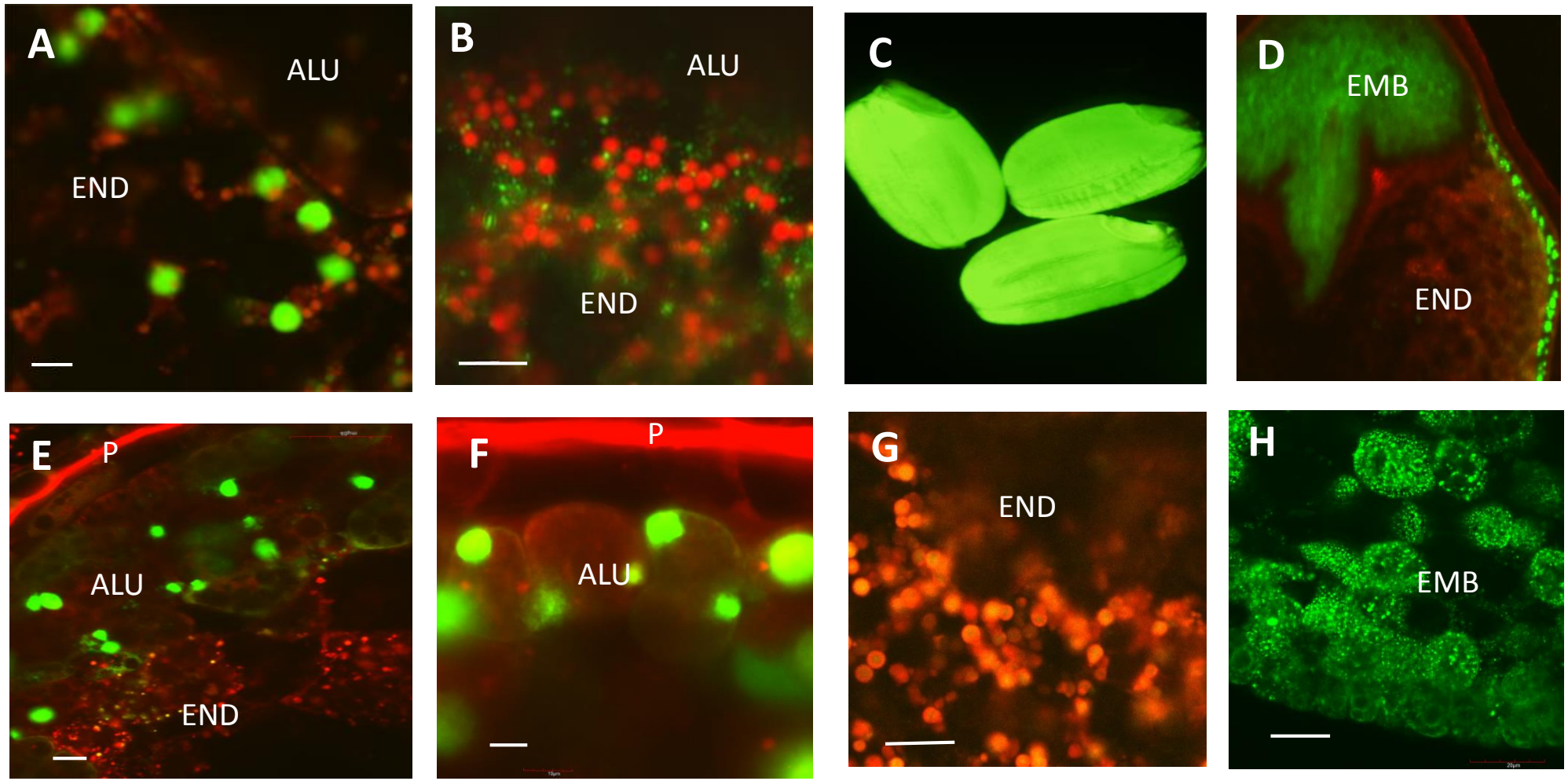
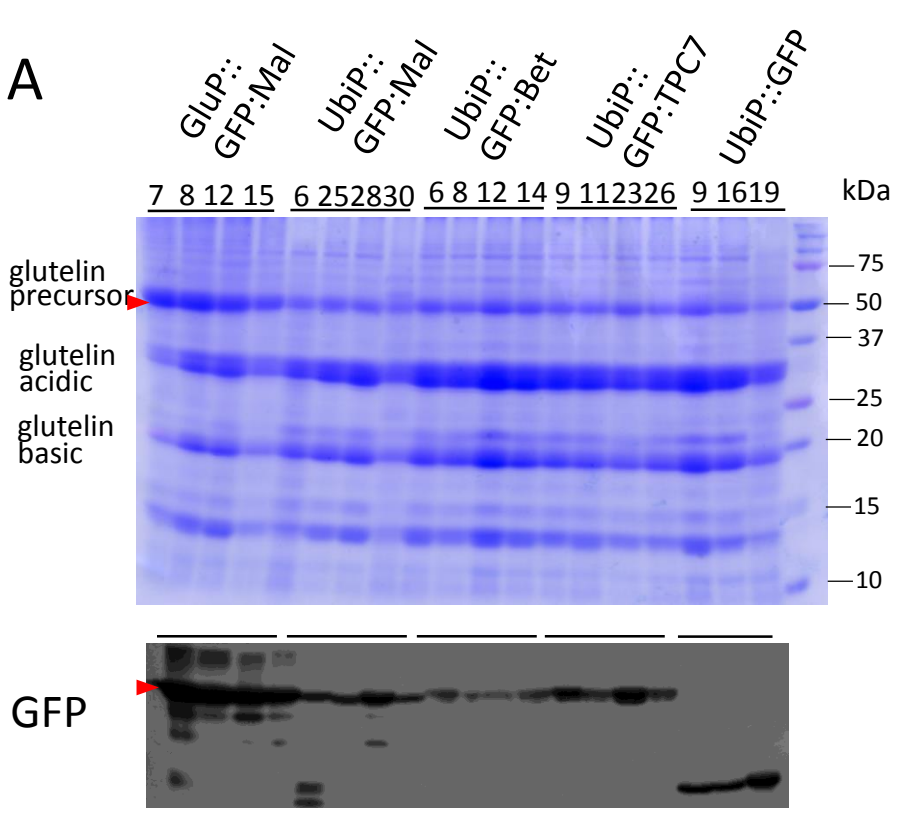


