How is embryo size genetically regulated in rice?

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SUMMARY

It is unclear how embryo size is genetically regulated in plants. Since cereals have a large persisting endosperm, it is expected that embryo size is affected by endosperm development. Nine single recessive mutations, four reduced embryo mutations representing three loci, REDUCED EMBRYO1, REDUCED EMBRYO2 and REDUCED EMBRYO3, four giant embryo mutations derived from a single locus GIANT EMBRYO, and one endospermless mutation endospermless1-2 were analyzed. Every reduced embryo mutation caused reduction of all the embryonic organs including apical meristems and the enlargement of the endosperm. The giant embryo mutants have a reduced endosperm and an enlarged scutellum. However, shoot and radicle sizes were not affected. All the reduced embryo and giant embryo mutations did not largely affect postembryonic development. Accordingly, the expression of genes analyzed are seed-specific. In reduced embryo and giant embryo mutations, abnormalities were detected in both embryo and endosperm as early as 2 days after pollination. endospermless1-1 resulted in an early loss of endosperm, yielding a giant embryo, suggesting that embryo growth was physically limited by the endosperm. A double mutant between giant embryo-2 and club-shaped embryo1-1, which has a normal endosperm and a minute undifferentiated embryo, resulted in a club-shaped embryo1-1 embryo and a reduced endosperm of giant embryo-2, indicating that GIANT EMBRYO regulates the endosperm development. Double mutants between giant embryo-2 and three reduced embryo mutants exhibited the reduced embryo phenotype in both embryo and endosperm, suggesting that reduced embryo mutations cause the enlarged endosperm. Further, a double mutant of reduced embryo3 and endospermless1-1 showed the enlarged embryo in endospermless seed. This confirms that reduced embryo3 does not regulate embryo size but enlarges endosperm size. Together with the results of the other double mutant analysis, REDUCED EMBRYO1, REDUCED EMBRYO2, REDUCED EMBRYO3 and GIANT EMBRYO are concluded to regulate endosperm development.

Key words: rice, embryo size, endosperm, reduced embryo, giant embryo

INTRODUCTION

Plant embryogenesis is the first developmental phase during which morphogenetic events occur to establish fundamental body plan and two apical meristems. Recent studies using various embryo mutants in rice (Nagato et al., 1989; Kitano et al., 1993; Hong et al., 1995a), maize (Clark and Sheridan, 1991; Sheridan and Clark, 1993) and Arabidopsis (Errampalli et al., 1991; Jurgens et al., 1991; Castle and Meinke, 1993) indicate that complicated regulatory processes are operating during embryogenesis, including pattern formation, organ determination, positional regulation, size regulation and morphogenesis. Among them, apical-basal pattern formation has been intensively studied in Arabidopsis (Mayer et al., 1993; Berleth and Jurgens, 1993), but the mechanisms of other regulatory processes are almost unknown.

The regulation of plant body size is a very interesting topic in biology and practical agriculture. Internode elongation, which primarily determines plant height, has been analyzed in detail, using a number of dwarf mutants. During embryogenesis, both shoot and root apical meristems are differentiated. Therefore, embryo size reflects the sizes of the two apical meristems and other embryonic organs, suggesting that embryo size would seriously affect the postembryonic development of plant. Nevertheless, embryo size has not been a target of genetic research, and the regulatory mechanism which controls embryo size is not known because of the lack of embryo size-related mutants. In this paper, embryo size mutants refer to those in which all the embryonic organs are formed but the sizes of one or more organs are modified, resulting in the alteration of embryo size. Therefore, a small embryo mutant which has some organ(s) missing should not be categorized as an embryo size mutant, rather it should be called an organ-determination mutant.

Embryo size is regulated in a nearly constant manner in each species. For example, the mature embryo of rice is around 2 mm in length which varies slightly among cultivars. This means that we can not expect a wide variation in embryo size among existing cultivars. Recently, both reduced embryo and giant embryo mutants have been identified in rice, in which
shoot and radicle are normally differentiated (Kitano et al., 1993; Hong et al., 1995a). These embryo size-related mutants would be useful for revealing how embryo size is controlled.

