Arabidopsis GLUTAMINE-RICH PROTEIN23 Is Essential for Early Embryogenesis and Encodes a Novel Nuclear PPR Motif Protein That Interacts with RNA Polymerase II Subunit III

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Precise control of gene expression is critical for embryo development in both animals and plants. We report that Arabidopsis thaliana GLUTAMINE-RICH PROTEIN23 (GRP23) is a pentatricopeptide repeat (PPR) protein that functions as a potential regulator of gene expression during early embryogenesis in Arabidopsis. Loss-of-function mutations of GRP23 caused the arrest of early embryo development. The vast majority of the mutant embryos arrested before the 16-cell dermatogen stage, and none of the grp23 embryos reached the heart stage. In addition, 19% of the mutant embryos displayed aberrant cell division patterns. GRP23 encodes a polypeptide with a Leu zipper domain, nine PPRs at the N terminus, and a Gln-rich C-terminal domain with an unusual WQQ repeat. GRP23 is a nuclear protein that physically interacts with RNA polymerase II subunit III in both yeast and plant cells. GRP23 is expressed in developing embryos up to the heart stage, as revealed by β-glucuronidase reporter gene expression and RNA in situ hybridization. Together, our data suggest that GRP23, by interaction with RNA polymerase II, likely functions as a transcriptional regulator essential for early embryogenesis in Arabidopsis.

INTRODUCTION

In flowering plants, embryogenesis is a highly orchestrated process of cell division, differentiation, growth, and pattern formation (Mansfield and Briarty, 1991; Mayer and Jürgens, 1998; Berleth and Chatfield, 2002). A minimal set of ~750 nonredundant Arabidopsis thaliana genes is thought to be required to coordinate these embryonic developmental events (McElver et al., 2001; Tzafrir et al., 2003). Therefore, it is of fundamental importance to understand the molecular mechanisms coordinating such a large number of genes (Jürgens, 2001; Willemsen and Scheres, 2004). In the process of identifying all Arabidopsis genes essential for seed development, an initial set of 250 embryo-defective genes (EMBs) essential for normal embryo development has been reported (McElver et al., 2001; Tzafrir et al., 2004). These genes function in various processes, including cell growth, metabolism, transport, transcription, and translation. Among the 250 EMB genes, ~15% are predicted to be transcription factors based on gene ontology analysis (Tzafrir et al., 2004). To date, only a small number of transcription factors essential for embryo development have been identified by forward genetic approaches. These include AB3 (Parcy et al., 1994), STM (Long et al., 1996), MP (Hardtke and Berleth, 1998), PEI1 (Li and Thomas, 1998), LEC1 (Lotan et al., 1998), CUC1 to CUC3 (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003), and PLT1 (Aida et al., 2004). Loss-of-function mutations in these genes caused defects during different stages of embryogenesis. A summary of the phenotypes of emb mutants can be found at www.seedgenes.org. In only a few mutants was embryo development arrested before the 16-cell dermatogen stage. This is a critical stage at which the transition from pattern establishment to its refinement occurs. Examples of mutants arrested very early during embryogenesis include fac1, a zygotic embryo-letal mutant (Xu et al., 2005), prp1a, a mutation in the RNP1 subunit of the 26S proteasome (Brukhin et al., 2005), titan mutants, in which genes proposed to be involved in chromosome maintenance and microtubule assembly are disrupted (Liu and Meinke, 1998; McElver et al., 2000; Liu et al., 2002; Tzafrir et al., 2002), and opt3, a mutation of a peptide transporter gene (Stacey et al., 2002). Interestingly, among the predicted EMB genes there are 17 putative pentatricopeptide repeat (PPR)–containing genes, of which 7 were shown to be essential for late embryogenesis (Tzafrir et al., 2004; Cushing et al., 2005).

PPR genes belong to one of the largest gene families, but their function in plants remains to be elucidated (Aubourg et al., 2000; Small and Peeters, 2000; 2004). A typical PPR motif, consisting of 35 amino acids, is a macromolecular binding motif that is arranged in tandem with 2 to 26 copies in a PPR protein (Small and Peeters, 2000). Recent genetic and biochemical analysis showed that PPR proteins bind in a sequence-specific manner to RNA or DNA with their PPR motif (Barkan et al., 1994; Ikeda and Gray, 1999; Lahmy et al., 2000; Small and Peeters, 2000; Mancebo et al., 2001; Tsuchiya et al., 2002; Meierhoff et al., 2004). Various PPR genes belong to one of the largest gene families, but their function in plants remains to be elucidated (Aubourg et al., 2000; Small and Peeters, 2000; 2004). A typical PPR motif, consisting of 35 amino acids, is a macromolecular binding motif that is arranged in tandem with 2 to 26 copies in a PPR protein (Small and Peeters, 2000). Recent genetic and biochemical analysis showed that PPR proteins bind in a sequence-specific manner to RNA or DNA with their PPR motif (Barkan et al., 1994; Ikeda and Gray, 1999; Lahmy et al., 2000; Small and Peeters, 2000; Mancebo et al., 2001; Tsuchiya et al., 2002; Meierhoff et al., 2004).
Isolation of the RESULTS essential role during early embryo development in GRP23 is a potential transcriptional regulator that plays an both yeast and plant cells. Together, our findings suggest that RNA polymerase II subunit III via its C-terminal WQQ domain in its C terminus. We showed that GRP23 interacts physically with GRP23 encodes a nuclear PPR protein with 14 WQQ repeats at glutamine-rich protein23 (grp23), in which embryo development was arrested before the 16-cell dermotagen stage and in some cases already at the zygote stage. In many cases, the cell division pattern was shown to be aberrant. GRP23 encodes a nuclear PPR protein with 14 WQQ repeats at its C terminus. We showed that GRP23 interacts physically with RNA polymerase II subunit III via its C-terminal WQQ domain in both yeast and plant cells. Together, our findings suggest that GRP23 is a potential transcriptional regulator that plays an essential role during early embryo development in Arabidopsis.

RESULTS

Isolation of the set09078 Embryo-Lethal Mutant

To understand the molecular mechanisms that control gametophyte and embryo development, we performed a genetic screen for mutants with a distorted Mendelian segregation and reduced seed set, we identified an early embryo-lethal mutant, designated glutamine-rich protein23 (grp23), in which embryo development was arrested before the 16-cell dermotagen stage and in some cases already at the zygote stage. In many cases, the cell division pattern was shown to be aberrant. GRP23 encodes a nuclear PPR protein with 14 WQQ repeats at its C terminus. We showed that GRP23 interacts physically with RNA polymerase II subunit III via its C-terminal WQQ domain in both yeast and plant cells. Together, our findings suggest that GRP23 is a potential transcriptional regulator that plays an essential role during early embryo development in Arabidopsis.

