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1 **EXPRESSION OF A NEW CHIMERIC PROTEIN WITH A HIGHLY**
2 **REPEATED SEQUENCE IN TOBACCO CELLS**

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34 **Abstract**

35

36 In wheat, the high molecular weight (HMW) glutenin subunits are known to contribute to
37 gluten viscoelasticity and show some similarities to elastomeric animal proteins as elastin.
38 Combining the sequence of a glutenin with that of elastin is a way to create new chimeric
39 functional proteins, which could be expressed in plants. The sequence of a glutenin subunit
40 was modified by the insertion of several hydrophobic and elastic motifs derived from elastin
41 (elastin-like peptide, ELP) into the hydrophilic repetitive domain of the glutenin subunit to
42 create a triblock protein, the objective being to improve the mechanical (elastomeric)
43 properties of this wheat storage protein.

44 In this study, we investigated an expression model system to analyze the expression
45 and trafficking of the wild type HMW glutenin subunit (GS_W) and an HMW glutenin subunit
46 mutated by the insertion of elastin motifs (GS_M -ELP). For this purpose, a series of constructs
47 was made to express wild-type subunits and subunits mutated by insertion of elastin motifs in
48 fusion with green fluorescent protein (GFP) in tobacco BY-2 cells. Our results showed for the
49 first time the expression of HMW glutenin fused with GFP in tobacco protoplasts. We also
50 expressed and localized the chimeric protein composed of plant glutenin and animal elastin-
51 like peptides (ELP) in BY-2 protoplasts, and demonstrated its presence in protein body-like
52 structures in the endoplasmic reticulum. This work therefore provides a basis for heterologous
53 production of the glutenin-ELP triblock protein to characterize its mechanical properties.

54

55 **Keywords:** glutenin . elastin . biopolymer . triblock protein . endoplasmic reticulum . BY-2
56 cells.

57

58 **Abbreviations**

59	BiP	Binding Protein
60	BY-2	Bright Yellow-2
61	ELP	Elastin-Like Peptide
62	ER	Endoplasmic Reticulum
63	GFP	Green Fluorescent Protein
64	GS_W	Wild type Glutenin Subunit
65	GS_M	Modified Glutenin Subunit
66	GS_M -ELP	Modified Glutenin Subunit-Elastin Like Peptide
67	HMW	High Molecular Weight

68	PB	Protein Body
69	PDI	Protein Disulphide Isomerase
70		

71 **Introduction**

72

73 Agricultural products, and particularly plant proteins, are candidates for the preparation of
74 renewable and biodegradable materials. Among these, wheat gluten presents the advantage of
75 being a commercially available raw material. The properties of wheat gluten films have been
76 widely studied and the potential of these proteins is now well defined (Popineau et al. 2001;
77 Mangavel et al. 2002). Wheat gluten proteins are prolamin storage proteins, comprised of
78 monomeric gliadins and polymeric glutenins, synthesized and stored in the endosperm cells of
79 the seeds. They exhibit unusual viscoelastic properties when hydrated and are largely
80 responsible for the rheological behaviours of wheat flour dough (Shewry et al. 2002; Shewry
81 and Halford 2002).

82 Wheat gluten proteins are typical secretory proteins, and processing within the
83 endoplasmic reticulum (ER) would include folding and intra-chain disulphide bond
84 formation, as well as formation of the inter-chain bonds that stabilize the glutenin polymers
85 (Shewry et al. 2003). Non-covalent interactions (notably hydrogen bonds) between gliadins
86 and glutenin polymers may also be established, resulting in protein precipitation and the
87 formation of hydrated protein particles within the secretory pathway. It is probable that the
88 folding and assembly of the gluten proteins is assisted by ER luminal proteins such as the
89 enzyme protein disulphide isomerase (PDI) and the molecular chaperone binding protein
90 (BiP) although this is still not conclusively established (Grimwade et al. 1996; DuPont et al.
91 1998). It has been reported that some prolamins accumulate in the ER before being deposited
92 into protein bodies (PBs) (Galili et al. 1993). These PBs are derived from the ER and it has
93 been suggested that they are transported directly to the storage vacuole bypassing the Golgi
94 (Levanony et al. 1992; Galili et al. 1993; Vitale and Galili 2001). However, other reports
95 show the trafficking of some prolamins *via* the Golgi particularly during the early stages of
96 seed development (Parker and Hawes 1982; Shy et al. 2001; Loussert et al. 2008; Tosi et al.
97 2009). In mature seeds, the prolamins form a matrix resulting from the disappearance of the
98 PB structure (Rubin et al. 1992).

99 The glutenin proteins are elastomeric, similar to silk protein, abductin or elastin
100 protein. In spite of their different amino acid sequences, all of these proteins present common
101 characteristics such as short repeated sequence conferring elasticity and the ability to form
102 stable intermolecular links (Tatham and Shewry 2000). Our experimental approach exploits a
103 sequence of the animal elastic protein, elastin. This protein exhibits mechanical properties that

104 are already used in the polymer industry with applications in biomedicine and
105 nanobiotechnology (Rodriguez-Cabello et al. 2009, Arias et al. 2006). As an additional
106 beneficial effect, elastin-like peptide (ELP) fusions have also been shown to significantly
107 enhance the accumulation of a range of different recombinant proteins in transgenic tobacco
108 leaves (Patel et al. 2007; Floss et al. 2008) and seeds (Scheller et al. 2006). Although it is
109 thought that ELP tags confer increased stability or solubility to their fusion partner, the means
110 by which ELP increases the production yield of recombinant proteins *in planta* has not yet
111 been established. The ability of ELP fusions to induce PB formation in plant leaves was
112 recently shown (Conley et al. 2009).

113 The two proteins, elastin and glutenin, have similar mechanical properties, but differ
114 in their molecular characteristics. Both proteins are proline-rich, with the ability to self-
115 assemble and form supramolecular secondary structures consisting of helices and spirals as a
116 result of their highly repetitive sequences (Shewry et al. 2002; Miao et al. 2003). The
117 introduction of several elastic hydrophobic motifs of elastin into glutenin could modify gluten
118 viscoelasticity by the formation of a triblock protein consisting of one hydrophobic elastin
119 block into a hydrophilic domain. The structure of this triblock protein could result in a
120 microstructure of separated phases where the hydrophobic parts could bind together separated
121 by hydrophilic domains. Such a triblock protein could improve the water sensitivity and
122 mechanical properties by the formation of elastic hydrophobic microdomains (Wellner et al.
123 2006; Wright and Conticello 2002) . This macrostructure type has been previously considered
124 for elastin-mimetic triblock polymers (Wright and Conticello 2002). Triblock copolymers are
125 already used as an interface agent to allow polymer mixing and to obtain stable composite
126 materials (Kumar et al. 2006).

