Gene-gene interactions between mutants that accumulate abnormally high amounts of proglutelin in rice seed

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We had previously identified eight mutants, esp2 and glut1 to 7, which accumulated abnormally high amounts of proglutelin, the major storage protein in rice seeds. Analysis of their seed proteins by SDS-PAGE, their levels of the luminal chaperone BiP and gene-gene interactions indicated that these mutants fell into four classes. The most epistatic class consisted of esp2, which encodes a defective protein disulfide isomerase (PDI). A second class consisting of Glup1, Glup2 and glup7 was hypostatic to esp2, and showed abnormally high levels of BiP, suggesting that maturation and export of proglutelins from the ER are inhibited in this class of mutants. The third class containing glup4, Glup5 and glup6 mutations was hypostatic to esp2, Glup1, Glup2 and glup7. Since the glut4 allele encodes the small GTPase Rab5a, which participates in the trafficking of proglutelin from Golgi apparatus to the protein storage vacuole (PSV), this third class of mutants is likely affected in this process. Lastly, glut3, which encodes a vacuolar processing enzyme, which proteolytically processes proglutelin into acidic and basic subunits within the PSV, was hypostatic to the other mutants. Overall, these gene relationships are consistent with the sequential intracellular transport and processing of proglutelin and provide novel insights on the trafficking of proglutelin to the PSV.

Key Words: endoplasmic reticulum, genetic analysis, glutelin, intracellular transport, mutant, seed.

Introduction

During seed development, most seed plants preferentially accumulate one type of storage protein: salt-soluble proteins such as the 11S and 7S globulins or alcohol-soluble prolamin in protein storage vacuoles (PSVs). However, rice accumulates three kinds of storage proteins, glutelins, globulins, and prolamins and deposits them into two separate compartments of the endomembrane system (Krishnan and White 1995, Tanaka et al. 1980). Acid soluble glutelins, the dominant storage protein, are deposited together with globulins, a minor storage protein, in PSVs to form PB-II (Krishnan et al. 1992, Tanaka et al. 1980). The glutelin polypeptides are synthesized on the endoplasmic reticulum (ER) membrane as a precursor form (proglutelin) of 57 kilodaltons (kD) (Yamagata et al. 1982). Within the ER, proglutelin forms one or more intracellular disulfide bond (Satoh-Cruz et al. 2010a), is glycosylated (Kishimoto et al. 1999), and assembles to form a tetramer (Sugimoto et al. 1986) before it is exported from the ER to the PSVs (Krishnan et al. 1986, Yamagata and Tanaka 1986), where it is posttranslationally processed to acidic and basic subunits in PSV (Yamagata et al. 1982). These subunits are bound together by an interchain disulfide bond and are accumulated as a hexamer in the PSV (Kumamaru et al. 2010, Sugimoto et al. 1986). The alcohol-soluble prolamins, the second most abundant storage protein in rice, are also synthesized on the ER but are then sequestered within this compartment to form PB-I.

The trafficking of storage protein from the ER to the PSV is mediated by the Golgi apparatus (Chrispeels 1983). In maturing bean and pea cotyledons, the storage proteins are concentrated as dense vesicles which are routinely observed in the vicinity of the Golgi apparatus. In addition, electron-dense inclusions are observed at the ends of the cis-cisternae of the Golgi (Chrispeels 1983, Hohl et al. 1996, Okita and Rogers 1996), suggesting that the dense vesicles are formed very early within the Golgi, which are then released at the trans-cisternae. These dense vesicles are then transported via an intermediate prevacuolar compartment to the PSV (Hinz et al. 2007). Golgi-associated dense vesicles containing glutelin and globulin are readily evident in developing rice endosperm cells supporting a role for the Golgi apparatus in routing these storage proteins to the PSV (Krishnan et al. 1990, 1992). An alternative direct ER to PSV pathway
bypassing the Golgi (Hara-Nishimura et al. 1998) has also been proposed in rice (Takahashi et al. 2005). However, other than this single study, PAC-like vesicles similar to that observed in pumpkin have not been seen in other microscopic studies on rice protein body biogenesis (Krishnan et al. 1990, 1992, Ogawa et al. 1989).