Embryo Development Is Arrested before the Globular Stage in set09078

To determine when embryo development was disrupted in the mutant, siliques at ~7 d after pollination from heterozygous set09078 plants were dissected and cleared by the whole-mount clearing method using Herr's solution (1971). Cleared ovules were examined with a Zeiss Axioskop II microscope using differential interference contrast optics. The results showed that embryo development was arrested at the early globular stage in approximately one-quarter of the ovules, whereas the remaining 75% had already developed to the heart stage (Figure 1C). Although the normal embryos continued to develop, the arrested embryos subsequently became shrunken and finally degenerated (data not shown). The mutant embryos never reached the heart stage but arrested by the early globular stage. Despite the aberrant embryo development phenotype, no developmental defect was observed during endosperm development in the mutant ovules.

To further investigate earlier developmental defects during embryogenesis, we first studied embryo development in wild-type plants. Siliques of wild-type plants at different times after pollination were made transparent with the whole-mount clearing method and examined microscopically. The typical embryo development of Arabidopsis has been reported previously (Mansfield and Briarty, 1991; West and Harada, 1993; Berleth and Chatfield, 2002). Briefly, the zygote elongates along the micropyle–chalazal axis and then undergoes an asymmetric transverse cell division, giving rise to a small apical cell and a large basal cell (Figure 1D). Subsequently, the apical cell undergoes a series of mitotic divisions to form a 16-cell dermotagen embryo, including an outer layer of eight protodermal cells and eight inner cells (Figure 1E). Thereafter, cells of the upper tier and the lower tier will adopt oriented divisions either perpendicular or parallel to the apical–basal axis in a predestined pattern to increase cell number. As cell divisions progress, embryo polarity is further refined at the early heart stage (Figure 1F), and polar growth along the apical–basal axis results in the subsequent formation of late heart (Figure 1G), torpedo, and mature embryos. Concurrently, the basal cell goes through a series of transverse divisions to form a suspensor composed of a single file of 6 to 11 cells.

We then determined the stage at which the set09078 embryos are arrested during embryogenesis. Siliques of the heterozygous set09078 plants grown under the same conditions as the wild type, 275 of 1045 ovules were small, shrunk, and aborted (Figure 1B). This finding indicates that approximately one-quarter of the ovules (275:1045) could not develop into mature seeds.

To investigate whether the mutation also affected gametophyte functions, reciprocal crosses between set09078 and wild-type plants were performed. When heterozygous set09078 plants were used as pollen donors, the F1 progeny segregated with a ratio of 0.95:1 (202 Kanr:212 Kans), and when heterozygous set09078 plants were used as egg donors, the F1 progeny segregated at 0.99:1 (190:192). Both ratios were close to 1:1 (P > 0.9), suggesting that both the male and female gametophytes are functionally normal and the mutation can fully transmit to the next generation. Together, these data indicated that the phenotype conferred by set09078 is attributable to a single recessive embryo-lethal mutation.

2003; Mili and Pinol-Roma, 2003; Nakamura et al., 2003; Williams and Barkan, 2003; Lurin et al., 2004; Schmitz-Linneweber et al., 2005). A few functions have been identified, such as those that enable the restoration of cytoplasmic male fertility (Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Kazama and Toriyama, 2003; Koizuka et al., 2003; Laforest et al., 2003; Akagi et al., 2004; Komori et al., 2004; Klein et al., 2005) and organelle biogenesis (Barkan et al., 1994; Fisk et al., 1999; Hashimoto et al., 2003; Yamazaki et al., 2004; Kotera et al., 2005). In a screen for mutants that caused distorted Mendelian segregation and reduced seed set, we identified an early embryo-lethal mutant, designated glutamine-rich protein23 (grp23), in which embryo development was arrested before the 16-cell dermotagen stage and in some cases already at the zygote stage. In many cases, the cell division pattern was shown to be aberrant. GRP23 encodes a nuclear PPR protein with 14 WQQ repeats at its C terminus. We showed that GRP23 interacts physically with RNA polymerase II subunit III via its C-terminal WQQ domain in both yeast and plant cells. Together, our findings suggest that GRP23 is a potential transcriptional regulator that plays an essential role during early embryo development in Arabidopsis.
observed. These resulted in the formation of an elongated apical cell or embryo proper (Figures 1J and 1N) instead of the typical globular embryo in wild-type plants (Figure 1E). A notable phenotype of the set09078 mutant is that ~19% of the mutant embryos displayed aberrant positioning of the division planes and asynchronous divisions (Figures 1O to 1Q). Furthermore, asynchronous divisions of the embryo proper (Figure 1K) and suspensor cells (Figure 1L) were observed. Despite the dramatic early embryo development defect in the mutant, the number of endosperm nuclei and the length of suspensor cells appeared to
be comparable to those of the wild type at the same stage. These results suggested that the mutation has pleiotropic effects but that the gene plays an essential role specifically during early embryo development.

**The Defective Embryos Were Caused by a Ds Insertion into GRP23**

To identify the gene disrupted in the mutant, thermal asymmetric interlaced PCR was used to obtain the genomic sequences flanking the Ds element in set09078 (Liu et al., 1995; Grossniklaus et al., 1998). Analysis of the flanking sequences revealed that a Ds element was inserted at nucleotide position 222 bp downstream of the ATG initiation codon of the intronless At1g10270 gene (Figure 2A). The insertion resulted in a 9-bp nucleotide duplication at the insertion site (data not shown). Based on the Arabidopsis genome sequence, there is only a single copy of the At1g10270 gene. DNA gel blot hybridization using a Ds-specific probe showed that only a single Ds element was inserted in the mutant genome (see Supplemental Figure 1 online). A database search further identified two independent T-DNA insertion lines (SALK_128329 and SALK_074740) available from the ABRC (Ohio State University). T-DNAs were present at different positions in At1g10270 in these two lines (Figure 2A). The T-DNA insertion sites were confirmed by PCR analysis using a T-DNA left border primer (LBb1) (Alonso et al., 2003) and a gene-specific primer. Phenotypic characterization showed that the seed abortion ratios were ~23% (170:728; P > 0.25) for SALK_074740 and 24% (168:698; P > 0.25) for SALK_128329. These ratios are consistent with the expected 25% seed abortion. Microscopic analysis revealed that the embryonic defects in these aborted seeds were similar to those in grp23 (data not shown). The segregation of T-DNA insertion was determined by PCR analyses using LBb1 and the gene-specific primer, because the NPTII gene conferring kanamycin resistance was silenced in the SALK lines (Alonso et al., 2003). In the selfed progeny of SALK_074740, 29 of 43 siblings contained T-DNA and 14 did not. For the selfed SALK_128329 progeny, 33 of 48 siblings contained the T-DNA insertion and 15 did not. Both lines showed a 2:1 ratio of T-DNA segregation, suggesting that the homozygous T-DNA insertion mutants were also as embryo-lethal as set09078. Together, these data strongly suggest that the embryo phenotype is tightly linked to the disruption of At1g10270 function.