127 The aim of this work is to study the expression of a new chimeric triblock protein
128 based on a HMW glutenin subunit and ELP, in fusion with green fluorescent protein (GFP) in
129 BY-2 suspension-cultured tobacco cells. Previous studies demonstrated the possibility to
130 express glutenins of high or low molecular weight (Shani et al. 1994; Robert et al. 1989;
131 Shimoni et al. 1997; Lombardi et al. 2009) and elastin-like motifs (Patel et al. 2007; Floss et
132 al. 2008; Conley et al. 2009) in tobacco plants. Our results demonstrated the ability to express
133 and identify the subcellular localization of the new chimeric protein in fusion with GFP in a
134 tobacco cell system.

135 **Materials and methods**

136

137 Plant Material

138

139 Tobacco *Nicotiana tabacum* L. cv *Bright Yellow* (BY-2) cells was maintained at 24°C on an
140 orbital shaker (120 rpm) in Linsmaier and Skoog modified medium (Nagata et al. 1992) in the
141 dark. This culture was maintained by weekly dilution of the cells by the medium (0.5:30
142 [v/v]).

143

144 Cloning of 1Dy10 cDNA

145

146 The cloning of 1Dy10 cDNA from a mRNA library obtained from endosperm of wheat grain
147 cultivar Recital aged of 313°D was achieved by PCR with 1Dy10 specific primers (5'-
148 AAGTCGACCACCCACAACACCGAGCACC-3'; 5'-AACCCGGGAGTTCTATCACTGG
149 CTAGCC-3') carrying the restriction sites SalI and SmaI, respectively.

150

151 GS_W-GFP, GS_M-GFP, GS_M-ELP-GFP fused genetic constructs

152

153 To study the subcellular compartmentalization of 1Dy10 glutenin subunit (GS) fused proteins,
154 three different constructs based on a GFP fusion were used. The pRTd35S-GFP vector was
155 designed especially as a reporter gene with enhanced green fluorescence. Expression of the
156 *GFP* gene is driven by a double 35S Cauliflower mosaic virus (CaMV) promoter, and GFP
157 protein synthesis is induced by a viral translation enhancer. In order to introduce the GS
158 cDNA in GFP upstream, the SalI and SmaI restriction sites are introduced in pRTd35S-GFP
159 between GFP open reading frame and the viral translation enhancer sequence by PCR using
160 primers 5'-AAGGAATCCAGAGTCGACACTCCCGGGTCCATGGGTAAAGGAGAAGA
161 ACT-3' and 5'-ACTCTAGAGGATCCTTAGAGATCTAGTTCA-3'.

162 The first construct corresponded to the GS_W-GFP fusion, for which the complete cDNA of
163 1Dy10 encoded for a wild-type glutenin subunit (GS_W) was used. To produce the GS_W-GFP
164 fusion protein, the GS cDNA without a STOP codon was amplified by PCR with the same
165 forward primer previously described and the reverse primer 5'-
166 AATCCCGGGCTGGCTAGCCG ACAATGCGTC-3' carrying the SmaI restriction site. The
167 PCR product corresponding to GS without a STOP codon was cloned as a SalI-SmaI fragment

168 in the new Sall-SmaI sites of pRTd35S-GFP vector under the control of the CaMV 35S
169 promoter and terminator.

170 For the second construct, the GS cDNA without a STOP codon was mutated by directed
171 mutagenesis with the QuickChange® Site-Directed Mutagenesis Kit (Clontech) in order to
172 introduce a BglIII restriction site. The mutagenesis PCR was performed with the forward
173 primer 5'-CCAAGACAATGGCAAAGATCTGGACAAGGGCAACAAG-3' and the reverse
174 primer 5'-CTTGTTGCCCTTGTCCAGATCTTTGCCATTGTCTTGG-3', allowing the
175 introduction of the BglIII restriction site (underlined sequence) into the repeated domain of GS
176 in order to obtain the GS mutated fragment corresponding to the GS_M-GFP fused protein.

177 For the third construct, the GS mutated fragment was used to introduce a fragment of 630pb
178 encoding the 40xELP sequence. The ELP nucleotide sequence used in this work encodes the
179 VPGVG pentapeptide (Scheller et al. 2004). It was provided into the pRTRA-40xELP
180 plasmid containing two BglIII sites, which are used for 40xELP sequence insertion into the
181 modified glutenin subunit sequence, the GS_M-GFP construct. The correct sequence of
182 constructs GS_W-GFP, GS_M-GFP, GS_M-ELP-GFP and the open reading frame conservation in
183 the gene junction were checked by sequencing of both strands in each construct.

184

185 BY-2 protoplasts preparation and transient transformation

186

187 Protoplasts were isolated from tobacco BY-2 cells cultured as previously described, four days
188 after subculture in fresh medium. Protoplast preparation and transformation using a
189 polyethyleneglycol-based technique were performed essentially according to the method of
190 Neuhaus and Boevink (2001). Overnight digestion of cell walls was achieved with 0.1%
191 pectolyase and 1% cellulase (Duchefa, Onozuka R-10) at 28°C in the dark with gentle
192 shaking. Approximately 7.5×10^5 protoplasts were transformed with 20 µg of plasmid DNA
193 and 20 µg of carrier DNA to a medium containing 40% PEG-6000, 0.1 M Ca(NO₃)₂, 0.4 M
194 mannitol, and 0.1% Mes, pH 8.0. Observation by confocal microscopy was made 48 h after
195 transformation, and protoplasts were maintained at 26°C in the dark without shaking.

196

197 Immunolocalization on BY-2 protoplasts

198

199 The transiently transfected protoplasts were recovered by three volumes of W5 medium
200 (Neuhaus and Boevink, 2001) and spinning for 10 min at 60 g. The fixative buffer (3.7%

201 paraformaldehyde, 0.5 M mannitol, 50 mM HEPES pH 5.8) added to the protoplasts pellet.
202 Protoplasts were kept in fixative buffer one hour at room temperature, gently mixed
203 occasionally, and transferred to 4°C overnight. The fixed protoplasts were rinsed three times
204 for few minutes and then for one hour in fresh mannitol buffer (0.5 M mannitol, 50 mM
205 HEPES pH 5.8) before using them for the immunolabelling step.
206 The protoplasts were incubated for 10 min exactly in 0.5 % Triton X-100 at room temperature
207 to perforate the membranes. The cells were washed quickly with IL (ImmunoLabelling)
208 buffer (PBS 1x, 0.5 % BSA, 0.05 % Tween-20, filter sterilized through a 0.45 µm filter) and
209 washed twice more for 5 min. The protoplasts were blocked with IL buffer plus 1 % BSA for
210 15 min at room temperature. The protoplasts suspensions were aliquoted into individual tubes,
211 one for each primary antibody, rabbit polyclonal anti-R2-HMW (Denery-Papini et al. 1996),
212 and rabbit anti-BiP (Pedrazzini et al. 1997). The supernatant was removed, and cells were
213 incubated for 1 h at room temperature with primary antibody diluted at 1/500 (rabbit anti-
214 glutenin or rabbit anti-BiP antibody). The protoplasts were rinsed quickly with 100 µl of IL
215 buffer, and washes were repeated three times for 10 min each. After removing supernatant,
216 the secondary antibody was added at a 1/100 dilution (Alexa Fluor 546 goat anti-rabbit) for 1
217 h at room temperature. The protoplasts were quickly rinsed with 100 µl of IL buffer, and the
218 washes were repeated three times for 10 min each with 100 µl of fresh IL buffer. Cells were
219 analyzed with a Zeiss LSM 410 microscope at 546 nm excitation for Alexa Fluor 546.
220 Emissions were detected using a 550–614 nm band-pass filter.