Many factors are involved in the synthesis, intracellular transport, and accumulation of glutelin within the PSV. As proglutelin is initially synthesized on the ER as a larger precursor form, proglutelin, which is then proteolytically processed in acidic and basic subunits at its final destination the PSV, anymutations affecting the intracellular trafficking of glutelin to the PSV and its processing at this site would be predicted to accumulate proglutelin in the cell. Analysis of mutants, which over-accumulate proglutelin, will aid in identifying these factors and in elucidating their function.

We have characterized eight independent rice mutants that accumulate large quantities of the 57-kD proglutelin polypeptide. The mutants were named as endosperm storage protein mutant 2 (esp2) (Kumamaru et al. 1987, 1988) or glutelin precursor mutant (glup) 1 to 7 (Kumamaru et al. 2007, Satoh et al. 1994, 1995, 1999, Tian et al. 2001, Ueda et al. 2004a, 2004b). The genes for three of these mutants esp2, glup3 and glup4 have been identified. TheEsp2 gene encodes the protein disulfide isomerase 1-1 (PDI 1-1), which catalyzes the formation of intramolecular disulfide bonds and serves as a molecular chaperone within the endoplasmic reticulum (Satoh-Cruz et al. 2010a, Takemoto et al. 2002). The Glup3 gene encodes a vacuolar processing enzyme (VPE), an activity which proteolytically processes proglutelin into acidic and basic subunits within the PSV (Kumamaru et al. 2010), while Glup4 gene encodes the small GTPase Rab5a, which participates in the trafficking of proglutelin from Golgi apparatus to the PSV (Satoh-Cruz et al. 2010b). The identification of the genes responsible for the other glup mutations and where they function in the synthesis, the transport, and the accumulation of the proglutelin remain to be resolved.

In order to elucidate the genetic control of processes centering on the biosynthesis, intracellular transport, and accumulation of glutelin, gene-gene interactions among the various proglutelin accumulating mutants were analyzed. Such genetic relationships together with storage protein and BiP accumulation patterns indicate that the eight proglutelin-overaccumulating lines fall into four classes and provide new insights on the processes affected during the trafficking of proglutelin to the PSV.

Materials and Methods

Plant Material

The highly proglutelin over-accumulating mutant lines, CM1787 and EM44 (esp2), EM61 (Glup1), EM305 (glup2), EM856 (glup3), EM956 (glup4), EM675 (Glup5), EM939 (glup6), and CM935 (glup7), produced by N-methyl-N-nitrosourea (MNU) mutagenesis and the Chinese variety HO1084 (glup3) (Kumamaru et al. 1987, 1988, 2010, Satoh et al. 1994, 1999, Tian et al. 2001, Ueda et al. 2004a, 2004b) were used as materials (Fig. 1).

To investigate the gene-gene interaction between the proglutelin mutants, crossing were made among the eight mutant lines. F1 plants were grown in the field and the F2 seeds were obtained from the self-pollination of progeny from the crosses between the mutant lines. The proteins from individual F2 seeds were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Western analyses

SDS-PAGE of the crude protein extract from individual F2 seeds and western blotting were performed as described previously (Kumamaru et al. 1990, Takemoto et al. 2002). Storage proteins from mature seeds were extracted with 700 μl of 4% (w/v) SDS, 4 M urea, 5% (v/v) 2-mecaptoethanol (ME), and 0.125 M Tris-HCl, pH 6.8. Five μl of extract was resolved on 14% polyacrylamide gels as described by Laemmli (1970). After electrophoresis, gels were stained with Coomassie Brilliant Blue. For western-blot analysis, proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes, which were then
incubated with various antibodies. Antibody-antigen reactions were visualized using a commercial ECL detection kit (GE Healthcare, Slough, UK).