Fig. 2


A
GluP::GFP:Mal
UbiP::GFP:Mal
UbiP::GFP:Bet
UbiP::GFP:TPC7
UbiP::GFP
7 8 12 15
6 252830
6 8 12 14
9 112326
9 1619
kDa
glutelin precursor
glutelin acidic
glutelin basic
75
50
37
25
20
15
10
GFP

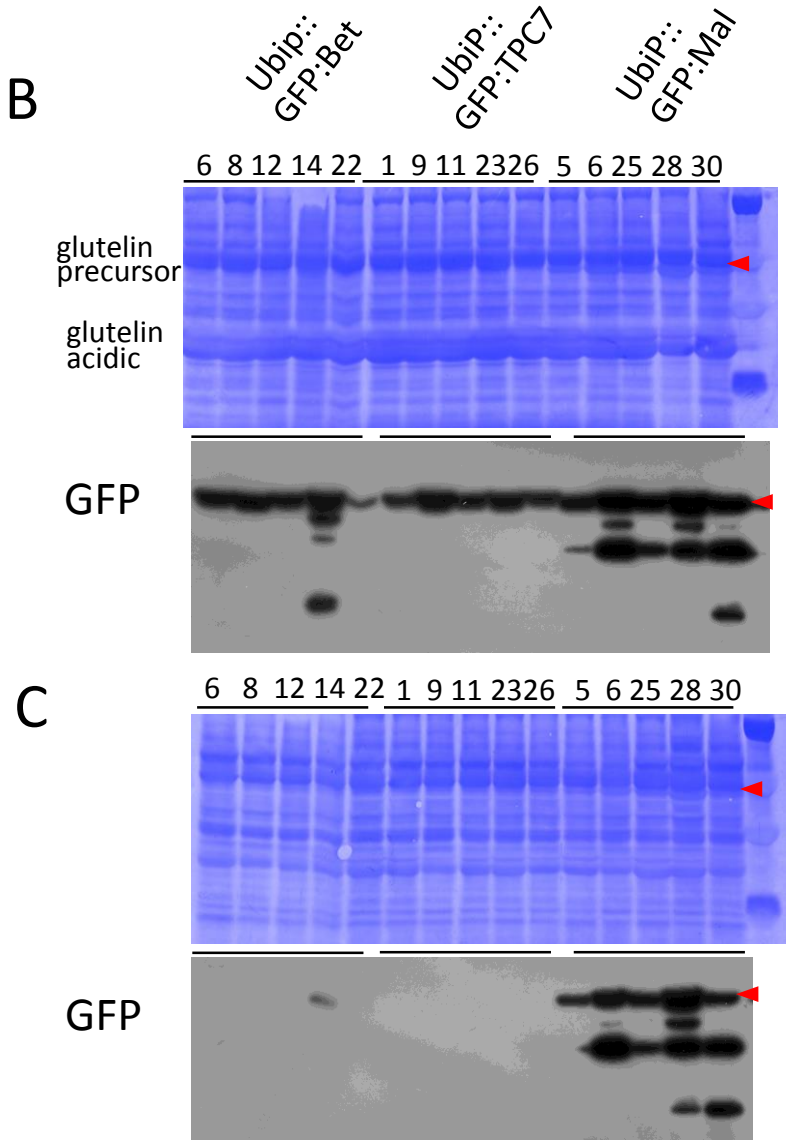
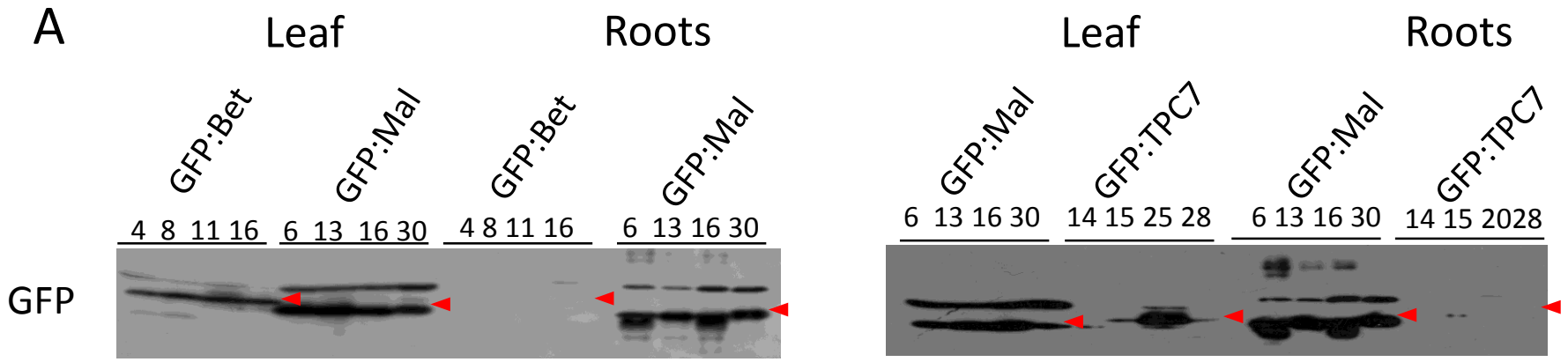