221

222 Confocal Imaging

223

224 Confocal microscopy experiments were performed with the facilities of platform
225 Biopolymers, Interactions and Structural Biology, INRA Nantes. An inverted Zeiss LSM 410
226 Axiovert confocal laser scanning microscope was used to examine the subcellular localization
227 of GFP fluorescence. The samples were examined with a water-immersed x40/NA objective.
228 GFP was excited at 488 nm with a blue argon ion laser, and emission was collected via a
229 photomultiplier through a 522-nm band-pass filter. Alexa Fluor 546 was excited at 546 nm.
230 Emissions were detected using a 550–614 nm band-pass filter.

231

232 **Results**

233

234 Construction of glutenin fluorescent proteins

235

236 HMW glutenin subunit (GS) 1Dy10 (Fig. 1a), has a signal peptide consisting of the first 21
237 amino acids, which is cleaved off during protein synthesis in the endosperm of wheat. It is
238 highly homologous with the signal peptides of other HMW GS (Kreis et al. 1985; Halford et
239 al. 1987). The primary structure of the mature 1Dy10 subunit consists of three domains, a
240 non-repetitive N-terminal domain of 104 residues, a repetitive central domain of 502 residues
241 and a non-repetitive C-terminal domain of 42 residues. Seven cysteine residues are present in
242 1Dy10 with five in the N-terminal region, one in the C-terminal region and one at the end of
243 the repetitive region. The central repetitive domain of 1Dy10 consists of nonapeptides
244 (consensus GYYPTSLQQ), hexapeptides (consensus PGQGQQ), and tripeptide (GQQ).

245 The hydrophobic domains responsible for elasticity of elastin contain a repeated motif
246 VPGVG (Urry and Parker 2002). To modify the elasticity of the HMW glutenin, 40 tandem
247 repeats of the elastin-like peptide motif VPGVG (ELPx40) (Fig. 1b) were inserted in the
248 HMW glutenin sequence. Because the ELPx40 sequence was strictly repeated, PCR
249 technology could not be used for the cloning and insertion into GS sequence, so cloning with
250 restriction sites was used.

251 In order to introduce the ELPx40 sequence into the GS repetitive domain, the GS
252 cDNA was modified by directed mutagenesis to insert one BglII restriction site. Because of
253 the high degree of repetition in this domain, its modification by PCR resulted in the loss of
254 315 bp of repeated domain and in a new and unique restriction site BglII. This unique BglII
255 site was used to introduce the ELPx40 nucleotide sequence of 630 pb in the modified GS
256 (GS_M). The GS_M protein therefore consisted of 105 fewer amino acids than the wild-type GS
257 (GS_W) protein (Fig. 1a between double arrows). This loss was replaced by 210 amino acid of
258 ELPx40 (40xVPGVG, Fig. 1b) in the GS_M-ELP protein. The GS_M sequence was used for
259 protoplasts transformation as control in comparison with the GS_W sequence.

260 The GS_W (Fig. 2a), GS_M (Fig. 2b) and GS_M-ELP (Fig. 2c) nucleotide sequences were
261 obtained without STOP codons and fused upstream of the Green Fluorescent Protein (GFP)
262 sequence in order to allow the accessibility and functionality of the glutenin N-terminal signal
263 peptide. These three sequences were introduced into the pRTd35S-GFP plasmid under the
264 control of the double cauliflower mosaic virus 35S promoter and terminator. These GFP

265 fusions were expressed in BY-2 protoplasts in order to determine their subcellular
266 localization.

267

268 Expression and localization in BY-2 cells

269

270 We first investigated the possibility to express the GS_W , GS_M and GS_M -ELP proteins in fusion
271 with GFP and then determined their subcellular localization in BY-2 cells. After transient
272 transformation of BY-2 protoplasts and 48 h of incubation in the dark, expression of the
273 fluorescent proteins was observed by confocal microscopy. The efficiency of the transient
274 BY-2 protoplast transformation was checked with the expression of GFP alone (Fig. 3a).
275 Confocal microscopy revealed greater fluorescence of the GFP alone in the nucleus than in
276 the cytoplasm. This agrees with the rapid import of GFP into the nuclear compartment as
277 described previously (Chiu et al. 1996).

278 For each fusion protein, the cellular sections illustrated were chosen after three-
279 dimensional reconstruction of whole BY-2 cells in order to select the confocal plane that best
280 revealed the nuclei and the ER network (Fig. 3b-g).

281 With GS_W -GFP expression, localization of the fluorescent signal was observed to be
282 more concentrated around the nucleus, where the ER network is very dense (Fig. 3b). No
283 fluorescence was observed in the vacuole and the nucleus. In addition, in the confocal plane
284 passing through the protoplast surface, the fluorescence signal formed a network
285 characteristic of the ER (Fig. 3c). In these two planes, a number of discrete spots were
286 observed by the emission of high fluorescence intensity and identified as protein body-like
287 structures. We estimated the size of these as about 0.5 μ m. The probable localization of GS_W -
288 GFP in the ER and the observation of body-like structure in BY-2 protoplasts are consistent
289 with the endogenous trafficking of wheat prolamins in endosperm cells of wheat seeds.

290 The fluorescent signal observed with the expression of the GS_M -GFP in tobacco
291 protoplasts was also concentrated around the nucleus and vacuoles (Fig. 3d) but absent from
292 these compartments. On peripheral cell focal plane, the fluorescent signal strongly suggested
293 localization of GS_M -GFP in the ER network (Fig. 3e). On the other hand, the fusion protein
294 was mainly not aggregated into protein body-like structures (PB-like structures), contrasting
295 with those observed with the full length GS_W -GFP fusion protein (Table 1, Fig. 4).

296 Expression of the GS_M -ELP-GFP fusion protein also showed localization of the
297 fluorescent signal around the nucleus and vacuole, and organized into the ER network (Fig.
298 3f, g). PB-like structures were present in cells expressing the GS_M -ELP-GFP protein.