To further determine whether the embryo-lethal phenotype that we observed is indeed the result of a Ds insertion within At1g10270, genetic complementation was performed. A 5577-bp genomic DNA fragment encompassing from 2262 bp upstream of the ATG initiation codon to 576 bp downstream of the TAG stop codon of At1g10270 was amplified by PCR and cloned into pCAMBIA1300. This construct was introduced into heterozygous set09078 plants by Agrobacterium tumefaciens-mediated infiltration (Bechtold and Pelletier, 1998). In total, 18 independent transgenic T1 plants were obtained by double selection on Murashige and Skoog (MS) medium plates containing 20 μg/mL hygromycin and 50 μg/mL kanamycin. All T1 plants were allowed to self-fertilize, and their progeny were scored for the segregation of normal and aborted seeds. We found that 17 transformants displayed obvious restoration of seed set, and five independent lines were then randomly chosen for a more detailed statistical analysis. This showed that the ratio of normal to aborted seeds in T1 plants is close to 15:1 (see Supplemental Table 1 online). This finding indicated that the transgene successfully complemented the mutation. In addition, several T3 plants that are completely resistant to kanamycin and hygromycin were obtained, and they all showed full seed set (data not shown). Together, these data demonstrated that the embryo-lethal phenotype was indeed caused by the loss of function of At1g10270. Furthermore, they showed that the 5577-bp fragment harbors all of the genetic components required for the function of this gene.

At1g10270 encodes a putative protein of 913 amino acids with an estimated molecular mass of 102 kD and a pl of 7.67. Sequence analysis revealed that there is a basic region with a putative nuclear localization signal (ERRRRRKRLR) at its N terminus. Downstream of the basic region, there is a classic Leu zipper motif in which periodic repetitions of Leu residues at every seventh position are present (Figure 2B). Interestingly, there is a 25-amino acid region rich in Pro residues (8:25) separating the nuclear localization signal and the Leu zipper domain (Figure 2B). Therefore, At1g10270 encodes a putative basic domain/leucine zipper (bZIP) transcriptional regulator. In addition, At1g10270 contains nine tandem PPR motifs downstream of the Leu zipper domain at its N terminus and a Gln-rich domain at its C terminus (Figures 2B and 2C). Therefore, we designated it GRP23, for glutamine-rich PPR protein. The six PPR motifs were arranged in tandem from amino acid residues 181 to 576 (Figures 2B and 2C). The PPR motif, usually consisting of ~35 amino acids, appears to have a structural similarity to the tetratricopeptide repeat (TPR) motif, which in general is 34 amino acids long and is involved in protein–protein interaction (Small and Peeters, 2000). There are three TPR motifs overlapping within the second, fourth, and eighth PPR repeats, respectively (Figure 2B).

Besides the nine PPR motifs at the N terminus of GRP23, there are 79 Gln residues from amino acid 630 to 905 in the C-terminal region (Figures 2B and 2C). This indicates that the C terminus is a distinct Gln-rich domain (29% Gln). Interestingly, there are 14 discernible repeats (Wx2Qx4Qx2) with regularly spaced Trp and Gln residues within the Gln-rich region. Each repeat contains 11 amino acids, and each starts with a Trp (Figure 2B). Database search results suggest that it is an unusual structural motif unique to the GRP23 protein. We named this repeat the WQQ repeat.

National Center for Biotechnology Information BLAST analysis reveals a large family of related proteins that have high homology with GRP23 at the amino acid level. Most of these proteins are annotated as PPR-containing proteins. Individual alignment of GRP23 with these proteins did not reveal any long stretches of sequence identity or similarity to the Gln-rich domain; rather, they shared conserved amino acids with the PPR domain of GRP23. An alignment between GRP23 and its top three hits is shown in Figure 3. Among these three proteins, ACT11109.4 from Cucumis melo is most similar to GRP23, showing 62% identity and 76% similarity at the amino acid level. AAV32241.1 from Oryza sativa shares 48% identity and 66% similarity to GRP23. Another notable homolog is At3g49240 from Arabidopsis; loss
Figure 2. Molecular Characterization of GRP23 Protein.
of function of At3g49240 also displayed an embryo-defective phenotype (Cushing et al., 2005). At3g49240 encodes a predicted polypeptide of 629 amino acid residues containing 11 PPR motifs arranged in tandem. It shares 34% identity and 51% similarity with GRP23 at the amino acid level in the PPR region. There is little homology in the C terminus. These analyses indicated that GRP23 is a novel PPR protein.

**GRP23 Is a Nuclear Protein**

The presence of the putative N-terminal nuclear localization signal and the bZIP domain suggests that GRP23 is a putative nuclear transcription regulator. To define its subcellular localization, C-terminal translational fusion of GRP23 with YELLOW FLUORESCENT PROTEIN (YFP) driven by the constitutive 35S promoter of Cauliflower mosaic virus was cloned into pCAMBIA1300 to yield P35S-GRP23-YFP. The P35S-GRP23-YFP construct was subsequently introduced into wild-type plants using Agrobacterium-mediated vacuum infiltration (Bechtold and Pelletier, 1998). Confocal laser scanning microscopy revealed that in the root cells of transgenic plants harboring the P35S-GRP23-YFP transgene, the YFP fluorescence colocalized with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI) fluorescence, a DNA-specific dye (Figure 4). This indicated that GRP23 is indeed targeted to the nucleus. To determine whether the fusion protein is still functional, the P35S-GRP23-YFP transgene was further introduced into grp23 mutant plants by crossing. All of the F2 plants heterozygous for the Ds insertion exhibited obvious rescue of the embryo-defective phenotype (data not shown), indicating that the P35S-GRP23-YFP fusion functionally complemented the mutant phenotype. Together, these results indicated that GRP23 is indeed a nuclear protein.