**Antibody**

An antibody against BiP was raised against a 14 amino acid synthetic peptide of rice BiP as described in Satoh-Cruz et al. (2010a).

**Results**

Fig. 1 shows the distribution of seed proteins as viewed by SDS-PAGE (A) and of proglutelin as determined by immunoblot analysis (B) of seed extracts from the 8 independent mutants which over-accumulate proglutelin. In all mutants, the amounts of 57-kD proglutelin protein are significantly larger than wild-type (WT) (Fig. 1A). Immunobloting analysis using the antibody against the glutelin acidic subunit confirms that the amounts of proglutelin were increased in these 8 mutant lines (Fig. 1B).

Although these 8 mutant lines shared a common phenotype of over-accumulating proglutelin levels compared to WT, there were differences in the relative amounts of proglutelin and glutelin subunits accumulated as well as for the other storage proteins globulin and prolamin. In addition to excess proglutelin amounts, esp2 showed reduced levels of acidic and basic glutelin subunits, the various prolamin polypeptides, and the 26 kD α-globulin compared to WT. The glup4, Glup5 and glup6 seed proteins show a protein pattern similar to esp2 in having reduced levels of acidic and basic glutelin subunits, prolamins, and the 26 kD α-globulin. However, proglutelin levels in these three glup mutants were significantly lower than esp2 seed. By contrast, the acidic and basic glutelin subunits, all prolamins classes, and the 26 kD α-globulin were nearly the same in Glup1, glup2 and glup7 seeds as in WT. The glup3 seeds show reduced levels of acidic and basic glutelin subunits but normal levels of prolamin.

Protein extracts prepared from F2 seeds derived from the crosses between esp2 line and the seven glup/Glup mutant lines were analyzed by SDS-PAGE (Table 1). In the cross combination with Glup1 line, analysis of the protein patterns by SDS-PAGE indicated that the wild-type, Glup1 type, and esp2 type segregated to 57, 19 and 24, respectively in F2 seeds. This segregation pattern fitted a 3:9:4 ratio and indicated that the esp2 gene is epistatic to the Glup1 mutant gene. Likewise, F2 seeds of the cross combinations of esp2 with glup2, glup3, glup4 and glup6 showed a segregation ratio of 9:3:4 for wild-type, glup mutant type, and esp2 type. Again, esp2 gene is epistatic to these mutant genes. In the cross combination with glup7 line, the segregation of wild-type, glup7, and esp2 types did not fit the 9:3:4 ratio. The number of esp2 type, however, was more than glup7 type with a χ² value of 3.11 (0.10 > P > 0.05), a result indicating that the esp2 gene is epistatic to the glup7 gene. The Glup5 type shows co-dominant inheritance as a cross between wild-type and Glup5 lines yields a 1:1 ratio (Ueda et al. 2004a, Supplemental Table 1). In the cross combination with Glup5 and esp2 lines, wild-type, Glup5, and esp2 types in the F2 seeds segregated to 27, 36 and 27, respectively. The segregation distribution fitted the expected 6:6:4 ratio significantly, indicating that the esp2 gene is epistatic to the Glup5 gene. Overall, these gene-gene interaction results indicate that esp2 gene is epistatic to all of the glup genes.

The esp2 seeds lack the ER-localized PDI1-1 which results in the accumulation of mis-localized proglutelin with prolamins within the ER (Satoh-Cruz et al. 2010a, Takemoto et al. 2002). Hence, the epistatic nature of esp2 to the other glup genes supports the view that PDI1-1 functions early in the maturation and trafficking of proglutelins and that disulfide-bond formation with the ER is one of the first events of proglutelin maturation (Satoh-Cruz et al. 2010a). Moreover, the hypostatic relationship of the seven glup/ Glup mutant lines to esp2 suggests that these genes are involved in maturation steps subsequent to disulfide bond formation within the ER or downstream transport processes beginning with ER export.