In many dicotyledonous species such as Arabidopsis thaliana, the endosperm degenerates at an early stage of development and as a consequence is absent in mature seed. In contrast, mature cereal seeds are mostly occupied by endosperm, suggesting a possibility that endosperm may physi- cally restrict the size of the embryo. In fact, spontaneous endospermless seeds, probably due to unfavorable physiological conditions, frequently have giant embryos. Recently an interesting mutant of rice has been detected whose embryo and endosperm development are affected by temperature (Hong et al., 1995b). In this mutant, embryo and endosperm sizes are negatively correlated. This indicates that the embryo and endosperm interact developmentally. Accordingly, the effect of endosperm should be taken into consideration during the analysis of embryo size determination processes of cereals.

In the present paper, we describe genetic factors which regulate embryo size, using a number of mutants associated with embryo and endosperm sizes.

**MATERIALS AND METHODS**

We used four reduced embryo mutants and four giant embryo mutants of rice affecting embryo size (Kitano et al., 1993; Hong et al., 1995), all of which were derived from a cultivar Taichung 65 mutagenized with methyl-nitrosourea. Among the four reduced embryo mutants, reduced embryo1-1 (re1-1) and reduced embryo1-2 (re1-2) are allelic, whereas re2 and re3 represent independent loci (Hong et al., 1995).

Four giant embryo mutants, giant embryo2-2 (ge-2), ge-3, ge-4 and ge-5 are all allelic to previously reported ge (Hong et al., 1995a; Satoh and Omura, 1981). In addition, we used another newly identified endospermless mutant, which has early loss of endosperm and a giant embryo. This is the result of a single, recessive mutation and was isolated from M2 lines of cultivar Taichung 65 mutagenized with n-methyl-n-nitrosourea. Allelism tests revealed that this mutation was allelic to a previously identified but unnamed endospermless mutation (Kageyama et al., 1991). Then the previous and the present mutations were designated as endospermless1-1 (enl1-1) and endospermless2-2 (enl2-2) respectively.

All the mutations used are single and recessive, and they are viable in a recessive homozygous state. Since the enl1 embryo is viviparous and is dead in the mature seed probably due to desiccation, recessive homozygous plants of enl1 were obtained by culturing nearly mature embryos at 20-30 days after pollination (20-30 DAP).

Embryonic phenotypes were characterized at various stages of development using a standard paraffin or plastic embedding method. All seed samples were fixed in FAA (formalin : glacial acetic acid : 70% ethanol, 5:5:90). For paraffin sections, seed samples were dehydrated in a graded ethanol series, embedded in paraffin and then sectioned at 12 μm. For plastic sections, samples were rinsed in 0.1 M phosphate buffer (pH 7.0), dehydrated in a graded acetone series and embedded in methacrylate resin. Acrytron E (Mitsubishi Rayon Co. Ltd.), which was polymerized at 45°C and sectioned at 5 μm.

Germination of each mutant was examined in more than 100 seeds inoculated on filter paper in Petri dishes at 30°C. Germination test was replicated for 2 years. For the examination of postembryonic growth, the height of ten plants of each mutant was measured every 10 days after germination. As heterozygous plants of each mutant were indistinguishable from the original cultivar, Taichung 65, we used normal Taichung 65 plants as the wild-type control instead of sibing plants of each mutant.

For determination of genic interactions, ge-2 was crossed with re1-1, re2 and re3, and phenotypes of F2 seeds were examined. To clarify the gene function of these embryo size mutants, we crossed them with other embryo mutants such as globular embryo 1 (gle1) which produced an undifferentiated minute globular embryo, club-shaped embryo 1-1 (cle1-1) which produced an undifferentiated minute club- shaped embryo, and shoot position 1 (shp1) which produced a small embryo with an apically displaced shoot and underdeveloped scutellum (Kitano et al., 1993). To examine the interaction between embryo and endosperm, enl1-1 was crossed with re3.