299 Although we observed a certain disparity between the different cells, PB-like structures were
300 generally more numerous than those bodies observed with GS_M-GFP expression. However,
301 they were less numerous, smaller and less individualized in comparison with those observed
302 with GS_W-GFP expression (Table 1, Fig. 4). Interestingly the fluorescence was low with the
303 GS_M-ELP-GFP construct compared to those obtained with the two others constructs, which
304 could be due to a lower expression level (Fig. 5).

305

306 Checking *in vivo* integrity of fusion between GS proteins and GFP

307

308 In order to ensure that the observed green fluorescent signal corresponded to GS fusion
309 proteins and to ensure of the integrity of the fusion proteins, we carried out an
310 immunolocalization using specific HMW glutenin subunit (HMW GS) antibodies. Transient
311 transformed protoplasts were first fixed before being subjected to immunolocalization (see
312 Material and Methods).

313 GFP alone was expressed in BY-2 protoplasts and used as negative control. This
314 showed no fluorescence after hybridization with the HMW GS antibody, which confirmed the
315 absence of endogenous HMW-GS-like sequences in tobacco BY-2 cells and the specificity of
316 the HMW GS antibody (Fig. 6a-c).

317 As previously demonstrated with non-fixed cells, protoplasts expressing all three
318 glutenin fusion proteins (GS_W-GFP, GS_M-GFP and GS_M-ELP-GFP) showed that the
319 fluorescent signal was more concentrated around the nucleus and at the cell periphery,
320 strongly suggesting an ER localization (Fig. 6e, h, k). No fluorescence was present in the
321 vacuole and the nucleus compartments. Some protein body-like structures were also observed
322 with the GS_W-GFP but not in a significant manner with GS_M-ELP-GFP and GS_M-GFP
323 expression. In this experiment, cells were submitted to a fixation treatment before being
324 immunolabelled, which could explain that PB-like structures were less visible than in living
325 cells, especially with GS_M-GFP and GS_M-ELP-GFP constructs with which PB-like structures
326 were seen to be less numerous and smaller than with GS_W-GFP. In addition, weaker
327 fluorescence intensity was also observed in the case of the GS_M-ELP-GFP as shown
328 previously.

329 After immunolocalization with the HMW GS antibody, protoplasts transformed with
330 the three GS constructs showed the same pattern of fluorescence as that obtained with the
331 GFP fluorescence (Fig. 6d, g, j), and merging of these patterns showed perfect co-localization

332 between the green fluorescence fusion protein and the HMW GS antibody hybridization (Fig.
333 6f, i, l). This confirmed that the integrity of fusion proteins was preserved.

334

335 Co-localization of the GS fusion protein with BiP

336

337 In order to confirm that the fluorescence observed with all the GS constructs was specifically
338 localized in the ER and therefore whether the 1Dy10 HMW glutenin and its variants were
339 retained in the ER or transported out of this compartment when expressed in tobacco
340 protoplasts, we performed immunolocalization with the transformed tobacco protoplasts using
341 a specific ER marker (Fig. 7). We chose to use an anti-BiP antibody as an ER marker because
342 the BiP is a typical ER-resident protein (Napier et al. 1992). BiP is a member of the heat-
343 shock protein-70 family and is involved in the folding and assembly of proteins in the ER.
344 BiP has been implicated in PB biogenesis (Zhang and Boston 1992; Li et al. 1993) and has
345 been shown to interact with storage proteins containing PBs in a manner that is distinct from
346 its normal chaperone activity (Frigerio et al. 2001). It is probable that the folding and
347 assembly of the gluten proteins is assisted by ER luminal proteins including BiP (Grimwade
348 et al. 1996; DuPont et al. 1998).

349 Forty-eight hours after transformation of protoplasts with GS_W-GFP, GS_M-GFP or
350 GS_M-ELP-GFP constructs, the cells were fixed and immunohybridized with anti-BiP
351 antibody. They were then observed by confocal microscopy at two different wavelengths to
352 detect the GFP signal in the green channel (Fig. 7e, h, k) and Alexa Fluor secondary antibody
353 signal for BiP localization in the red channel (Fig. 7d, g, j).

354 As previously, we used the GFP alone as negative control. As foreseen, protoplasts
355 transformed with GFP alone showed a difference in compartmentalization between GFP and
356 BiP protein expression (Fig. 7a, b, c). We observed a clear fluorescent signal in the nucleus
357 and a lower signal in the cytoplasm for GFP while BiP showed no fluorescent signal in the
358 nucleus and a strong signal in ER compartment.

359 The three fluorescent fusion proteins were found to be confined to the ER region,
360 which was also stained by the anti-BiP antibody (Fig. 7). The subcellular co-localization of
361 GS_W-GFP (Fig. 7d- f), GS_M-GFP (Fig. 7g-i) and GS_M-ELP-GFP (Fig. 7j-l) proteins with the
362 BiP therefore confirmed their ER unique localization. As seen previously, PB-like structures
363 were especially formed in protoplasts transformed with GS_W-GFP. These PB-like structures
364 co-localized with the BiP marker, indicating that BiP was also present in these bodies.

365 Whereas the GS_w-GFP protein was essentially present as PB-like structures, the BiP staining
366 is more diffuse in the ER compared to the GS_w-GFP.

367 **Discussion**

368

369 There are currently few reports of prolamin fusions with fluorescent protein (Foresti et al.
370 2008; Saito et al. 2009), although GFP has been widely employed as a marker protein to
371 demonstrate passage of protein through the plant secretory pathway. To our knowledge, this is
372 the first reported example of fluorescent protein fusion with a HMW glutenin. We can
373 conclude that fusion with GFP does not modify the glutenin subcellular localization in our
374 cell model since glutenin fused to fluorescent protein is retained in the ER and forms PB-like
375 structures that are similar to the reports of endogenously expressed prolamins in wheat
376 endosperm cells (Levanony et al. 1992; Shewry et al. 1995). Whereas the size of the PB-like
377 structures formed by GS subunit expression in tobacco cells was smaller than that observed in
378 wheat endosperm cells, they could be compared to those which were present at the early
379 stages of development in wheat seeds (Loussert et al. 2008). These similarities between this
380 heterologous expression system and expression in wheat endosperm show that the expression
381 in tobacco protoplast is a suitable model to study the HMW glutenin subunit trafficking.
382 Moreover, the fusion protein GS-GFP retained its integrity during its trafficking in tobacco
383 cells, since we demonstrated a perfect co-localization of the immunolabelling with glutenin
384 and GFP antibodies in the transformed tobacco protoplasts.

