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2 **Difference in accumulation pattern of the allergens within the same PR10 family in**
3 **transgenic rice**
4
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6
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34 **Difference in accumulation pattern of the allergens within the same PR10 family in** 35 **transgenic rice**

36 37 **Highlight**

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

40 41 **Abstract**

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

57
58 **Key words:** Apple allergen, Bet v 1, PR10, protein body, Mal d 1, transgenic rice

59

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-allergic 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).

86 Mal d 1 is encoded by a multigene family composed of 31 members, which are divided
87 into four groups based on amino acid sequence similarities, Mal d 1.01, Mal d 1.02, Mal d
88 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
89 predominantly expressed in apple fruit (peel and pulp) (Marzban *et al.*, 2005). Amino
90 acid sequence identities between Mal d 1 isoforms and Bet v 1 range from 55 to 68 % (Ma
91 *et al.*, 2006).

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

101 We previously generated transgenic rice seeds containing versatile hypo-allergenic
102 derivatives of major birch pollen allergen Bet v 1 referred to as tree pollen chimera7 (TPC7)
103 or TPC9, which have been developed as allergy vaccines (tolerogens) against the major
104 allergens of trees belonging to the Fagales order Bet v 1 family for allergen-specific
105 immunotherapy (Wang *et al.*, 2013). When these native Bet v 1 and its derivatives (TPC7
106 and TPC9) were expressed as secretory protein in rice seeds under the control of the
107 endosperm-specific glutelin *GluB-1* promoter by ligating the glutelin *GluB-1* signal peptide
108 and the KDEL ER retention signal to their N- and C-termini, they were deposited in
109 remarkably huge spherical ER-derived PBs designated as TPC7 bodies with the size of
110 >10 μm (Wang *et al.*, 2013; Ogo *et al.*, 2014). Furthermore, their accumulation levels
111 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 genes 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

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

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

146

147 **SDS-PAGE and immunoblot analysis**

148 Individual mature grain (four grains per independent transgenic line) and leaves and
149 roots frozen in liquid nitrogen were ground into a fine powder using a Multibeads shocker
150 (Yasui Kikai, Osaka, Japan). Total proteins were extracted from the powder of a mature
151 grain, leaves and roots with 400 µl (grain) or 200 µl (leaves and roots) of urea-SDS buffer
152 (50 mM Tris-HCl, pH 6.8, 8M urea, 4% SDS, 5% 2-mercaptoethanol (2-MER), 20% glycerol)
153 as described previously (Tada *et al.*, 2003). After separation of total protein extract (2-5 µl)
154 by electrophoresis on 13% SDS-PAGE, proteins were visualized by Coomassie Brilliant
155 Blue (CBB)-R250 staining or were transferred to PVDF membranes (Millipore, Billerica, MA,
156 USA) for immunodetection with rabbit anti-GFP or appropriate antibodies. Rabbit antibodies
157 against TPC7, rice glutelin A (GluA), and rice chaperones of binding protein 1 (BiP1) and
158 4&5 (BiP4/5), protein disulfide isomerase-like 1-1 and 2-3 (PDIL1-1 and PDIL2-3), and
159 calnexin (CNX) were prepared as described previously (Yasuda *et al.*, 2009; Oono *et al.*,
160 2010; Wakasa *et al.*, 2011; Wang *et al.*, 2013). For immunodetection, membranes were
161 incubated with rabbit antibodies, followed by horseradish peroxidase-conjugated
162 anti-rabbit secondary IgG antibodies (dilution 1:5000) (Cell Signaling Technology, Danvers,
163 MA, USA). Bands were visualized using Clarity Western ECL detection kit (Bio-Rad).