When malformed proteins accumulate in the ER lumen, the unfolded protein response is generated where molecular chaperones such as BiP are induced (Okushima et al. 2002). In esp2, BiP levels are significantly elevated (Takemoto et al. 2002). Therefore, to determine whether the unfolded protein response was induced in the Glup/glup mutants we assessed the relative levels of BiP accumulated in their seeds compared to WT. Fig. 2 shows results of an immunoblot

### Table 1. Segregation of phenotypes in F1 and F2 seeds derived from the cross between esp2 line and other proglutelin accumulating mutant lines

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>Phenotype of F1 seeds</th>
<th>Segregation types in F2 seeds</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant type</td>
<td>esp2 type</td>
</tr>
<tr>
<td>EM61 (Glup1) × CM1787 (esp2)</td>
<td>19</td>
<td>57</td>
<td>24</td>
</tr>
<tr>
<td>EM305 (glup2) × CM1787 (esp2)</td>
<td>52</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>HO1055 (glup3) × CM1787 (esp2)</td>
<td>112</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td>EM956 (glup4) × EM44 (esp2)</td>
<td>43</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>EM675 (Glup5) × CM1787 (esp2)</td>
<td>27</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td>EM939 (glup6) × CM1787 (esp2)</td>
<td>36</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>CM935 (glup7) × PM162 (esp2)</td>
<td>65</td>
<td>15</td>
<td>20</td>
</tr>
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</table>

*a the type of the counterpart parent with esp2 line.
analysis of seed protein extracted from the seven mutants using BiP antibody. BiP levels in glup4, Glup5 and glup6 lines were similar to that detected in WT, while those in glup7 were noticeably higher. By contrast, Glup1 and glup2 showed significantly elevated BiP levels similar to that observed in esp2. These results indicate that the over-accumulation of proglutelin within ER induces an unfolded protein response in Glup1, glup2 and glup7. Therefore, these lines contain mutations that likely affect the folding of proglutelin within the ER whereas the remaining glup/Glup mutations affect processes at or downstream of ER export.

The relative distributions of storage proteins (Fig. 1) and BiP (Fig. 2) suggest that the glup/Glup mutants fall into two separate classes. The glup types are grouped together. Moreover, some progeny in the F2 population showed the ‘additive’ phenotype where proglutelin levels were significantly higher than the parents. As described earlier, Glup5 shows co-dominant inheritance, a trait which complicated the analysis. In F2 seeds of the cross between glup4 and Glup5 lines, the segregation of wild-type, glup4/Glup5 type, and additive type fit the ratio of 6 : 8 : 2 significantly. In the cross between glup4 and glup6 lines, the segregation of wild-type, glup4/glup6 type, and additive type fit the ratio of 9 : 6 : 1 significantly. Although a clear result could not be obtained in the study of progeny generated from the cross between Glup5 and glup6, the results suggest that glup4, Glup5 and glup6 genes act additively and the products from these genes have a similar function or participate in same process during the synthesis, the intracellular transport, and the accumulation of the proglutelin.

In glup4 lines, the small GTPase Rab5a was rendered non-functional (Satoh-Cruz et al. 2010b). The available evidence indicates that Rab5a participates in the trafficking process in the ER.

Table 2. Segregation of phenotypes in F1 and F2 seeds derived from the cross between Glup1, glup2 and glup7 lines

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>Segregation types in F2 seeds</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant type</td>
</tr>
<tr>
<td>CM935 (glup7) × EM61 (Glup1)</td>
<td>Mutant type</td>
<td>24</td>
</tr>
<tr>
<td>CM935 (glup7) × EM305 (glup2)</td>
<td>Wild type</td>
<td>67</td>
</tr>
</tbody>
</table>

⁺ Glup1 or glup7 types, ² glup4 or glup2 types.