385 As described in this work, the GS_W-GFP, GS_M-GFP and GS_M-ELP-GFP fusion
386 proteins are located in the ER, despite the differences in the size and sequence of the repeated
387 domain. However, differences were revealed between their forms of accumulation. After
388 deletion of 105 amino acids from the repeated sequence of the GS_W, the truncated protein
389 seemed to possess a weaker ability to aggregate, since a small amount of fluorescent protein
390 body-like structures was observed. This deletion from the repeated sequence then reduces the
391 ability of the glutenin to form protein body-like structures in the ER. Whereas mechanisms
392 allowing the assembly of prolamins in the ER and the formation of protein bodies are still
393 unknown, our results suggest the importance of an intact repetitive domain for glutenin
394 accumulation. It has been suggested that accumulation in the ER lumen could be a
395 consequence of the ability of glutenins to form insoluble aggregates (Shewry 1999). Non-
396 covalent interactions, notably hydrogen bonds formed by the repeated glutamine-rich domain,
397 may be established, resulting in protein precipitation and the formation of hydrated protein
398 particles (Rubin et al. 1992). Other sequences present in cereal storage proteins may favour
399 aggregation and protein body formation. For example, the PPPVHL repeat domain of ZeraTM,
400 derived from maize γ -zein (Torrent et al. 2009), adopts a polyproline II conformation forming

401 an extended amphipathic helix, which is able to self-assemble and form cylindrical micelles
402 (Kogan et al. 2002). This intramolecular interaction among seed storage proteins appears to
403 be essential for their aggregation and the biogenesis of PBs (Kogan et al. 2001). Our study
404 therefore suggested that the decrease in GS_M-GFP protein bodies could be due to the loss of
405 repetitive motifs in the glutenin sequence, and thus the ability to form hydrogen bonds, as
406 suggested by Wan et al. (2005) and Feeney et al. (2003).

407 The GS_M-ELP-GFP protein contains 40 repeats of the VPGVG pentapeptide replacing
408 the 105 amino acids lost in the GS_M-GFP protein. While we observed a cell-to-cell variation
409 in the subcellular distribution of the fusion proteins, our experimental and quantitative data
410 supported the fact that in a general manner, more protein body-like structures were observed
411 in the case of GS_M-ELP-GFP expression compared to the GS_M-GFP expression. This result
412 suggested that the addition of 40xELP to the GS_M sequence seemed to improve its ability to
413 aggregate. A recent report also showed that an elastin-like polypeptide (28xVPGVG) tagged
414 with KDEL (an ER retention signal), significantly enhanced accumulation of recombinant
415 protein in the ER (Conley et al. 2009, for review see Floss et al. 2010). They suggested that
416 this induction of PB formation resulted from the accumulation of heterologous protein
417 reaching a critical local concentration exceeding the normal solubility limit, which
418 subsequently triggered the aggregation and assembly into spherical PBs. Floss et al. (2009)
419 investigated the localization of recombinant antibodies fused to 100xELP after seed-specific
420 expression in tobacco. Complete full-length ELPylated recombinant antibodies were localized
421 in small, putative ER-derived protein bodies found throughout the cytosol but also in the
422 protein storage vacuole. An ELPylated scFv expressed in seeds was shown to form protein
423 bodies in the cytoplasm. The authors hypothesize that ELP retard the trafficking of the fusion
424 proteins along the endomembrane system. Furthermore, the ELPs are hydrophobic sequences
425 already reported to possess autoassembly, properties responsible in part for its entropic elastic
426 force (Urry and Parker, 2002). The relatively high content of hydrophobic amino acids
427 suggests that ELPs may aggregate with themselves as a means of avoiding the hydrophilic
428 conditions in the aqueous lumen of the ER to protect the hydrophobic ELP from the aqueous
429 phase, as seen previously in the case of maize zein (Kogan et al. 2002). Analogous to seed
430 storage proteins, mammalian-derived ELP also possesses the ability to self-aggregate and
431 undergo co-acervation *via* an ordered process, leading to the formation of a stable
432 supramolecular structure (Miao et al. 2003). As the temperature rises, the soluble ELPs
433 polypeptide collapses from an extended chain into an insoluble twisted filament structure
434 consisting of β -spirals comprising type II β -turns (Reiersen et al. 1998; Lee et al. 2001). This

435 process could be also explained by the development of closer associations between single β -
436 spiral strands of ELP and the subsequent formation of interstrand β -sheet structures. These
437 structures might mediate close association or aggregation of different ELP chains (Serrano et
438 al. 2007). The temperature used for plant cells culture by Conley et al. (2009) should not have
439 induced the aggregation of a 28xELP tag, however, the concentration of ELP is also known to
440 affect the precipitation behaviour (Meyer and Chilkoti 2004; Ge and Filipe 2006). Although
441 not proven, high local concentrations and long stretches of ELP-fusion proteins in the ER may
442 play a role in their aggregation and subsequent formation into novel PBs.

443 The GS_M-ELP-GFP protein seemed to form protein body-like structures but these were
444 smaller and less numerous than those formed with GS_W-GFP protein expression. In our study,
445 the propensity of ELP to induce PB-like structures seemed to be partially restored but could
446 not completely compensate for the ability of the GS_W-GFP protein to form PB-like structures.
447 The ELP motifs were inserted into the repeated sequence of the glutenin. This localization
448 may explain the less numerous PB-like structures with the chimeric protein because in this
449 case the ELP motifs were in a specific environment that could influence its structure and
450 physical-chemical properties. The expected physical-chemical properties of the GS_M-ELP-
451 GFP triblock protein could induce an aggregate structure that differs from that adopted by the
452 wild-type glutenin subunit. Moreover we used a double 35S promoter that leads to an
453 expression level weaker than in the work of Conley (2009), where they used the p19
454 suppressor of gene silencing, which has been found to significantly increase the production
455 levels of recombinant proteins in plants (Voinnet et al. 2003; Sudarshana et al. 2006). The
456 expression level of the 28xELP-KDEL was enhanced in the presence of p19 by approximately
457 30-fold after agroinfiltration in tobacco leaves compared to the expression without p19
458 (Conley et al. 2009). The use of p19 suppressor could lead to an increase in the expression
459 level of our chimerical protein in tobacco cells and enhance the PB-like structures formation,
460 in order to produce the GS_M-ELP protein and study its mechanical properties.

461 We also noted a lower fluorescence level for the protoplasts transformed with the
462 GS_M-ELP-GFP construct compared to the protoplasts expressing the wild GS_W-GFP and
463 GS_M-GFP, which could be related to the protein stability. This is the first example where the
464 fusion of a protein with an ELP sequence reduces the accumulation level of the fusion protein.
465 However, in contrast to other ELP fusion proteins described in the literature, the ELP
466 sequence in our case was located into the glutenin sequence and not at its extremity, the
467 objective being to obtain a triblock protein. This could explain the relatively low level of
468 glutenin-ELP-GFP expression. Indeed, the protein stability of GS_M-ELP-GFP protein could

469 be disturbed by the presence of exogenous sequences inside the glutenin sequence, which can
470 induce ER-associated protein degradation (ERAD). This quality control in the ER prevents
471 the arrival of incorrectly or incompletely folded proteins at their final destination and targets
472 permanently misfolded proteins for degradation. Such proteins have a high affinity for the ER
473 chaperone BiP and are finally degraded via retrograde translocation from ER lumen back to
474 the cytosol. ERAD is currently thought to constitute the main disposal route for misfolded
475 proteins (Anelli and Sitia 2008). The protein degradability hypothesis could explain the low
476 fluorescence and low amount of PB-like structures observed with the GS_M-ELP-GFP protein
477 expression. Further investigations could focus on the location of ELP sequences at an
478 extremity of the glutenin, and determine the expression level and PB formation resulting from
479 such a fusion protein.