164

165 **Confocal laser scanning microscopy and electron immunomicroscopy**

166 Developing seeds were harvested 15–20 days after flowering (DAF) from various transgenic
 167 lines and then cross-sectioned with a DTK-1000 Microslicer (Dosaka EM) to approximately
 168 150 μm in thickness for indirect immune-histochemical analysis (Yasuda *et al.* 2006).
 169 Mouse anti-GFP monoclonal antibody, rabbit anti-CNX, anti-PDIL1-1, anti-PDIL2/3,
 170 anti-BiP1-1, anti-BIP4/5, anti-TiP3 and anti-RM1 (14kDa prolamin) polyclonal antibodies
 171 were used to investigate their intracellular localization by Alexa-488 conjugated
 172 anti-mouse IgG antibody and Alexa-546 conjugated anti-rabbit IgG antibody. After
 173 immunostaining of samples, rhodamine B was used to stain ER-derived protein bodies
 174 (PB-Is). Samples cross-sections were spread on glass slides, sealed with a cover glass, and
 175 observed with a confocal laser scanning microscope (FLUOVEIW; OLYMPUS, Tokyo,
 176 Japan).

177

178 **RNA extraction and RT-PCR analysis**

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

182

183 **Extraction of recombinant proteins from mature seeds**

184 Leaves and roots were collected from each transgenic rice plant and frozen instantly in
 185 liquid nitrogen and then powdered using a Multibeads shocker. Pulverized seed,
 186 leaves and roots were treated with ten volume of various extraction buffer (200 μl) for
 187 20 min by vortex, and then centrifuged at 15000 rpm for 10 min. The supernatant was
 188 transferred to new tube and then equal volume of 2x sample buffer (125 mM Tris-HCl
 189 (pH6.8), 20% glycerol, 4%SDS) without 2-MER was added. Some aliquot (5ul) was

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

191

192 **Results**

193 **Endosperm-specific expression of Mal d 1 in transgenic rice**

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

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

212 Subcellular localization of GFP:Mald1 with the presence or absence of the KDEL signal
 213 was investigated in maturing transgenic seeds by immuno-histological analysis using
 214 confocal laser scanning microscopy. As shown in Fig. 2A, the GFP:Mald1 containing the
 215 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 μ m 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

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

252 When the transcript (mRNA) levels in leaf or roots between the Mal d 1 and Bet v 1 were
253 examined by RT-PCR, there was little difference in their transcript levels between them,
254 indicating that their accumulation levels are determined by post-transcriptional level (Fig.
255 4B).

256

257 **Subcellular localization of GFP:Mald1**

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

268 prolamins and small granules with a size of $\leq 1 \mu\text{m}$ (Fig. 2F, Fig. S3L). In the embryo of
 269 maturing seed, it was predominantly localized in ER lumen (Fig. 2H, Fig. S3H,K). To
 270 investigate the participation of chaperon proteins in huge PB formation, intracellular
 271 localization of several chaperon proteins was analyzed. As shown in Fig. S3 (A,B,D,I), BIP1,
 272 BiP4/5, PDIL1-1 and PDIL2-3 were co-localized in huge peripheral PBs in the aleurone cells,
 273 whereas they are also localized in ER lumen and PB-Is in the starchy endosperm. When
 274 antibodies against CNX (Fig. S3F), OsTiP3 (Fig. S3G) and Cys-rich 14kDa prolamin (RM1)
 275 (Fig. S3SL) were used as markers for subcellular compartments of ER, protein storage
 276 vacuole (PB-II) and ER-derived PBs (PB-Is), respectively, they were not observed to be
 277 over-lapped with huge PBs. These results indicate that huge PBs accumulating
 278 GFP:Mald1 are formed by aid of BiPs and PDILs chaperone proteins.

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

284

285 **Identification of regions of Mal d 1 responsible for stable accumulation in vegetative** 286 **tissues**

287 To investigate which domain(s) in the Mal d 1 molecule is involved in high and stable
 288 accumulation in vegetative tissues, the Mal d 1 was progressively deleted from the N
 289 or C termini and their resultant regions were fused to the GFP reporter as shown in Fig.
 290 5A. They were expressed under the control of the maize ubiquitin constitutive promoter.

291 When these fusion product levels were examined in vegetative tissues (leaf and roots) by
 292 immunoblotting using anti-GFP antibody, the regions of the Mal d 1 between positions 1-120,
 293 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.