**GRP23 Is Expressed Preferentially in Gametophytes and Young Embryos**

We next investigated the temporal and spatial expression pattern of the GRP23 gene. At first, we used RT-PCR with total RNA from different organs, including roots, stems, leaves, inflorescences, siliques, and seedlings. As shown in Figure 5A, after 35 cycles of PCR amplification, a single band with the expected size was detectable in RNAs from roots, stems, leaves, inflorescences, siliques, and seedlings. The highest expression level was in inflorescences, siliques, and roots, and very low expression occurred in stems, seedlings, and leaves (Figure 5A). Consistently, microarray data available at the GENEVESTIGATOR (http://www.genevestigator.ethz.ch) showed the highest expression level of GRP23 in seeds and moderate levels in shoot apices and inflorescences (Figure 5B). A lower level of expression occurred in other tissues.

To further investigate its expression pattern, especially during embryogenesis, we used a PGRP23-GUS (for β-glucuronidase) reporter system to monitor GRP23 expression. The 2718-bp promoter region upstream of the ATG start codon plus the 347 bp of the first exon of GRP23 was used to drive the GUS reporter gene. In addition, the 576-bp 3' fragment immediately downstream of the stop codon was also cloned behind the GUS coding sequence. Twelve independent transgenic lines expressing the PGRP23-GUS fusion protein were analyzed. A high level of GUS activity was detected in ovules of young siliques (Figures 6A and 6C), inflorescences (Figure 6B), and pollen grains (Figure 6C). In embryo sacs, GUS activity was detected at all stages of female gametophyte development from one nucleate to the mature unfertilized embryo sac (Figures 6E and 6F). Strong GUS staining was seen in the zygote (Figure 6G), and ~12 h after fertilization, GUS activity was detected in the apical cell, basal cell, and endosperm cells (Figure 6H). Before the globular stage, strong GUS activity was observed mainly in the embryo proper (Figures 6I and 6J). Besides in the embryo proper, weak GUS activity was also detected in the suspensor and the endosperm at the heart stage. In addition, GUS activity was observed in the apical root meristem, lateral root primordia, shoot apex, and leaf primordia (Figure 6D). These data indicated that GRP23 is expressed mostly in pollen grains and embryos, with low levels of expression in endosperm cells during sexual reproduction and in actively dividing cells during vegetative growth.

To validate the GUS expression pattern, we further performed RNA in situ hybridization. The gene-specific antisense and sense RNA probes were labeled with digoxigenin. Specific mRNA signals could be detected at different stages during early embryogenesis. Figure 6K shows that a moderate hybridization signal was observed in the embryo proper and suspensor cell. Prominent signals were detected at the eight-cell embryo proper, suspensor, and developing endosperm cells (Figures 6L to 6N). Strong signals could be detected up to the heart stage (Figure 6O). No signal above background was observed in the control experiment when the sense RNA probe was used (Figure 6P).

In summary, GRP23 is expressed preferentially both in gametophytes and in embryos and endosperm cells during embryogenesis. This expression pattern of GRP23 is in agreement with its essential role during early embryogenesis. In addition, its expression in meristematic cells suggests that GRP23 plays a role in actively dividing cells during vegetative development.

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**Figure 2.** (continued).

(A) Diagram of the insertion positions of Ds and T-DNAs in GRP23. The hatched box indicates the predicted open reading frame of the GRP23 gene. Ds insertion caused a 9-bp nucleotide duplication in set09078. The nucleotide numbers are consistent with those in BAC clone F14N23.

(B) Predicted GRP23 amino acid sequences. The insertion positions of Ds in set09078 and T-DNA in SALK_128329 and SALK_074740 are indicated by arrowheads. The basic region and Leu zipper motif are shown in boldface italic and underlined, respectively. The shaded amino acids representing the nine PPR motifs and the three overlapping TPR motifs are underlined. Boxed columns show the conserved Trp (W) and Gln (Q) residues in the WQQ repeats. Q amino acids are highlighted in boldface.

(C) Scheme of the GRP23 protein domains. The bZIP domain and nine PPR repeats are indicated. The white open boxes represent the WQQ motifs in the Gln-rich region.
Figure 3. Alignment of the GRP23 Protein with Its Homologs from Cucumis melo, Oryza sativa (cv japonica), and Arabidopsis thaliana.

Identical amino acids are shown with white letters in black boxes, and similar amino acids are shown with shaded boxes.
GRP23 Interacts Physically with RNA Polymerase II in Yeast and Plant Cells

To further investigate the biochemical function of GRP23, a two-hybrid screen was performed using the full-length GRP23 coding sequence fused to the GAL4 DNA binding domain as bait (pBD-GRP23). Of 1.2 × 10^6 transformants screened, four clones were identified, all of which contained the same gene, encoding the 36-kD subunit III (RBP36B) of the DNA-directed RNA polymerase II (Ulamasov et al., 1996). Reconfirming experiments showed that yeast cells cotransformed with pAD-RBP36B and pBD-GRP23 grew well in Trp-, Leu-, and His-dropout medium supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT) (Figure 7). Meanwhile, they were able to produce an intense β-galactosidase signal (Figure 7E). In addition, both GRP23 and RBP36B showed a similar expression pattern in planta (Figure 5). These data suggest that GRP23 interacts physically with RBP36B in yeast cells and potentially in plants.

We then further identified which domain of GRP23 is required for its interaction with RBP36B. We constructed two deletion fragments of GRP23 (Figure 7A) and analyzed their ability to interact with RBP36B in yeast. The PPR domain (residues 1 to 579) and the WQQ repeat–containing Gln-rich domain (residues 602 to 913) (Figure 7A) were cloned into pGBK7, giving rise to the plasmids pBD-PPR and pBD-WQQ, respectively. Yeast cells cotransformed with pAD-RBP36B and pBD-WQQ were still able to grow well on selective medium and produced an intense β-galactosidase signal (Figure 7). By contrast, yeast cells cotransformed with pAD-RBP36B and pBD-PPR could not grow on the same selective medium (Figure 7D). These results showed that GRP23 containing only its Gln-rich domain, by deleting the PPR domain, retained the ability to interact with RBP36B, whereas deletion of the Gln-rich domain almost totally abolished this ability. These data indicated that the Gln-rich domain of

**Figure 5.** Expression Patterns of the GRP23 and RBP36B Genes.
(A) Tissue-specific expression of GRP23 and RBP36B using RT-PCR analysis. RT-PCR was performed on total RNAs from different tissues, including roots (Rt), stems (St), leaves (Lf), inflorescences (Fr), siliques (Se), and seedlings (Sd), with (+) or without (−) reverse transcriptase (R.T.) as indicated. After 35 cycles, the resulting products were stained with ethidium bromide and analyzed by gel electrophoresis. ACTIN2 RNA was used as an internal template control.
(B) and (C) Expression profiles of GRP23 (B) and RBP36B (C) in various organs. The y axis represents the expression level. Data used in this analysis were retrieved from the public GENEVESTIGATOR microarray data set (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004).
Figure 6. Temporal and Spatial Patterns of GRP23 Gene Expression.