B
Ubip::GFP:Bet
UbiP::GFP:TPC7
UbiP::GFP:Mal
6 8 12 14 22
1 9 11 2326
5 6 25 28 30
glutelin precursor
glutelin acidic
GFP
C
6 8 12 14 22
1 9 11 2326
5 6 25 28 30
GFP

Fig. 3


A
Leaf
Roots
GFP:Bet
GFP:Mal
GFP:Bet
GFP:Mal
4 8 11 16
6 13 16 30
4 8 11 16
6 13 16 30
GFP
Leaf
Roots
GFP:Mal
GFP:TPC7
GFP:Mal
GFP:TPC7
6 13 16 30
14 15 25 28
6 13 16 30
14 15 2028

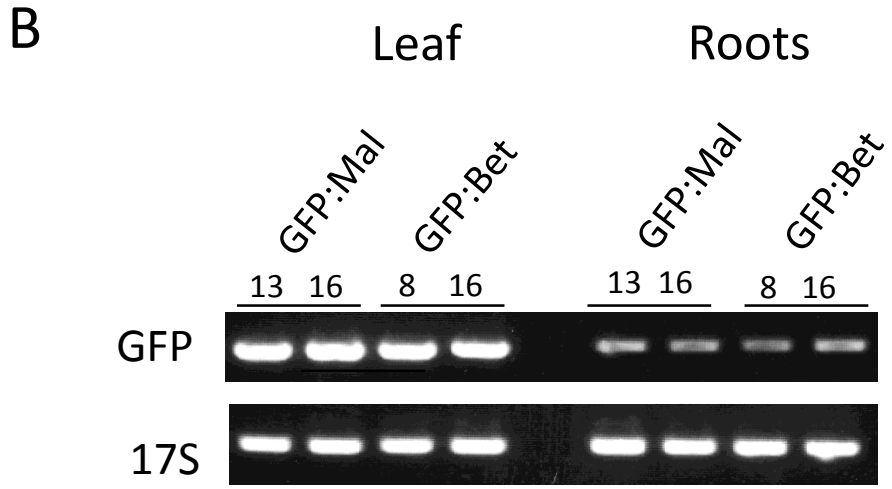

B
Leaf
Roots
GFP:Mal
GFP:Bet
GFP:Mal
GFP:Bet
13 16
8 16
13 16
8 16
GFP
17S

Fig. 4

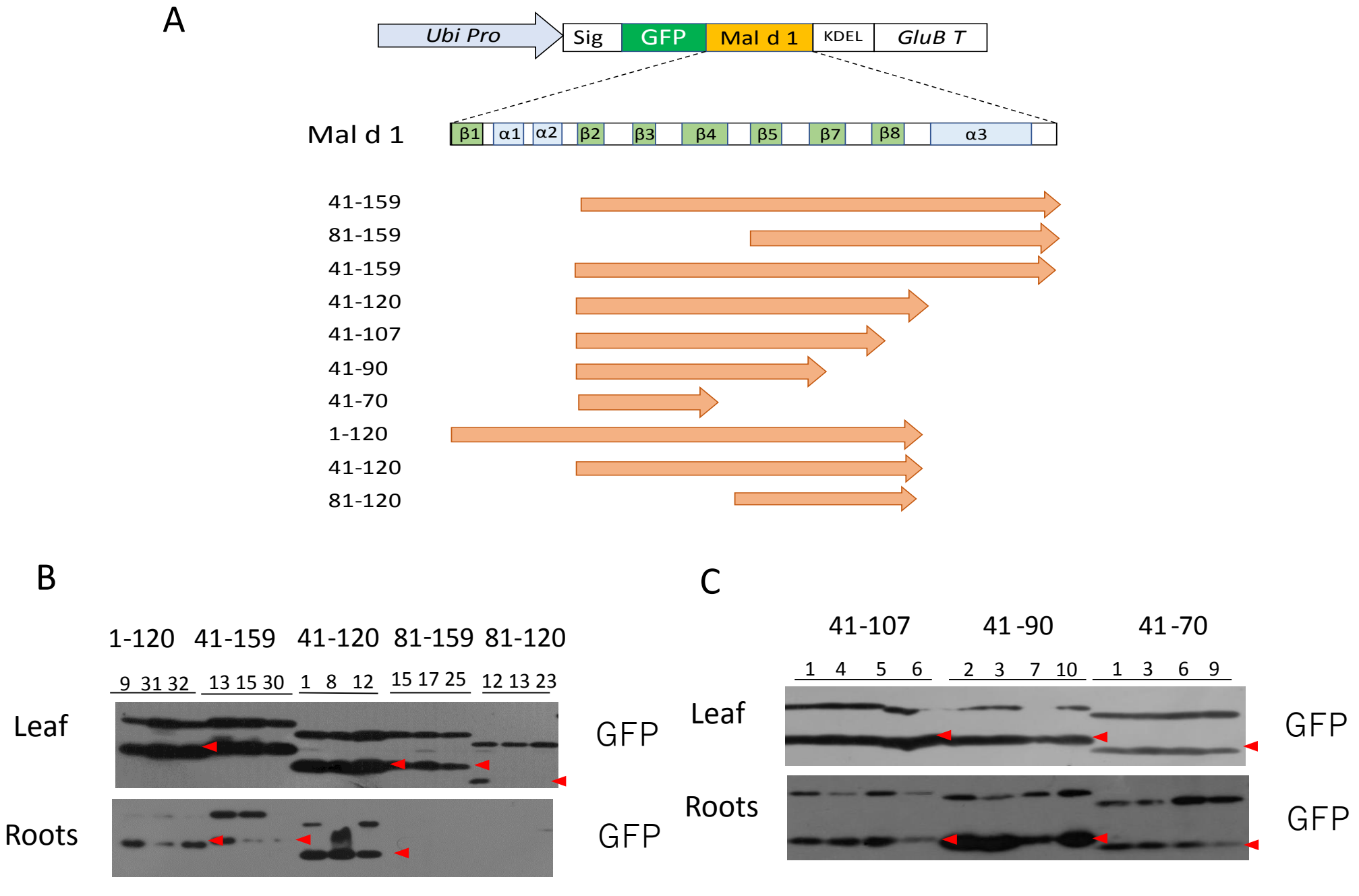

A
Ubi Pro
Sig
GFP
Mal d 1
KDEL
GluB T
Mal d 1
β1
α1
α2
β2
β3
β4
β5
β7
β8
α3
41-159
81-159
41-159
41-120
41-107
41-90
41-70
1-120
41-120
81-120
B
1-120
41-159
41-120
81-159
81-120
9 31 32
13 15 30
1 8 12
15 17 25
12 13 23
Leaf
GFP
Roots
GFP
C
41-107
41 -90
41 -70
1 4 5 6
2 3 7 10
1 3 6 9
Leaf
GFP
Roots
GFP