480 The ER-resident chaperone BiP has been shown to be associated with prolamin PBs in
481 rice (Muench et al. 1997) and wheat (Levanony et al. 1992). In addition, BiP has been
482 suggested to be involved in PB biogenesis because of its role in retention of prolamins in the
483 ER lumen by facilitating their folding and assembly into insoluble PBs in developing seeds
484 (Zhang and Boston 1992; Li et al. 1993) and leaves of transgenic plants (Bagga et al. 1997;
485 Mainieri et al. 2004). Previous studies identified specific BiP binding motifs within storage
486 proteins, such as zein (Randall et al. 2005) and phaseolin (Foresti et al. 2003), using BiP
487 scoring software (Blond-Elguindi et al. 1993). Furthermore, BiP has been shown to interact
488 with seed storage proteins in a specific manner, which is unique from its normal chaperone
489 activity (Mainieri et al. 2004; Frigerio et al. 2001; Randall et al. 2005). Although Conley et al.
490 (2009) identified a strong BiP-binding motif repeated throughout the ELP sequence, the co-
491 immunoprecipitation analysis revealed no specific interactions between ELP and the ER-
492 chaperone BiP. In the present study, BiP was co-localized with the different fusion proteins,
493 suggesting that BiP may play a role in the trafficking of the GS variants in tobacco
494 protoplasts, as discussed previously (Zhang and Boston 1992; Li et al. 1993). Further
495 investigations could focus on the involvement of BiP protein and other proteins such as the
496 protein disulphide isomerase, which were suggested to play a major role in the prolamin
497 assembly during their trafficking.

498

499 To conclude, in addition to show the ER localization and the formation of PB-like
500 structures when an HMW-GS of wheat seed was expressed in fusion with a fluorescent
501 protein in tobacco cells model, we demonstrated the expression of a new chimeric triblock
502 protein based on glutenin and elastin-like motifs and showed its subcellular localization in

503 tobacco cells. These experiments open the way to investigate the possibility to perform high
504 level production of this triblock protein in order to conduct structural and functional analysis,
505 and to characterize its specific mechanical and *ex vivo* associative properties.

506

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Figure legends

Fig. 1 1Dy10 subunit. **a** Amino acid sequence of 1Dy10 (GS_W) protein, the signal peptide and non-repeated sequence are framed respectively by a discontinue line and by a continue line. The repeated domain is underlined. The deleted sequence of the 1Dy10 mutated protein (GS_M) is localized between double arrows. **b** Amino acid sequence of ELP introduced into GS_M protein in replacement of sequence deleted between double arrows of amino acid sequence of GS_W protein (**a**).

Fig. 2 Schematic representation of the expression cassettes and designation of chimerical proteins expressed in transient transformed tobacco cells. **a** GS_W-GFP: 1Dy10 protein composed of a long repeated domain and flanked by two non repeated domains in N and C terminal extremities of 1Dy10 with respectively 5 and 2 cysteines (short lines). **b** GS_M-GFP resulting of a loss of sequence represented between two double arrows on A. into the repeated domain by directed mutagenesis to introduce a restriction site. **c** GS_M-ELP-GFP results of ELP (Elastin Like-Peptide) coding for 40 VPGVG repetitions introduced into the repeated domain of mutated Dy10 using the new restriction site BglII. These three proteins were fused to GFP protein at their C-terminal extremity and placed under the control of cauliflower mosaic virus 35S promoter and in the upstream cauliflower mosaic virus 35S terminator.

Fig. 3 Expression and subcellular localization of glutenin subunits in BY2 cells. Tobacco protoplasts were transiently transformed with a plasmid encoding the GFP (**a**), the GS_W-GFP (**b**, **c**), the GS_M-GFP (**d**, **e**) or the GS_M-ELP-GFP (**f**, **g**) proteins and observed after 48 h transfection. The subcellular distribution of these proteins was examined by confocal laser scanning microscopy using the 488-nm blue line excitation, and emission of the fluorescence was collected via a photomultiplier through a 522-nm band-pass filter. The subcellular distribution was observed with two different confocal plans crossing the cells surface (**c**, **e**, **g**) or into the cells through the nucleus (**b**, **d**, **f**). **n**, nucleus; **v**, central vacuole. Scale bar = 20 μ m. *Experiment repetitions among $n \geq 3$.*

Fig. 4 Confocal image illustrations corresponding to the different cell categories defined in Table 1 ((a): numerous and well defined PB-like structures; (b): few and/or less individualized PB-like structures; (c): no PB-like structures) for each construct.

Fig. 5 Fluorescence intensity quantification from cells expressing the chimerical proteins.

Fluorescence intensities were measured from confocal images of BY-2 protoplasts expressing GS_W-GFP, GS_M-GFP or GS_M-ELP-GFP proteins. They are expressed as the gray level mean per pixel.

Fig. 6 *In vivo* checking of fluorescence signal specificity due to fusion between the Dy10 chimerical protein and the GFP protein. Tobacco protoplasts were transiently transformed with a plasmid encoding the GFP (**a, b, c**), the GS_W-GFP (**d-f**), the GS_M-GFP (**g-i**) or the GS_M-ELP-GFP (**j-l**) proteins and fixed with 3% paraformaldehyde after 48 h transfection. The subcellular distribution of GFP protein and GS wild-type or mutated proteins was examined by confocal laser scanning microscopy using a combination of a rabbit anti-HMW antiserum. **a, d, g** and **j**: Reticular fluorescence of GS visualized by the anti-HMW and Alexa Fluor 546 goat anti-rabbit secondary antibody. **b, e, h** and **k**: Distribution pattern of the GFP fluorescent protein observed by confocal laser scanning microscopy using the 488-nm blue line excitation, and emission of the fluorescence was collected via a photomultiplier through a 522-nm band-pass filter. **c, f, i** and **l**: Overlaid images respectively of **a** (red) + **b** (green), **d** (red) + **e** (green), **g** (red) + **h** (green), **j** (red) + **k** (green), where the yellow color revealed the co-localization of green and red colors. **n**, nucleus; **v**, central vacuole. Scale bar = 20 μ m. *Experiment repetitions among $n \geq 3$.*