(A) to (J) Histochemical assays for the expression pattern of the $P_{GRP}$-GUS transgene revealed by the $P_{GRP23}$-GUS reporter.

(A) Young seeds in a silique expressing GUS. Bar = 1 mm.

(B) Micrograph showing GUS activity specifically detected in anthers and young ovules during different flower development stages. Bar = 1 mm.

(C) A stage 12 flower showing GUS activity in pollen grains (arrowhead) and embryo sacs at about the one- to two-nucleate stage (arrow). Bar = 100 μm.

(D) GUS staining of a 7-d-old seedling showing GRP23 expression in the shoot apex, leaf primordium, lateral root primordia, and root meristem (inset). Bar = 1 mm.

(E) GUS staining detected in an early two-nucleate embryo sac (arrow).

(F) A mature embryo sac expressing GUS.

(G) A zygote expressing GUS.

(H) Approximately 12 h after fertilization, GUS staining is detected in the apical cell, basal cell, and endosperm cells.

(I) Micrograph showing GUS activity in the embryo proper at the globular stage.

(J) Micrograph showing GUS activity in the embryo proper and weak GUS activity in the endosperm cells at the heart stage.

(K) to (P) RNA in situ hybridization confirmed the GRP23 expression during embryogenesis.

(K) to (O) Antisense probe hybridization to longitudinal sections of seeds at different developmental stages.

(K) Nomarski micrograph showing signal in the embryo proper and suspensor cell at the two- to four-cell stage.

(L) and (M) Nomarski micrographs showing strong signal in the embryo proper, suspensor, and endosperm cells at the eight-cell stage.
GRP23 is essential for the interaction with RNA polymerase II subunit III.

To further validate the yeast data, we used the bimolecular fluorescence complementation (BIFC) approach. BIFC detects protein interactions in living cells by relying on the fluorescence from the split YFP peptide fragments that are brought into close proximity by the two interacting proteins to which they are fused (Hu et al., 2002; Walter et al., 2004; Diaz et al., 2005). The full-length open reading frame sequence of GRP23 was cloned into pUC-SPYNE, and RBP36B was cloned into pUC-SPYCE, to produce GRP23-YFP\textsuperscript{N} and RBP36B-YFP\textsuperscript{C}, respectively. These two constructs were cobombarded into onion (Allium cepa) epidermal cells. If they interact physically, YFP fluorescence would be restored, and YFP fluorescence was indeed observed in the nucleus of onion epidermal cells when cobombarded with GRP23-YFP\textsuperscript{N} and RBP36B-YFP\textsuperscript{C} (Figure 7F). By contrast, YFP fluorescence was never detected in onion epidermal cells when cobombarded with either GRP23-YFP\textsuperscript{N} in combination with pUC-SPYCE or pUC-SPYNE in combination with RBP36B-YFP\textsuperscript{C} (data not shown). These data suggest that GRP23 interacts physically with RNA polymerase II in living onion cells.

In conclusion, we provided evidence that the nuclear protein GRP23 can interact physically with RNA polymerase II subunit III through its C-terminal WQQ repeat–containing Gln-rich domain in both yeast and plant cells. Together with the presence of the basic region adjacent to the Leu zipper domain, our data suggest that GRP23 could be a potential transcriptional regulator essential for embryogenesis.

**DISCUSSION**

Through genetic studies, we have shown that the loss of GRP23 function caused an early arrest of embryo development before the 16-cell dermatogen stage, a critical stage for pattern formation and its refinement (Mayer et al., 1991; Goldberg et al., 1994; Jürgens, 2001; Willemsen and Scheres, 2004). In addition, a notable phenotype of the grp23 mutant is that approximately one-fifth of the mutant embryos displayed an aberrant cell division pattern. It is not clear whether this is an indirect or direct cause of the loss of function of GRP23.

Several reports have shown that loss of function of the PPR gene in Arabidopsis often caused an embryo-lethal phenotype, although at relatively late stages of embryogenesis compared with grp23 (Tzafir et al., 2004; Cushing et al., 2005). In addition, they also showed dramatic morphological defects, such as enlarged shoot apices and stunted cotyledons (Cushing et al., 2005). As we did not observe such defects in grp23 mutants as a result of its early embryo lethality, the role of GRP23 in meristematic cells remains to be addressed. Nevertheless, these data suggest that PPR genes have an essential role in cell growth and embryo development. Our genetic analysis of GRP23 further supports such a role for PPR genes.

Despite the fact that GRP23 is expressed in gametophytes and endosperm, their development did not appear to be affected in the mutants. Although several PPR genes have been shown to control cytoplasmic male sterility (Kazama and Toriyama, 2003) and fertility restoration (Bentoilla et al., 2002; Brown et al., 2003; Deslore et al., 2003; Koizuka et al., 2003; Laforest et al., 2003; Akagi et al., 2004; Komori et al., 2004; Klein et al., 2005), it appears that the GRP23 function is not essential for gametophyte and endosperm development, which may partly be attributable to the genetic redundancy of PPR genes (Lurin et al., 2004; Cushing et al., 2005). Alternatively, there is a dosage effect for the gametophyte development for which only two or three cell divisions are required; they may receive sufficient GRP23 protein from their heterozygous maternal or paternal precursor cells.