Fig. 5

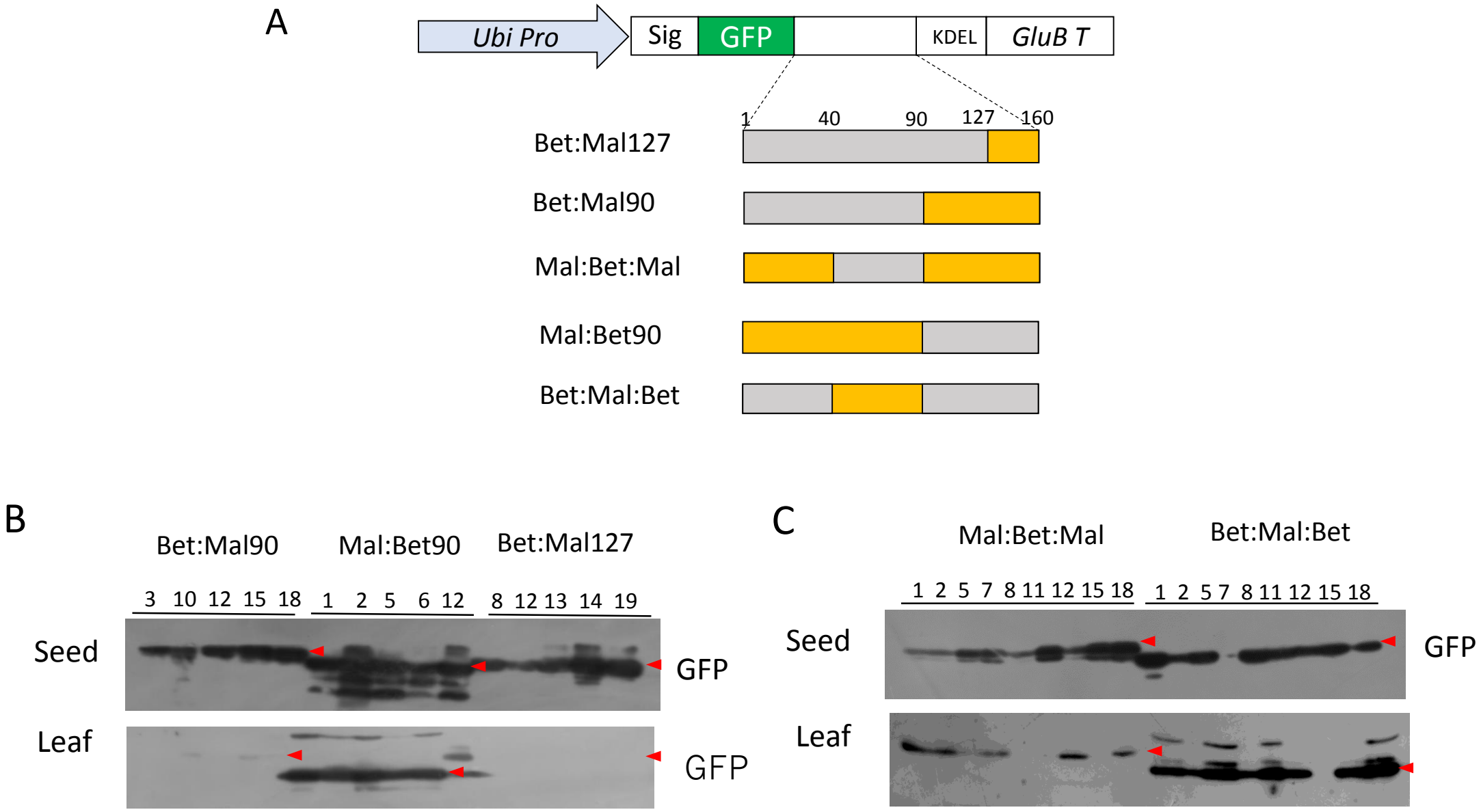
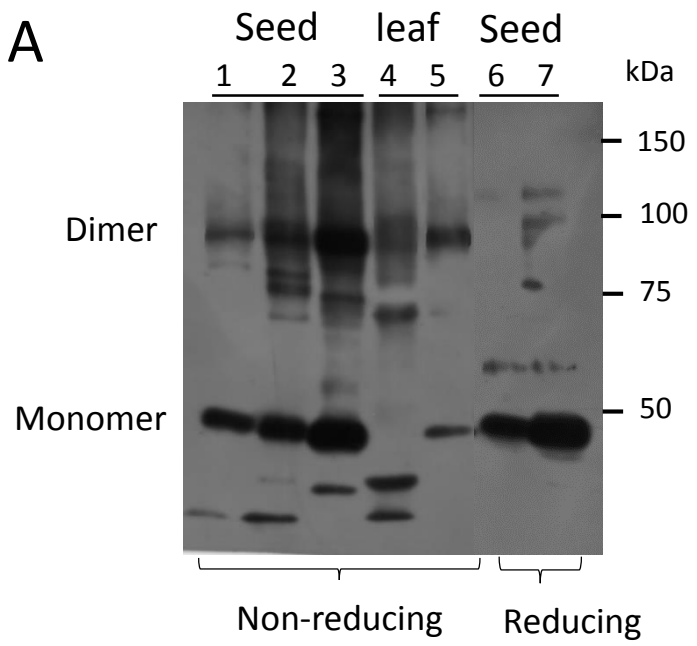