**RESULTS**

Phenotypes of reduced embryo and giant embryo mutations

Phenotypes of mature embryos

Mature embryos of four reduced embryo mutants are shown in Fig. 1. It is clear that in each mutant, embryo size was significantly reduced due to the reduction of each embryonic organ. Two alleles, re1-1 and re1-2, at the RE1 locus had indistinguishable phenotypes (Fig. 1B,C). Embryos of re2 and re3 also showed similar phenotypes (Fig. 1D,E) with re1-1 and re1-2. Embryos with morphologically aberrant or underdeveloped shoot and/or radicle were observed occasionally in re1-1 and re1-2, but frequently in re2 and re3. Detailed measurements revealed that the four mutants had similar sizes of organs (Table 1). Embryo length was reduced to 35-45% of the wild type, and embryo thickness to approx. 50%, although embryo size varied widely among seeds of each mutant. Similarly, the sizes of shoot apex and radicle were much reduced. Table 1 also indicates that size reduction of organs was due to the small number of cells, not due to the small cell size. Accordingly, these three loci, RE1, RE2 and RE3, would affect embryo size through regulation of the number of cells constituting all embryonic organs. It should be noted that in these four reduced embryo mutants, the endosperm occupies the rest of the space within the seed, indicating that these mutants have enlarged endosperm, as the seed size does not significantly differ from that of the wild type. Consequently, these reduced embryo mutations may be alternatively characterized as ‘enlarged endosperm mutations’.

Four giant embryo mutations located at the GE locus commonly caused a conspicuous enlargement of scutellum (Fig. 2B-E). Although most mutations examined did not seriously affect the differentiation of shoot and radicle, shoot development was more or less impaired at a low frequency.

Quantitative analysis of embryo size revealed that embryos of four mutants were nearly 1.25-fold longer than the wild type (Table 1). Since the sizes of shoot and radicle were not significantly affected, except for the slightly wider shoot apex in two mutants, the large embryo size was mostly due to the enlarged scutellum. Interestingly, the number of cells in the mutant embryo did not significantly differ from that in the wild-type embryo. This means that the larger scutellum was mainly caused by the enlargement of cells. It is considered that GE fundamentally affects the size of the scutellum and sometimes affects shoot and radicle development depending on the strength of allele. In contrast to the reduce embryo mutations, giant embryo mutations caused the endosperm size to be reduced. Accordingly, giant embryo mutations may be equivalent to reduced endosperm mutations.
How is embryo size genetically regulated?

The two types of embryo size mutants indicate that embryo size is controlled in both directions, reduction and enlargement, but the regulatory mechanism involved differs from each other. It is worthwhile to mention that in our embryo-size mutants, both embryo and endosperm sizes are simultaneously altered. However, we did not show whether the genes function in the embryo, endosperm or both. To elucidate this point, developmental analysis was conducted.

Developmental course of *rel-1* and *ge-2* seeds

As early as 2 DAP, *rel-1* embryos always showed distinguishable characteristics from the wild-type embryos. At 2

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**Table 1. Phenotypes of mature embryos in reduced embryo and giant embryo mutations**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Shoot apex</th>
<th>Radicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height (µm)</td>
<td>Diameter (µm)</td>
</tr>
<tr>
<td>Wild type</td>
<td>59±9</td>
<td>54±9</td>
</tr>
<tr>
<td><em>rel-1</em></td>
<td>34±3**</td>
<td>36±5**</td>
</tr>
<tr>
<td><em>rel-2</em></td>
<td>36±9**</td>
<td>37±8**</td>
</tr>
<tr>
<td><em>re2</em></td>
<td>34±10**</td>
<td>36±8**</td>
</tr>
<tr>
<td><em>re3</em></td>
<td>35±2**</td>
<td>41±3**</td>
</tr>
<tr>
<td><em>ge-2</em></td>
<td>64±4</td>
<td>63±9</td>
</tr>
<tr>
<td><em>ge-3</em></td>
<td>60±7</td>
<td>64±4*</td>
</tr>
<tr>
<td><em>ge-4</em></td>
<td>61±4</td>
<td>62±9</td>
</tr>
<tr>
<td><em>ge-5</em></td>
<td>65±9</td>
<td>64±6*</td>
</tr>
</tbody>
</table>

Size data are presented in µm ± s.d.
†Counted in median longitudinal sections.
*, **Significantly deviated from the wild type at 5% and 1% level respectively.

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**Fig. 1.** Mature embryos of *reduced embryo* mutants. (A) Wild type, (B) *re1*-1, (C) *re1*-2, (D) *re2*, (E) *re3*. All the embryonic organs including apical meristems are reduced in size in each of the mutants. The arrow indicates the shoot; the arrowhead, the radicle; s, scutellum. Bar, 0.5 mm.