**GRP23 Encodes a Nuclear PPR Protein That Is Essential for Early Embryogenesis of Arabidopsis**

Molecular analysis indicates that GRP23 encodes a protein containing nine PPR motifs at its N terminus and a Gln-rich domain at its C terminus. The nine PPR motifs are arranged in tandem between amino acid residues 181 and 576. The name PPR was coined to distinguish it from a TPR motif (Small and Peeters, 2000). Because the 35-amino acid repeats in the PPR domain do resemble TPR motifs, it is not surprising that three TPR motifs were found to overlap within the PPR domain in the GRP23 protein. The TPR motif usually consists of 34 amino acid residues and is present as 3 to 16 tandem repeats in a protein. Proteins containing TPRs are involved in a variety of biological processes, such as protein folding, cell cycle regulation, and transcriptional control (Goebi and Yanagida, 1991; Blatch and Lassle, 1999). PPR proteins are widely distributed in eukaryotes, especially in plants. In the Arabidopsis genome, >451 PPR genes have been identified and largely annotated with unknown functions (Aubourg et al., 2000; Small and Peeters, 2000; Lurin et al., 2004). Several loss-of-function mutations of the PPR family have been isolated, and they are mostly involved in RNA processing in mitochondria or chloroplasts (Barkan et al., 1994; Lahmy et al., 2000; Hashimoto et al., 2003; Kazama and Toriyama, 2003; Meierhoff et al., 2003; Nakamura et al., 2003; Lin et al., 2004; Yamazaki et al., 2004; Kotera et al., 2005; Schmitz-Linneweber et al., 2005). Recently, the identification of seven PPR proteins essential for embryogenesis among the predicted EMB gene data set further extended their function in a developmental context (Cushing et al., 2005). However, dramatic differences exist between the GRP23 and other PPR proteins characterized to date. PPR genes are usually expressed at a low level in all tissues, whereas GRP23 is expressed preferentially in the sexual...
organs, including both gametophytes and the developing embryos. Although the majority of PPR proteins are predicted to be targeted to organelles using the programs TargetP and Predotar (Emanuelsson and von Heijne, 2001; Lurin et al., 2004; Small et al., 2004), GRP23 is localized to the nucleus, most likely through the nuclear localization signal at the N terminus. The basic region contains a bipartite nuclear localization signal and is rich in Pro. This domain does not match with any of the classified bZIP group proteins of Arabidopsis (Jakoby et al., 2002). In addition, the typical L-x6-L-x6-L Leu zipper motif is followed

Figure 7. GRP23 Interacts with RBP36B in Both Yeast and Onion Cells.

(A) Scheme of GRP23 representing the nine PPR motifs as nine open boxes and the Gln-rich domain as a hatched bar. GRP23 deletion constructs containing either the PPR domain or the WQQ repeat domain are shown below. Numbers indicate the first and last amino acids of the peptides.

(B) GRP23 interacts with RBP36B in yeast cells. Yeast cells were cotransformed with pGBK7 and pAD-RBP36B (sector 1), pBD-GRP23 and pGAD-GH (sector 2), and pBD-GRP23 and pAD-RBP36B (sector 3). Transformants were streaked on a plate containing synthetic dropout selection medium that lacked Trp, Leu, and His supplemented with 5 mM 3-AT (SD/-Trp-Leu-His + 5 mM 3-AT).

(C) The WQQ repeat–containing Gln-rich domain of GRP23 interacts with RBP36B in yeast. Yeast cells were cotransformed with pGBK7 and pAD-RBP36B (sector 1), pBD-WQQ and pGAD-GH (sector 2), and pBD-WQQ and pAD-RBP36B (sector 3). Transformants were streaked on a SD/-Trp-Leu-His + 5 mM 3-AT plate.

(D) GRP23 deletions containing only its PPR domain failed to interact with RBP36B in yeast. Yeast cells were cotransformed with pGBK7 and pAD-RBP36B (sector 1), pBD-PPR and pGAD-GH (sector 2), and pBD-PPR and pAD-RBP36B (sector 3). Transformants were streaked on a SD/-Trp-Leu-His + 5 mM 3-AT plate.

(E) β-Galactosidase activities by o-nitrophenyl-β-D-galactopyranosidase assays of yeast cells cotransformed with pGBK7 and pGAD-GH (bar 1), pBD-GRP23 and pGAD-GH (bar 2), pBD-WQQ and pGAD-GH (bar 4), pBD-PPR and pAD-RBP36B (bar 5), pBD-WQQ and pAD-RBP36B (bar 6), and pBD-PPR and pAD-RBP36B (bar 7). These assays were repeated three times. Mean ± SD values are shown. mU, milliunits.

(F) BiFC visualization of GRP23 interaction with RBP36B in nuclei of onion cells (arrows). Left, YFP fluorescence image of onion epidermal cells cotransfected with GRP23-YFPN and RBP36B-YFPC; middle, bright-field image of the onion cells; right, merged image showing YFP fluorescence in nucleus. Bars = 5 μm.
immediately by an additional x5-L-x5-L-x5-L motif. These structural features suggest that GRP23 is a type of bZIP protein. Besides the nine PPR motifs at its N terminus, there is a distinct Gln-rich domain at the C terminus. There is a relatively conserved transcriptional activation function for the Gln-rich domain from yeast to human (Dynan and Tjian, 1983; Remacle et al., 1997; Escher et al., 2000; Petcherski and Kimble, 2000; Freiman and Tjian, 2002). This might be true for GRP23 as well. Intriguingly, there are 14 WQQ repeats in the C-terminal Gln-rich region. Although it is not known what role these structural motifs have, it would be interesting to explore the role of WQQ repeats in the future. Together, these data suggest that GRP23 represents a novel nuclear PPR protein whose function is essential for embryogenesis in Arabidopsis.

**GRP23 Most Likely Functions as a Transcriptional Regulator via Its Interaction with DNA-Dependent RNA Polymerase II**

Gln-rich domains represent one of the three classes of protein domains present in proteins that regulate the expression of specific sets of genes by selectively interacting with a core component of the transcriptional machinery, such as TFIID and TAFIIIs (Triezenberg, 1995; Remacle et al., 1997; Saluja et al., 1998). The sequences of Gln-rich proteins usually have very low homology, which makes it difficult to classify them. Nevertheless, many Gln-rich transcription regulators have been characterized from yeast to animal (Dynan and Tjian, 1983; Kunzler et al., 1994; Gomes et al., 1999; Zhang et al., 1999; Petcherski and Kimble, 2000; Shimohata et al., 2000; Dunah et al., 2002; Liu et al., 2002; Wang et al., 2005). The specificity protein (SP1), a distinctive Gln-rich domain-containing protein, was the first human transcriptional activator that was isolated (Dynan and Tjian, 1983). SP1 binds to GC-box DNA elements present in the promoters of specific genes. LAG-3 is a Gln-rich protein that is involved in Notch signaling in Caenorhabditis elegans (Petcherski and Kimble, 2000). To regulate transcription, LAG-3 forms a ternary complex together with the LAG-1 DNA binding protein and the intracellular domain of its receptor in C. elegans.