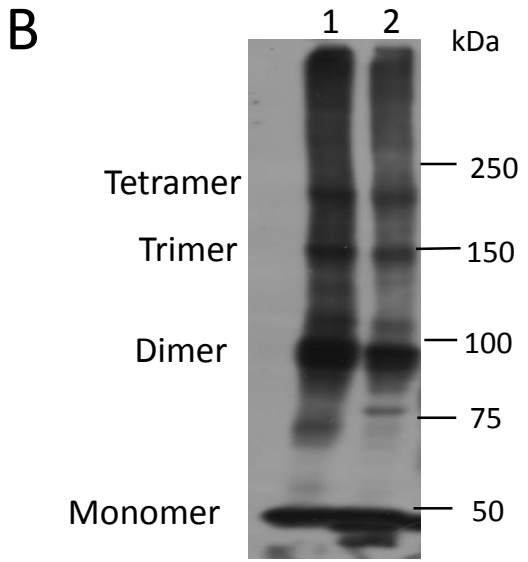
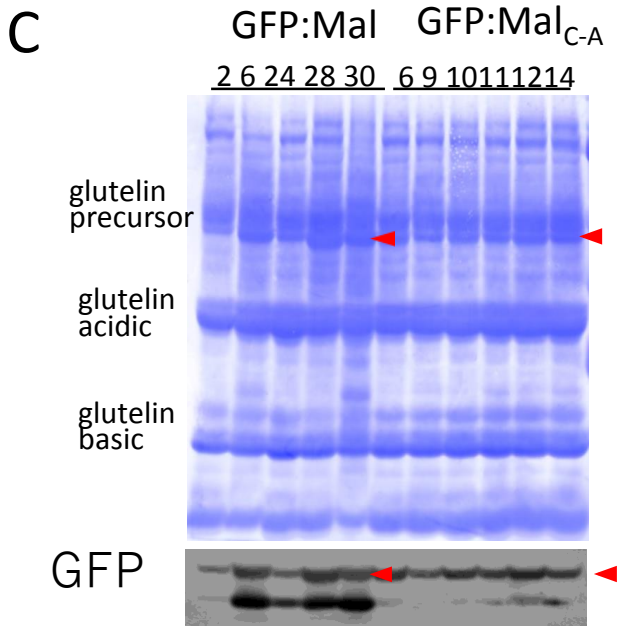