Fig. 7 Co-localization of GS-GFP fusion proteins with an endoplasmic reticulum marker, the protein BiP. Tobacco protoplasts were transiently transformed with a plasmid encoding the GFP (**a-c**), the GS_W-GFP (**d-f**), the GS_M-GFP (**g-i**) or the GS_M-ELP-GFP (**j-l**) proteins and fixed with 3% paraformaldehyde after 48 h transfection. The subcellular distribution of fusion GFP-proteins and ER marker BiP was examined by confocal laser scanning microscopy using a combination of a rabbit anti-BiP antiserum. **a, d, g** and **j**: Reticular fluorescence of endogenous BiP visualized by the anti-BiP and Alexa Fluor 546 goat anti-rabbit secondary antibody. **b, e, h** and **k**: Distribution pattern of the heterologous fluorescent proteins observed by confocal laser scanning microscopy using the 488-nm blue line excitation, and emission of the fluorescence was collected via a photomultiplier through a 522-nm band-pass filter. **c, f, i** and **l**: Overlaid images respectively of **a** (red) + **b** (green), **d** (red) + **e** (green), **g** (red) + **h** (green), **j** (red) + **k** (green), where the yellow color revealed the co-localization of green and red colors. **n**, Nucleus; **v**, central vacuole. Scale bar = 20 μ m. *Experiment repetitions among $n \geq 3$.*

Table

Table 1 Quantification of the transformed cells containing protein body-like structures

	cells containing:		
	numerous well defined PBs (a)	few and/or less individualized PBs (b)	no PBs (c)
G _{S_W} -GFP	100%	0%	0%
G _{S_M} -GFP	16.7%	33.3%	50%
G _{S_M} -ELP-GFP	33.3%	44.5%	22.2%

Analyzed cells were classified into three categories according to the protein body-like structures (PBs) they contained: the first category corresponds to cells containing numerous and well-defined PB-like structures; the second category corresponds to cells containing few and/or less individualized PB-like structures; and the third category corresponds to cells without PB-like structures.

Cells were provided from at least three individual protoplast transformation experiments, transformed with each of the three constructs. A total of 51, 48 and 54 cells were examined for the G_{S_W}-GFP, G_{S_M}-ELP and G_{S_M}-ELP-GFP constructs respectively.