**Fig. 2.** Mature embryos of *giant embryo* mutants. (A) Wild type, (B) *ge-2*, (C) *ge-3*, (D) *ge-4*, (E) *ge-5*. In each mutant, shoot (arrow) and radicle (arrowhead) are of normal size and only the scutellum (s) is enlarged. Bar, 0.5 mm.
DAP, embryonic development was delayed, as indicated by the small embryo size and small number of cells (Fig. 3F). Developmental retardation of the embryo then continued throughout seed development (Fig. 3F-J).

Endosperm development of re1-1 was also different from that of the wild type. At 2 DAP, the endosperm nuclei of re1-1 was not observed at the micropyler end (Fig. 3F). As shown in Fig. 3G, endosperm of re1-1 at 3 DAP was still at syncytial stage which is in contrast to a cellular endosperm in the wild-type seed (Fig. 3B). Difference of endosperm development between re1-1 and the wild type was also detected after 4 DAP. In the wild-type seed, a small space was always observed between embryo and endosperm (Fig. 3C-E). In contrast, endosperm was in direct contact with the embryo in re1-1 (Fig. 3H, I, J). All the other three reduced embryo mutants, re1-1, re2 and re3, also showed no space between embryo and endosperm at 5 DAP (Fig. 4). Accordingly, abnormalities of both embryo and endosperm development were confirmed from the early stage of seed development.

In ge-2 embryos, organ differentiation was delayed slightly (Fig. 3M-O). Enlargement of the scutellum, due to large cell size, was evident from 4 DAP (Fig. 3M) compared with that of wild type (Fig. 3C). Endosperm development was retarded from 2 DAP in ge-2 endosperm which was at syncytial stage (Fig. 3K), in contrast to the wild-type with cellularized endosperm (Fig. 3A). Fig. 3M and N also show a degradation of a larger number of endosperm cells located near the embryo in ge-2, which is not present in the wild-type embryo (Fig. 3C, D). Therefore, development of both embryo and endosperm in ge-2 was apparently distinguishable from that of the wild-type seed at this early stage.

Embryo and endosperm of both re1-1 and ge-2 showed developmental abnormalities from 2 DAP, suggesting that both RE1 and GE are expressed at 2 DAP. As both embryo and endosperm are concomitantly affected in re1-1 and ge-2, the functional domains of RE1 and GE are still unclear.

Postembryonic development
We analyzed how the embryo size mutations affected postembryonic development. Examination of the temporal...
change in germination rate of reduced embryo mutants revealed that *re1-1* and *re1-2* showed high germination rates (approx. 90%), whereas *re2* and *re3* showed lower germination rates (approx. 60%). Four giant embryo mutants (*ge-2, ge-3, ge-4, ge-5*) exhibited high germination rates (80-90%). In addition, these embryo size mutants also showed delay in germination. In wild type, it took only 12 hours to reach 50% germination, whereas it took 15-36 hours for the viable seeds of mutants to reach 50% germination. Among the mutants, *re2* and *re3* showed very low and much delayed germination, probably due to the defect in shoot and radicle differentiation as previously described. These results indicate that embryo size affects viability and germination depending on alleles which sometimes cause the defects in organ differentiation.

Postgermination analysis revealed that the initial growth of shoots of both reduced embryo and giant embryo mutants was delayed (Figs 5 and 6). However at maturity, about four months after germination, no significant difference in plant height was detected between the mutants and wild type (Figs 5 and 6). Therefore, it is considered that the embryo size-related genes function only during seed development and not after germination.