A
Ubi Pro
Sig
GFP
KDEL
GluB T
1
40
90
127
160
Bet:Mal127
Bet:Mal90
Mal:Bet:Mal
Mal:Bet90
Bet:Mal:Bet
B
Bet:Mal90
Mal:Bet90
Bet:Mal127
3 10 12 15 18
1 2 5 6 12
8 12 13 14 19
Seed
GFP
Leaf
GFP
C
Mal:Bet:Mal
Bet:Mal:Bet
1 2 5 7 8 11 12 15 18
1 2 5 7 8 11 12 15 18
Seed
GFP
Leaf

Fig. 6


A
Seed
leaf
Seed
1 2 3 4 5 6 7
kDa
150
100
75
50
Dimer
Monomer
Non-reducing
Reducing


B
1 2
kDa
250
150
100
75
50
Tetramer
Trimer
Dimer
Monomer


C
GFP:Mal
GFP:Mal C-A
2 6 24 28 30 6 9 10111214
glutelin precursor
glutelin acidic
glutelin basic
GFP

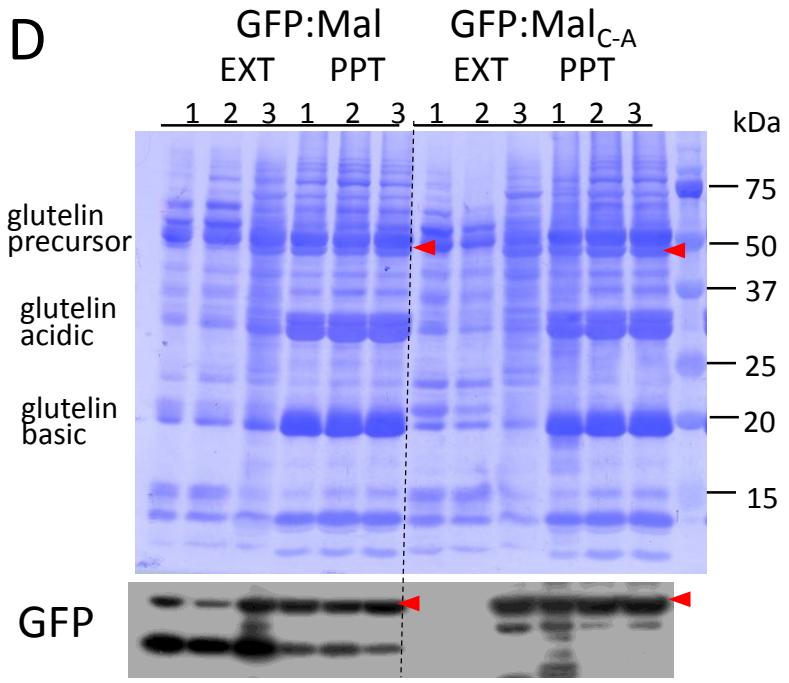

D
GFP:Mal
GFP:Mal C-A
EXT PPT EXT PPT
1 2 3 1 2 3 1 2 3 1 2 3
kDa
75
50
37
25
20
15
glutelin precursor
glutelin acidic
glutelin basic
GFP