320 **Domain swapping between the Mal d 1 and Bet v 1**

321 To identify the domain that is implicated in high accumulation in vegetative tissues of
 322 transgenic rice harboring GFP:Mald1, domain swapping was carried out between the Mal d
 323 1 and Bet v 1 as shown in Fig. 6A. When the C terminal regions of the Bet v 1 between
 324 positions 90-159 and positions 127-159 were exchanged with the corresponding regions of
 325 the Mal d 1, accumulation levels of these chimeric products (Bet:Mal90, Bet:Mal127) were
 326 very low in leaves like the native Bet v 1. That is, accumulation levels of these chimeric
 327 products were not significantly altered by fusion with the C terminal region of Mal d,
 328 indicating that the C terminal half region of the Mal d 1 has little enhancing effect. On the
 329 other hand, when the N terminal half region of Bet v 1 between positions 1 and 90 was
 330 exchanged with the corresponding region of the Mal d 1, much higher level of this chimeric
 331 product (Mal:Bet90) than the native Bet v 1 was observed in leaves. These findings
 332 indicated that the N terminal half region of the Mal d 1 is responsible for high
 333 accumulation of the Mal d 1 in vegetative tissues. Therefore, in order to ascertain that
 334 the region between positions 41-90 is critical for high accumulation in vegetative tissue,
 335 the corresponding region was mutually exchanged between the Mal d 1 and Bet v 1. The
 336 Bet:Mal:Bet chimeric product, in which the region between positions 41-90 was substituted
 337 with the corresponding region of the Mal d 1, gave rise to high levels of accumulation in
 338 leaves of most of transgenic lines, whereas the Mal:Bet:Mal chimeric product containing the
 339 Bet v 1 sequence between positions 41-90 resulted in lower levels of accumulation in
 340 most of transgenic rice leaves. These results indicated that the region between positions
 341 41-90 is mainly responsible for high accumulation in vegetative tissues of Mal d 1.

342

343 **Aggregation property of Mal d 1**

344 When the Mal d 1 containing KDEL ER retention tag was expressed under the control of the
 345 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 Mald1 like the Bet v 1 and TPC7. Therefore, the Cys residue in the Mald1 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-A} was extracted from
359 seed and leaf and then analyzed by native PAGE, dimeric form of the modified Mald1 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:Mal_{C-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

398 accumulating the Bet v 1 and its derivatives, TPC7 and TCP9. We previously reported that
399 high amounts of TPC7 (540 ug/seed) and native Bet v 1 were deposited in spherical
400 huge protein bodies in endosperm cells of transgenic rice seeds, when expressed as
401 secretory protein under the control of glutelin endosperm-specific promoter (Wang *et al.*
402 2013; Ogo *et al.* 2014).

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

413 As shown in Fig. 3, when the GFP:Betv1, GFP:TPC7 and GFP:Mald1 were expressed
414 as secretory protein by ligating the N terminal signal peptide and C terminal KDEL tag
415 under the control of the maize ubiquitin promoter, accumulation levels of GFP:Betv1 and
416 GFP:TPC7 were very low in various vegetative tissues such as leaf, root and seed embryo
417 except for seed (endosperm). In contrast, the GFP:Mald1 highly accumulated not only in
418 seed, but also in leaf and root. When intracellular localization of the GFP:Mald1 was
419 examined in maturing transgenic seeds, it is mainly deposited in huge PBs in aleurone
420 cells and in ER-derived PBs (PB-Is) containing prolamin storage proteins in starchy
421 endosperm cells. This accumulation pattern was remarkably different from that directed by
422 rice endosperm-specific glutelin promoter, in which the GFP:Mald1 is predominantly
423 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 μ m. 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 Lys97 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.

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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, hygromycin
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

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

703

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

711

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

718

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

723

724 **Figure 7. Comparison of aggregation property between GFP:Mald1 and GFP:Mald1_{C-A}.**
725 **(A)** Immunoblot analysis of GFP:Mald1 and GFP:Mald1_{C-A} expressed in transgenic rice
726 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:Mal_{C-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 μ m Thin bars= 5 μ m

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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 immunoblotting 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-conjugated 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 μ m. 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 μ m

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.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

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Fig. 5

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Fig. 6

Fig. 7

Fig. 8

Fig. 9