These mutant types could not be distinguished. AD indicates an additive effect where higher proglutelin levels are evident than either parent.

Table 3. Segregation of phenotypes in F1 and F2 seeds derived from the cross between glup4, Glup5 and glup6 lines

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>Segregation types in F2 seeds</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant type</td>
</tr>
<tr>
<td>EM956 (glup4) × EM675 (Glup5)</td>
<td>Mutant type</td>
<td>60</td>
</tr>
<tr>
<td>EM956 (glup4) × EM939 (glup6)</td>
<td>Wild type</td>
<td>34</td>
</tr>
</tbody>
</table>

⁺ Glup5 or glup4 types, ² glup4 or glup6 types.

These mutant types could not be distinguished. AD indicates an additive effect where higher proglutelin levels are evident than either parent.
of proglutelin from Golgi apparatus to PSV. Therefore, our gene-gene interaction results suggest that Glup5 and glup6 mutations also interfere with the transport of proglutelin from the Golgi apparatus to PSV.

To determine the genetic relationship between the two classes of Glup1-glup2-glup7 mutants, and glup4-Glup5-glup6 mutants, glup7 and glup4 lines were combined and protein samples of the F2 seeds were analyzed (Table 4). The results showed that the segregation of the wild-type, glup4, and glup7 types did not fit the 9 : 3 : 4 ratio, but the number of the glup7 type was more than glup4 type and the value of  was 4.64 (0.05 > P > 0.01), suggesting that the glup7 gene is epistatic to the glup4 gene. Although analysis of F2 progeny generated from the crosses between glup2 and glup4, or glup2 and glup6 yielded ambiguous results (data not shown), it is highly likely that the class consisting of Glup1-glup2-glup7 is epistatic to the class consisting of glup4-Glup5-glup6 mutants.

Finally, in order to determine the genetic relationship of glup3 to the other glup lines, protein from F2 seeds derived from initial crosses between glup3 and glup7 and between glup3 and glup4 lines were analyzed (Table 5). In both combinations, the segregation of wild-type, glup3 or glup7 types, glup3 type, and intermediate type fitted the expected 9 : 3 : 3 : 1 ratio significantly, suggesting that the intermediate type in both combinations is the double recessive type. Although the interpretation of the segregation of the intermediate type is difficult, since the glup3 mutant results in a loss of VPE activity which is responsible for the proteolytic cleavage of proglutelin within PSV (Kumamaru et al. 2010), the segregation ratio suggests that the cleavage of the proglutelin by VPE is the most downstream on the process in the synthesis, intracellular traffic, and accumulation of the proglutelin.

Based on the preceding results, the genetic relationship among these mutants is summarized in Fig. 3. This relationship suggests that each mutation affects a specific process in the biosynthesis, intracellular trafficking, and accumulation of glutelin from the ER to PSV. Among the mutations, esp2 mutation affects the most upstream process within the ER, the class consisting of Glup1, glup2 and glup7 mutations affect a process downstream of esp2 mutation within the ER, the class consisting of glup4, Glup5 and glup6 mutations located even further downstream with, while the glup3 mutation is located at or near a terminal process within the PSV.

**Discussion**

During seed development, rice glutelin polypeptides are initially synthesized on the ER membrane as a 57-kD proglutelin (Yamagata et al. 1982), which is then transported to the Golgi apparatus and finally to the PSVs (Krishnan et al. 1986, Yamagata and Tanaka 1986), where the proglutelin is cleaved to form acidic and basic subunits (Yamagata et al.
Glup4-Rab5a participates in the intracellular transport of Arabidopsis storage proteins have also been identified by glup6 glutelins from Golgi apparatus to PSV. As protein products may also participate in the trafficking of mulation of foreign protein within the ER induces an unfold-

Ara7, another Arabidopsis Rab5 homologue, is localized to the PVC and Golgi apparatus and acts on the vacuolar trafficking components of intracellular protein trafficking in plants. 