Fig. 7

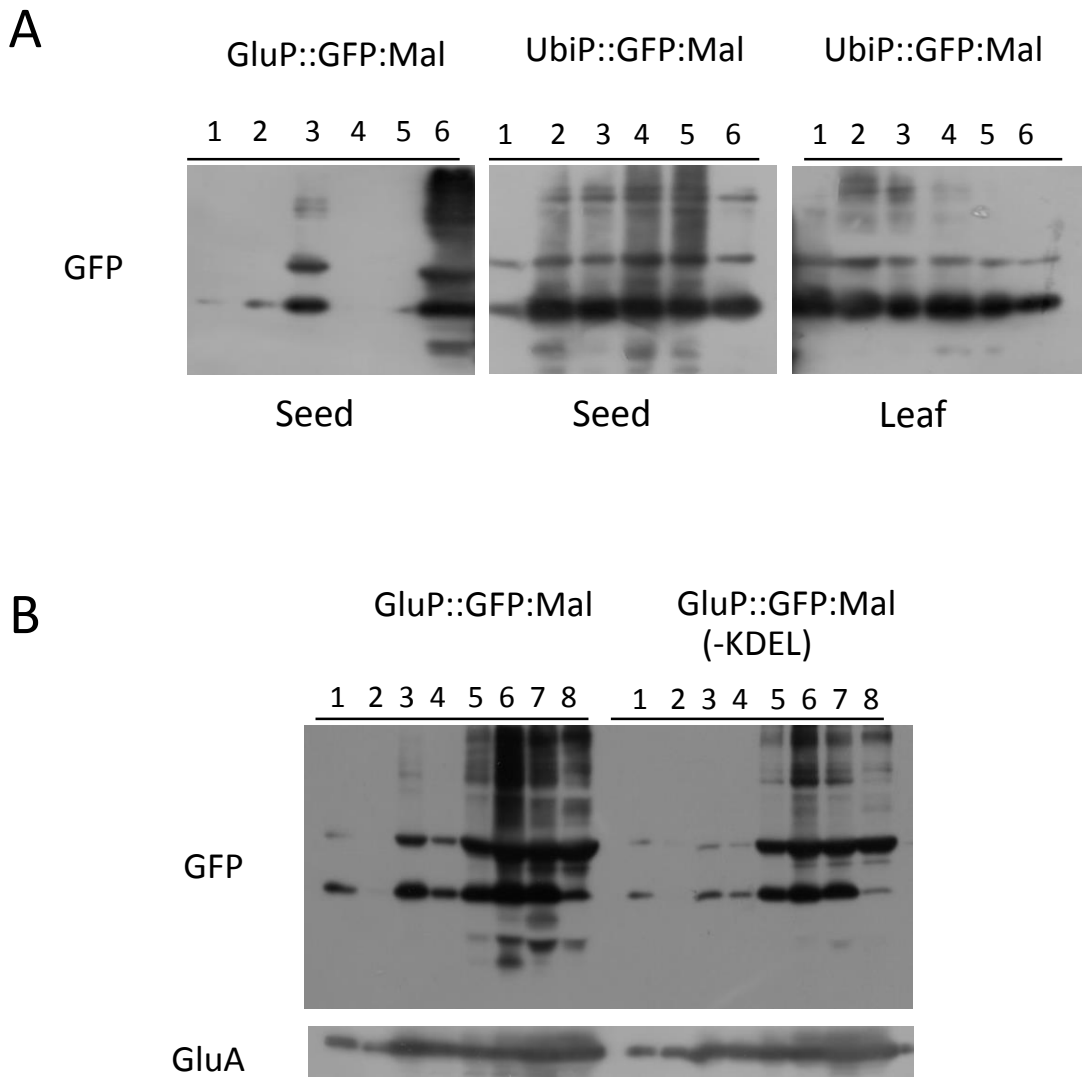

A
GluP::GFP:Mal
UbiP::GFP:Mal
UbiP::GFP:Mal
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
GFP
Seed
Seed