**Characterization of endospermless1-2 (enl1-2) mutants**

To clarify whether endosperm directly affects embryonic development, we identified a single recessive endospermless mutation, *enl1-2*. This mutation is probably leaky or environment-dependent, since the endospermless phenotype was manifested in only 10-20% of seeds set on homozygous plants. In spite of the low penetrance of the mutant phenotype, the frequency of heterozygous plants confirmed a single recessive mutation of *enl1-2* (data not shown). In this mutant, endosperm was normal at the early developmental stages (Fig. 7A), but degenerated before the onset of organ differentiation, resulting in an endospermless mature seed. In the *enl1-2* seed, a ‘giant’ embryo of nearly 3 mm in length was observed (Fig. 7B). This giant embryo has a shoot and radicle of normal size but has a large scutellum, as in *ge*. Although the shoot and radicle were morphologically normal in many embryos, some embryos had aberrant shoots. Before the degeneration of the endosperm, no abnormality was recognized in embryonic development. In addition, the scutellum became enlarged only after the loss of endosperm, indicating that this loss at an early developmental stage affects the scutellum size, but not shoot or radicle differentiation, as observed in *ge* mutations. Therefore, it is
suggested that the size of the embryo is physically restricted by the endosperm. Furthermore, embryos of this mutant did not become dormant and germinated viviparously on the panicle, but failed to sprout out of the glume (Fig. 7C).

Functional domain of RE1, RE2, RE3 and GE
To determine whether reduced embryo and giant embryo mutations primarily affect the embryo or endosperm, the functional domain of re1-1, re2, re3 and ge-2 was evaluated by using double mutants between them and other embryo mutants. In each combination, the same results were obtained in reciprocal crosses.

In the double mutants, ge-2 cle1-1 and ge-2 gle1, the embryo was similar to cle1-1 or gle1 whereas the endosperm was like that of a ge-2 type (Fig. 8). The reduction in the size of the endosperm resulted in a large space in the basal region of the double mutant seed in which minute cle1-1 or gle1 type embryos were located. This result clearly shows that ge-2 determines the size of endosperm, whereas cle1-1 and gle1 are associated with embryonic development irrespective of endosperm size. Therefore, it is conceivable that the giant embryo of ge-2 is a result of a reduced endosperm size.

In contrast, the double mutant, ge-2 re1-1, had a re1-1 type embryo and endosperm. If re1-1 is exclusively associated with embryo development rather than endosperm size, the endosperm of the ge-2 re1-1 double mutant must be a ge-2 type. Thus, the result indicates that re1-1 determines endosperm size and is epistatic to ge-2. The reduction in the size of the re1-1 embryo is a result of the limited space caused by the presence of large endosperm. The same results were obtained in the double mutants between ge-2 and re2 or re3.

The double mutant between re1 or ge-1 and shp1 was also analyzed. The embryo of shp1 seed is smaller than wild type but a little larger than re1-1. shp1 has a shoot and radicle of
normal size (Fig. 9C), and the endosperm which is not in direct contact with the embryo is normal. In the double mutant, re1-l and shp1 were additively expressed in embryo, i.e. a reduced embryo with an apically displaced shoot. In addition, the endosperm was a re1-l type (Fig. 9B). In contrast, the double mutant of ge-2 and shp1 had the reduced endosperm of ge-2 and a shp1 embryo (Fig. 9D). We also made double mutants between re2 or re3 and cle1-l. The re2 cle1-l seed had a cle1-l type embryo and an enlarged endosperm of re2 (Fig. 10B). The phenotype of re3 cle1-1 was the same as that of re2 cle1-1.

The above double mutant analyses indicate that both reduced embryo and giant embryo mutations affect endosperm development. However, it may be conceivable that reduced embryo mutations simultaneously cause both embryo reduction and endosperm enlargement. Therefore we made a double mutant between re3 and enl1-l. If re3 functions directly to reduce embryo size, the double mutant is expected to have a reduced embryo in endospermless seed. The results, however, show that the double mutant phenotype is the same as that of enl1-l (Fig. 7), i.e., a giant embryo in an endospermless seed (Table 2). This indicates that re3 does not regulate embryo size and that the re3 embryo is capable of enlarging if the endosperm does not physically limit the space. Based on these results, RE1, RE2, RE3 and GE are considered to regulate the size of the endosperm.