The glup4 mutant, which contains a defective GTPase Rab5a (Satoh-Cruz et al. 2010b) controls an intermediate between esp2 and glup3 (Fig. 3). These facts demonstrate that the gene-gene interaction in the present study and the function of the responsible gene products in other mutants. 

The glup4 mutant encodes a defective small GTPase Rab5a (Satoh-Cruz et al. 2010b). Rha1, an Arabidopsis protein that has amino acid sequence homology with the animal Rab5, is involved specifically in the trafficking of soluble proteins from the prevacuolar compartment (PVC) to the lytic vacuole in Arabidopsis protoplasts (Sohn et al. 2003). Ara7, another Arabidopsis Rab5 homologue, is localized to the PVC and Golgi apparatus and acts on the vacuolar trafficking pathway in tobacco leaf epidermal cells (Kotzer et al. 2004). Immunocytochemical studies have demonstrated that Ara7 and Rha1 have been shown to reside on the multi-vesicular PVC (Haas et al. 2007). Therefore it is likely that Glup4-Rab5a participates in the intracellular transport of proglutelin from Golgi apparatus to PSV. As Glup5 and glup6 mutants belonging to same class as glup4, their coded protein products may also participate in the trafficking of glutelins from Golgi apparatus to the PSV. 

Factors involved in the intracellular transportation of Arabidopsis storage proteins have also been identified by analyzing seed mutant which over-accumulate precursors. An Arabidopsis thaliana mutant, maigo2 (mag2), accumulated the precursors of two major storage proteins, 2S albumin and 12S globulin, in dry seeds. MAG2 functions in the transport of storage protein precursors between the ER and Golgi apparatus in plants (Li et al. 2006). A type I membrane protein, AtVSR1/AtELP, of Arabidopsis functions as a sorting receptor for storage proteins. The atvrs1 seeds have distorted cells and smaller protein storage vacuoles than do WT seeds, and atvrs1 seeds abnormally accumulate the precursors of two major storage proteins, 12S globulin and 2S albumin, together with the mature forms of these proteins (Shimada et al. 2003). These studies including the one described here demonstrate the utility and power of studying mutants that over-accumulate precursor form of the storage protein in identifying components of intracellular protein trafficking in plants. 

The class of Glup1-glup2-glup7 mutant genes is epistatic to the class of glup4-Glup5-glup6 mutant genes. The accumulation of foreign protein within the ER induces an unfold-
ed protein response resulting in the accumulation of the molecular chaperone BiP (Hagan et al. 2003, Takemoto et al. 2002, Yasuda et al. 2006). In esp2 seeds, proglutelin accumulates in the ER accompanied by elevated BiP levels, reflecting an unfolded protein response (Satoh-Cruz et al. 2010a). Glup1, glup2 and glup7 mutants contained high levels of BiP (Fig. 2), indicating that the unfolding protein response by ER stress has been induced. The presence of an unfolded protein response infers that proglutelin has not folded correctly and is retained within the ER in Glup1, glup2 and glup7 seeds. 

The ER is the organelle responsible for proper folding and delivery of proteins into the secretory pathway. Many seed proteins undergo post-translational modifications including glycosylation, folding, and oligomer assembly within ER (Kermode and Bewley 1999, Kostova and Wolf 2003). In addition to the formation of disulfide bond catalyzed by PDI, rice glutelins are O-linked glycosylated (Kishimoto et al. 1999), a modification which may be essential for maturation of the protein and competence for ER export. On the other hand, a direct loss of ER export may also be responsible for the retention of proglutelin within the ER and the induction of the unfolded protein response. Possible candidates for ER export includes those that function in the formation of COPII vesicles which move cargo from the ER to Golgi in eukaryotic cell (Sato and Nakano 2007). The gene products of Glup1, glup2 and glup7 mutants are likely to participate in the post-translational modification of the proglutelin within ER, or on their export from the ER.

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