Although the transcriptional activation function for proteins with a Gln-rich domain property in animal cells has been well documented, their biochemical function is poorly understood in higher plants (Ulmasov et al., 1999; Conner and Liu, 2000; Franks et al., 2002; Tiwari et al., 2003; Wilmortho et al., 2005). Before transcribing a gene, RNA polymerase II must be first recruited to form a complex with a general transcription factor. Also, some mediators and/or coactivators are required to initiate transcriptional activation and repression. Transcriptional regulators usually have two distinct domains: one responsible for specific DNA binding, and the other responsible for transcriptional activation. The domain structure of GRP23 suggests that it may bind DNA cis-regulatory elements through the N-terminal basic region of the bZIP domain or the PPR motifs. The physical interaction between the C-terminal WQQ domain and subunit III of DNA-dependent RNA polymerase II suggests that GRP23 may recruit RNA polymerase to control the expression of a subset of genes. Thus, it is reasonable to speculate that GRP23 may function as a putative transcription factor or activator through its Gln-rich domain at the C terminus and as a specific DNA binding player through its N-terminal bZIP domains (Jakoby et al., 2002). The role of the PPR motif is still not clear. It would be interesting to know whether the PPR motif itself can bind DNA or RNA, as PPR proteins have been shown to possess DNA or RNA binding activity (Barkan et al., 1994; Ikeda and Gray, 1999; Lahmy et al., 2000; Small and Peeters, 2000; Mancebo et al., 2001; Liu and McKeegan, 2002; Tsuchiya et al., 2002; Meierhoff et al., 2003; Mill and Pinol-Roma, 2003; Nakamura et al., 2003; Williams and Barkan, 2003; Lurin et al., 2004; Schmitz-Linneweber et al., 2005).

In conclusion, the nuclear protein GRP23 represents a novel PPR protein and a potential transcriptional regulator that is essential for early embryo development in Arabidopsis. It would be interesting to compare the expression profiles between the mutant and wild-type embryos using microarray technology and laser capture microdissection (Casson et al., 2005) or to over-express GRP23 and see what genes it activates. This would help to identify the downstream genes.

**METHODS**

**Plant Materials**

Seeds of Arabidopsis thaliana ecotypes Landsberg erecta and Columbia were sterilized with 20% bleach and 70% ethanol for 5 min, respectively, then washed five times in sterilized water and germinated on MS agar plates supplemented as required with 50 µg/mL kanamycin and/or 20 µg/mL hygromycin. Plants were grown at 22 ± 2°C in an air-conditioned greenhouse under a 16-h-light/8-h-dark cycle. Arabidopsis transformation was performed via Agrobacterium tumefaciens-mediated vacuum infiltration (Bechtold and Pelletier, 1998).

**Genetic Analysis**

The genetic screen of grp23 from Ds insertion lines was conducted as described by Sundaresan et al. (1999). Ds copy number was determined by DNA gel blot hybridization using a 750-bp Ds-5’ fragment as a probe. The T-DNA insertion lines SALK_074740 and SALK_128329 were obtained from the ABRC. PCR amplified by the T-DNA left border primer LBB1 (5’-GCCTGAGCCGCTTGCAGCT-3’) in combination with the At1g10270-specific primer P224 (5’-ATTTACCTCTCCAGGGTC-3’) was used to confirm T-DNA insertion positions in these two lines. Reciprocal crosses between wild type and the mutant were performed as described (Yang et al., 1999).

**Phenotypic Analysis**

To determine the embryo phenotype, siliques from grp23 heterozygous plants were dissected with hypodermic needles and cleared in Herr’s solution containing lactic acid:chloral hydrate:phenol:clove oil:xylene (2:2:2:1, w/w) (Herr, 1971; Vieille-Calzada et al., 2000). Embryo and endosperm development was studied microscopically with a Zeiss Axioskop II microscope equipped with differential interference contrast optics. The number of endosperm nuclei was scored, and the length of suspensor cells was measured carefully. Micrographs were obtained with a Nikon 4500 digital camera. Besides the embryonic phenotype, the morphology of the mutant plants in other tissues, including root, stem, and leaf, was also observed carefully.

**Molecular Cloning and Genetic Complementation**

The Ds flanking sequences were isolated by thermal asymmetric interlaced PCR as described previously (Liu et al., 1995; Grossniklaus et al.,
1998). To conduct the complementation experiment, a 5577-bp GRP23 genomic fragment from 2262 bp upstream of the ATG start codon to 576 bp downstream of the TAG stop codon was amplified by LA Taq polymerase (TakaRa) using primers P25268B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P3151B (5'-CCGCTGTCTCTCATCAGAGTCGTCAGAAGGAAC-3'). The resulting fragment was cloned into pCAMBIA1300 (http://www.cambia.org.au) to produce p13000-GRP23G. After sequence verification, this construct was introduced into heterozygous seto9078 mutant plants by Agrobacterium infiltration (Bechtold and Pelletier, 1998). Transgenic plants were obtained by double selection on MS agar plates containing 50 μg/mL kanamycin and 20 μg/mL hygromycin. T1 plants were allowed to set seed after self-fertilization. Siliques were dissected and cleared with Herr’s solution (Herr, 1971) to study the restoration of the embryo-leaflet phenotype.

\[ P_{5577\text{-}GRP23}\text{-}YFP \text{ Fusion Construct and Subcellular Localization} \]

A 763-bp EYFP fragment released from pEYFP (Clontech) by XbaI digestion was cloned into pWM101 [M. Xia, unpublished data; the 3SS enhancer promoter and poly(A) were inserted into the EcoRI and HindIII sites of pCAMBIA1300] to give rise to pWM-YFP. The full-length coding sequence of GRP23 was amplified by RT-PCR using primers P36 (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P2739B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') (HindIII site, underlined) in the correct direction. The resulting fragment was then cloned into pWM-YFP at the BamHI site (underlined) in the correct direction to yield P_{5577\text{-}GRP23}\text{-}YFP. Transgenic plants expressing the fusion gene were selected on MS agar plates containing 20 μg/mL hygromycin. YFP images of the transgenic root cells were captured with confocal laser scanning microscopy with a Zeiss confocal microscope. A 514-nm argon laser and a 560- to 600-nm filter set were used for YFP scanning. DAPI staining and scanning were performed as described previously (Shi et al., 2005).