Leaf
B
GluP::GFP:Mal
GluP::GFP:Mal
(-KDEL)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
GFP
GluA

Fig. 8

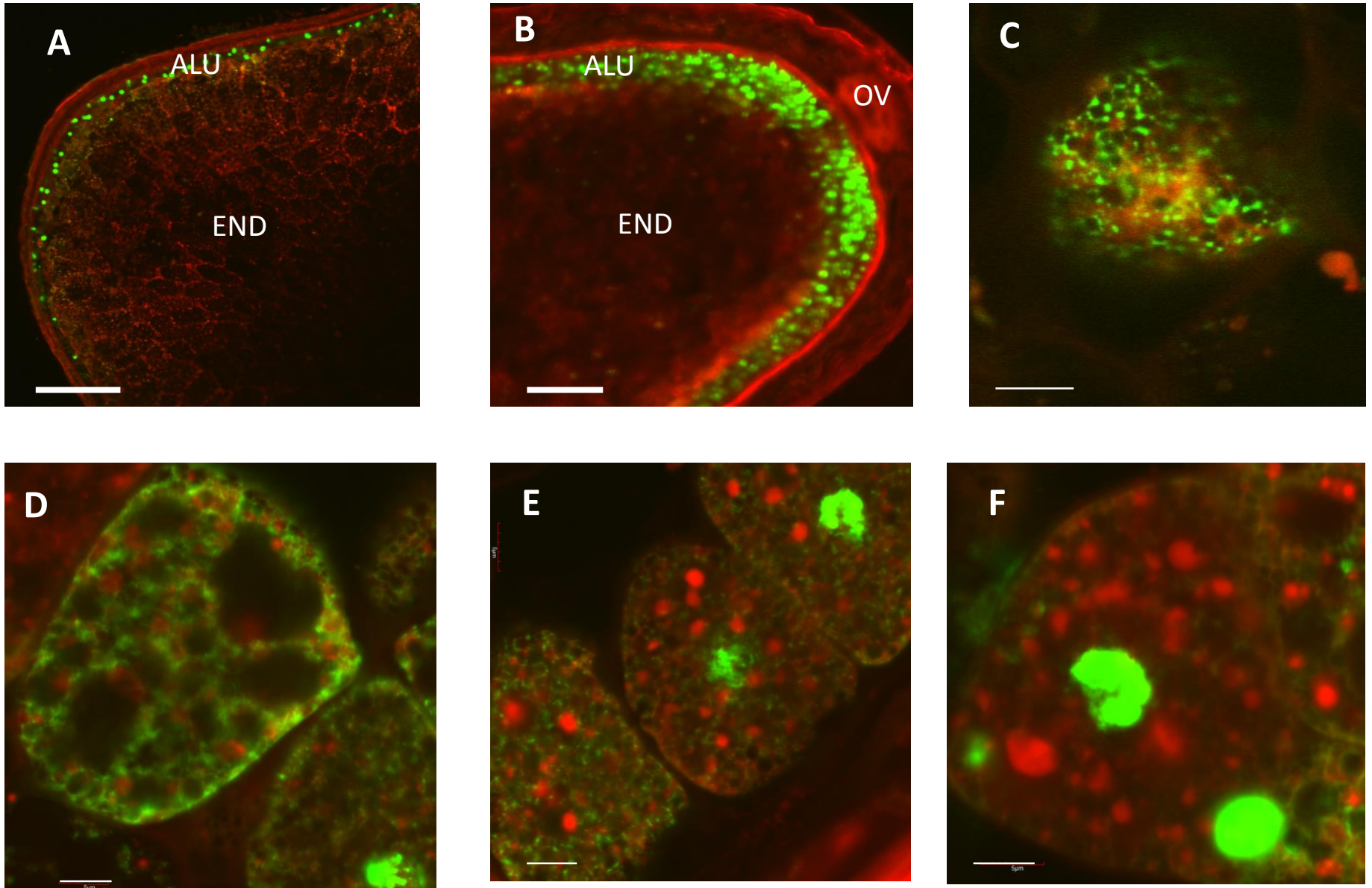


Fig. 9