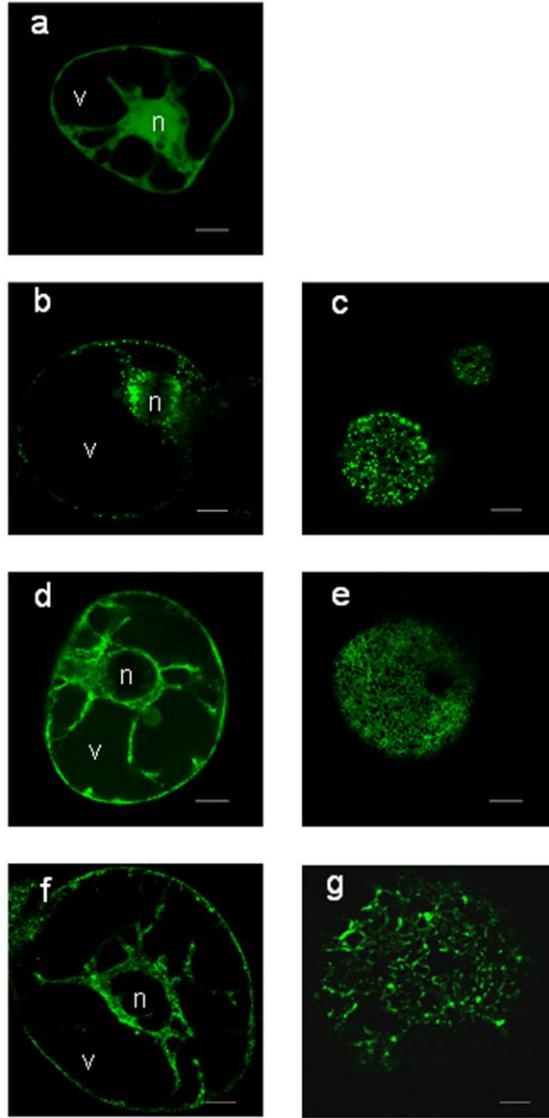


Figure 3

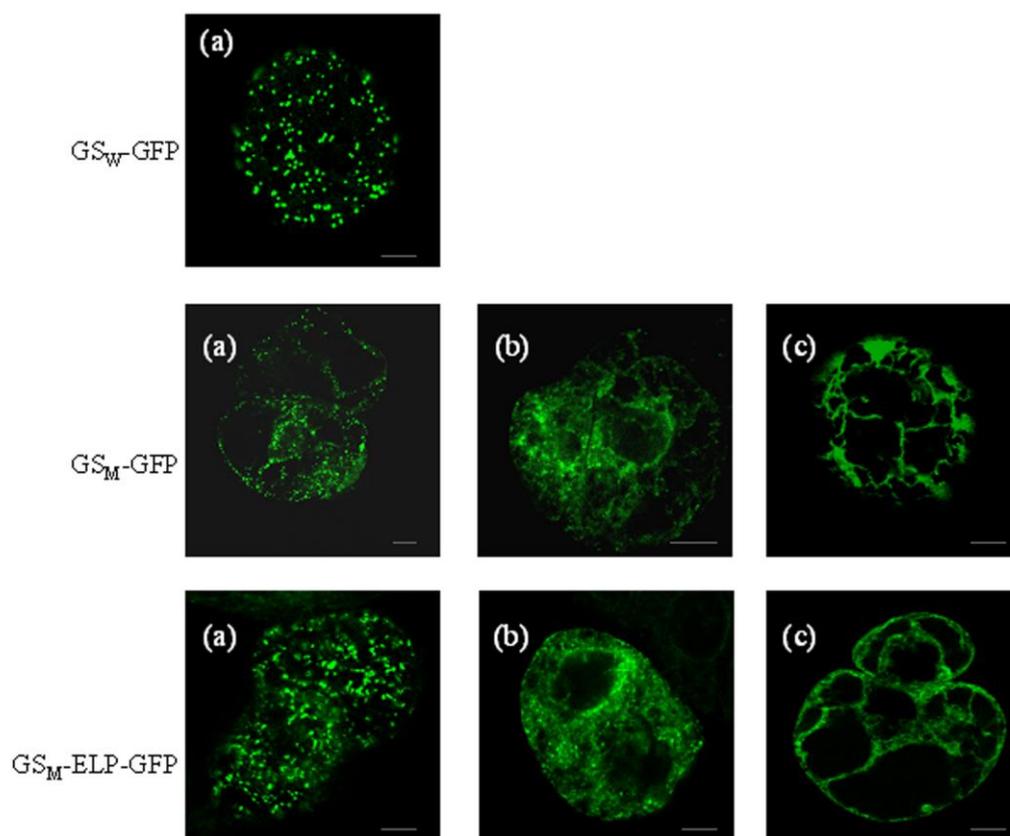


Figure 4

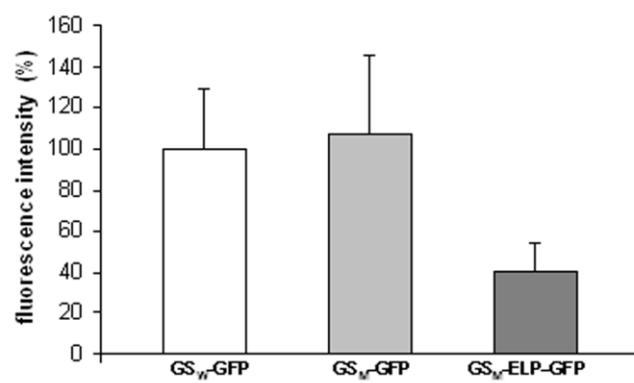


Figure 5

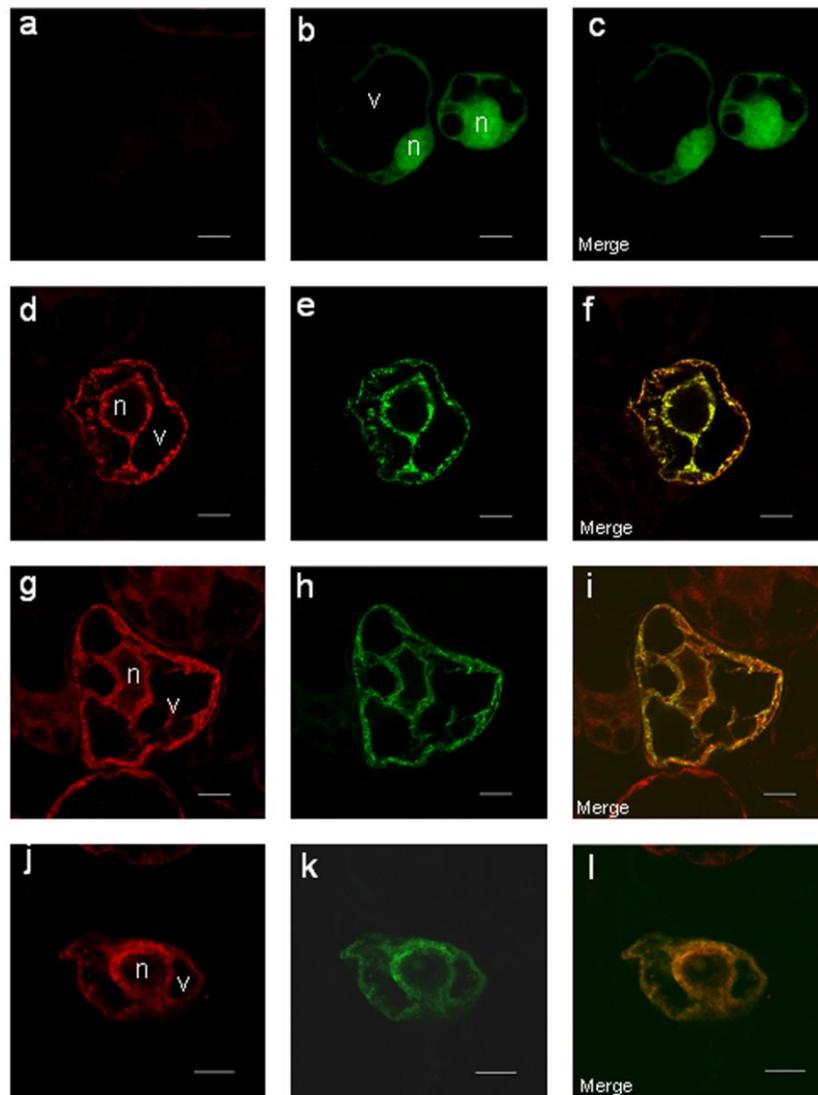


Figure 6

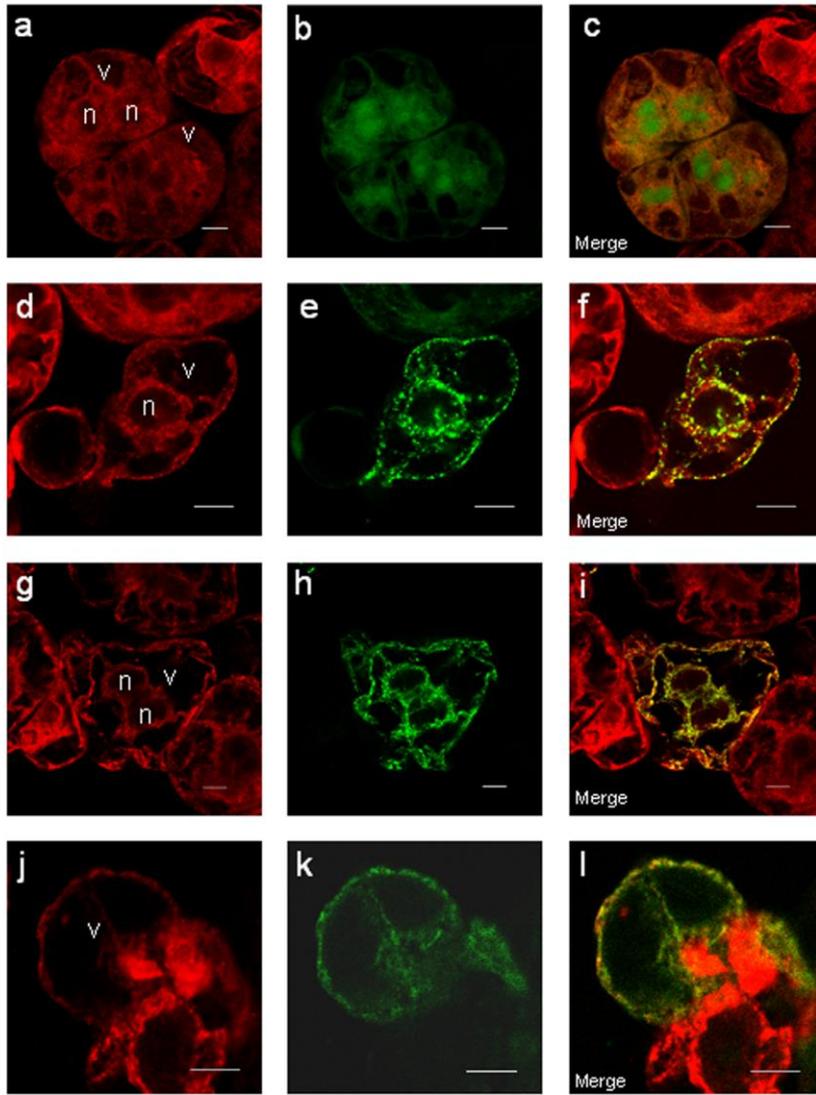


Figure 7