\[ \text{RNA Isolation and RT-PCR Analysis} \]

Total RNA was isolated from Landsberg erecta plants using TRizol reagent (Invitrogen). Total RNA (1 μg) was digested with RNase-free DNase (TakaRa) and used as a template to transcribe single-stranded cDNA by reverse transcriptase XL (Takara). cDNA was synthesized with the primers P1 (5'-GGATCCTGTCTCTCATCAGAGTCGTCAGAAGGAAC-3') and P3315B (5'-GGATCCTGTCTCTCATCAGAGTCGTCAGAAGGAAC-3') (HindIII site, underlined) in the correct direction. The resulting cDNA was used as a template to transcribe single-stranded RNA in the presence of RNase-free T4 RNA polymerase. PCR primers 5'-GAGTGGATCCATCAGAGTCGTCAGAAGGAAC-3' (internal controls). After 35 cycles of amplification, PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

\[ \text{Construction of the P}_{GRP23}\text{-}GUS \text{ Fusion and GUS Activity Assay of Transgenic Plants} \]

The fragment containing 2718 bp upstream of the ATG start codon plus the first 347 bp of the exon and the fragment containing the 576-bp sequence downstream of the TAG stop codon were amplified by PCR with primer pairs P25268B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P3472B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P2718B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P3151B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') (HindIII site, underlined) in the correct direction. The resulting cDNA was used as a template to transcribe single-stranded RNA in the presence of RNase-free T4 RNA polymerase. PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

\[ \text{Yeast Two-Hybrid Screen} \]

We performed the screen essentially as described previously (Xie et al., 1999). We used the yeast strain Saccharomyces cerevisiae HF7C [MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 gal80-538 lys2::GAL1UAS-GAL1TATA-HIS3 URA3::GAL4 17mers(×3)-CyC1TATA-LacZ], which contains the two reporter genes LacZ and HIS3. Yeast cells were first transformed with pBD-GRP23, a plasmid containing the full-length GRP23 coding sequence fused to the Gal4 DNA binding domain in the pGBKTK vector (a pTR1 selection marker). Then, they were sequentially transformed with the Arabidopsis cDNA library constructed in the Gal4 activation domain vector pGAD-GH (a LEU2 selection marker). This cDNA library, carrying 1.2 × 10^6 primary recombinants in pGAD-GH, was constructed from mRNA isolated from Arabidopsis Landsberg erecta suspension cells using a commercial kit (Stratagene). The transformed cells were plated on synthetic dropout selection medium that lacked Trp, Leu, and His supplemented with 5 mM 3-AT to reduce the appearance of false-positive colonies. The plates were incubated at 30°C for 3 to 8 d. β-Galactosidase activities were assayed as described (Gindullis et al., 1999).

To create pBD-GRP23, the open reading frame of GRP23 was amplified by PCR using the primers P25B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P2739B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') (HindIII site, underlined) in the correct direction. The resulting PCR product was cloned into the single BamHI site (underlined) in the pGBK7 vector in the correct direction. Similarly, the coding sequences of the PPR domain and the WQQ repeat–containing domain were amplified by using primers P36 (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') and P3315B (5'-GGATCCTGGATCCATCAGAGTCGTCAGAAGGAAC-3') (HindIII site, underlined) in the correct direction. The resulting cDNA was used as a template to transcribe single-stranded RNA in the presence of RNase-free T4 RNA polymerase. PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

\[ \text{RNA in Situ Hybridization} \]

For probe labeling, a 676-bp GRP23 cDNA fragment was amplified using primers P1 (5'-GGATCCTGTCTCTCATCAGAGTCGTCAGAAGGAAC-3') and P2739B (5'-GGATCCTGTCTCTCATCAGAGTCGTCAGAAGGAAC-3') (HindIII site, underlined) in the correct direction. The resulting cDNA was used as a template to transcribe single-stranded RNA in the presence of RNase-free T4 RNA polymerase. PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

\[ \text{DNA Is Essential for Early Embryogenesis} \]

dopsis wild-type plants by Agrobacterium-mediated vacuum infiltration (Bechtold and Pelletier, 1998). Transgenic plants were selected on MS agar plates containing 50 μg/mL kanamycin and stained in GUS staining buffer (10 mM EDTA, 0.1% Triton X-100, 4 mM FeCl3, 4 mM FeCl2, and 1 mg/mL X-Gluc [Sigma-Aldrich] in 50 mM sodium phosphate buffer, pH 7.0) for 3 d at 37°C. For ovules, stained pistils were cleared in 20% lactic acid and 20% glycerol, then observed with a Zeiss microscope and documented with a Nikon 4500 camera. For whole plants, stained seedlings were first decolorized with 95% ethanol and then observed and photographed with an Olympus SZX12 microscope equipped with a camera.
BiFC Assay

We performed BiFC assays in vivo as described (Walter et al., 2004). The open reading frame sequence of GRP23 was amplified by PCR with primers P18 (5′-CTGGGATCCATCTCCGTCACGTC-3′) and P2730X (5′-TCTCTCGAGGTCCTCAACTCAGACC-3′) and cloned into the plasmid pUC-SPY-SPY in the BamHI and XhoI sites (underlined), thus resulting in the plasmid pUC-SPYNE-GRP23 expressed as the GRP23-YFP fusion protein. Coding sequences of RBP36B (primers 5′-CTCAAGGCATGCATATGGGCACCG-3′ and 5′-GATTCTCAGTTGCGATGGAACG-3′) were also obtained by PCR and cloned into the plasmid pUC-SPY at the BamHI and XhoI sites (underlined) to give rise to the plasmid pUC-SPY-GRP23 expressed as the RBP36B-YFP fusion protein. pUC-SPYNE-GRP23 in combination with pUC-SPY-GRP23 were coated with gold particles, as were allion (Allium cepa) epidermis and bombarded using a gene gun (PDS-100/He Biolistic particle delivery system; Bio-Rad). Then, the bombarded samples were incubated on MS plates at 22°C for 24 h. Cells with YFP fluorescence were observed using confocal laser scanning microscopy as described previously.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_100901 for GRP23 (At1g10270) and NM_127100 for GRP23 (At2g15400).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. DNA Gel Blot of the Ds Insertion in grp23.

Supplemental Table 1. Seed Abortion Ratio Analysis in Complemented grp23 Plants (T1).

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