DISCUSSION

The interaction between embryo and endosperm has been investigated mainly from the nutritive aspects of endosperm as an embryo-nourishing tissue (Lopes and Larkins, 1993). Endosperm seems to be required for the nourishment of young embryo. Failure of endosperm development usually results in embryo abortion (Birchler, 1993). Dependency of early embryonic development on endosperm is implied in apomictic studies. In both autonomous and pseudogamous apomicts, endosperm formation seems to be indispensable for the maturation of apomictic embryos. In Citrus, adventitious embryos (nucellar origin) development seem to require endosperm formation, as a nutritive source (Moore, 1985). Similarly, in diplosporous Chinese chive (Allium tuberosum), apomictic embryos are aborted at an early stage, unless pollination takes place and endosperm is formed (Kojima and Kawaguchi, 1989). These aborted embryos in unpollinated ovules can be rescued by culturing on an enriched medium (Kojima and Kawaguchi, 1989), suggesting that the role of endosperm in embryonic development can be replaced by an enriched medium.

However, other functions of endosperm associated with embryo development are almost unknown. In cereals the endosperm may affect the developmental events of the embryo in some ways other than through nourishment. The present study shows that the endosperm development can restrict the size of the embryo. In an extreme case, for instance in enl1, a giant embryo was produced. In ge mutants which exhibit a reduced endosperm size, the giant embryos is a result of the enlargement of the scutellum, not an enlargement of the shoot and radicle. This indicates that there may be an optimal meristem size. However, in re1, re2 and re3 mutations, sizes of shoot and radicle meristems are much reduced in addition to the other embryonic organs. However, the reduction in meristem size is not a direct function of re genes, but a result of small available space. This indicates that meristem size can be reduced by external factors. Plants may adapt to a limited space by reducing the size of meristems and organs to establish their body plan. It is interesting to note that all five loci analyzed, ENL1, RE1, RE2, RE3 and GE, are considered to regulate endosperm development. In addition, EML1, reported by Hong et al. (1995b) appears to primarily affect endosperm development. Although no loci have been detected that directly regulate embryo size, Tamura et al. (1992) reported an embryo-specific gene affecting meristem size. A gene regulating the floral meristem size has been identified in rice (Nagasawa et al., 1996) and Arabidopsis (Clark et al., 1993). Accordingly, embryo size may be determined by the interaction between embryo-specific gene(s) and endosperm-specific genes regulating the endosperm development.

As can be seen in Fig. 3, abnormalities in endosperm were apparent as early as 2 and 3 DAP in both re1-l and ge-2 mutants. Similarly in the temperature-sensitive enl1 mutant of rice, seed phenotype was determined at 2 or 3 DAP (Hong et al., 1995b). These results suggest that developmental events at 2 or 3 DAP determine the final state of the endosperm. In rice, endosperm is cellularized at 2 or 3 DAP. Accordingly, cellu-

Table 2. Frequency of F2 seed phenotypes from an enl1-l × re3 cross

<table>
<thead>
<tr>
<th>Seed phenotype</th>
<th>Normal seed</th>
<th>Reduced embryo</th>
<th>Giant embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced endosperm</td>
<td>172</td>
<td>52</td>
<td>69</td>
</tr>
<tr>
<td>Giant embryo no endosperm</td>
<td>(9:3:4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>χ²</td>
<td>0.717</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 10. Seed phenotype of re2 and cle1-l. (A) re2, (B) re2 cle1-l, (C) cle1-l. In re2 cle1-l, the embryo is cle1-l type (arrowhead), but the endosperm is enlarged as in re2.
larization from syncytial stage would be a very important event for subsequent endosperm development.

In re and ge embryos, endosperm tissue in the basal region (embryo side) is specifically affected, where starch and other storage substances normally accumulate. This suggests that RE1, RE2, RE3 and GE function in the regulation of endosperm size in the basal region, whereas the development of other regions is regulated by other genes. Although there have been no reports suggesting a region-specific regulation of endosperm development, the present results indicate a differential expression of genes among regions in endosperm.

Since the double mutant phenotypes did not differ between reciprocal crosses, the genes analyzed are expressed at the postzygotic stage. In maize, maternal genotype affects the size and shape of endosperm (Brink and Cooper, 1947; Cooper, 1951; Birchler, 1980), probably due to parental imprinting (Lin, 1984). There are, however, no suggestions of imprinting of the present embryo (endosperm)-size related genes.

The embryo size-related genes characterized in this study are considered to regulate endosperm development. The investigation of these genes is very important for agriculture because cereal endosperms are the staple diet in many countries. Accordingly, further detailed analysis of these genes would be biologically and agriculturally interesting.

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