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GROWTH-REGULATING FACTOR4 of *Arabidopsis thaliana* Is Required for Development of Leaves, Cotyledons, and Shoot Apical Meristem†

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Previously, we identified a *GROWTH-REGULATING FACTOR* gene family, comprising nine members, which encodes putative transcription factors in *Arabidopsis thaliana*. The *grf1 grf2 grf3* triple mutants produced partially fused cotyledons and developed small leaves due to a reduction in cell numbers. To understand the functional role of another member of this gene family, *GRF4*, we have now identified a *grf4* null mutant and constructed a quadruple mutant by crossing it to the *grf* triple mutant. The quadruple mutant has much smaller leaves than its parental mutants, with this reduced size again due to fewer cells. Interestingly, the quadruple mutant displays not only a much stronger fusion of cotyledons but also the phenotype of the *shoot meristemless* mutant. The aberrant cotyledons of the quadruple mutants result from a fusion of cotyledon primordia during embryogenesis. These results suggest that GRF4 is required for both leaf cell proliferation and the embryonic development of cotyledons and the shoot apical meristem.

Keywords: cup-shaped cotyledon, GROWTH-REGULATING FACTOR, leaf and cotyledon development, *shoot meristemless,* transcription factors

We have previously identified and characterized the *GROWTH-REGULATING FACTOR* (*GRF*) gene family, which consists of nine members and encodes putative transcription activators (Kim et al., 2003; Kim and Kende, 2004). Compared with the single mutant, which has no apparently distinctive phenotype, the *grf1 grf2 grf3* (briefly, *grf1/2/3*) triple mutants develop small, narrow leaves whose areas are about 70% the size of wild-type leaves (Kim et al., 2003). This reduction in leaf size is due to fewer cells, which indicates that the *GRF1/2/3* genes act as positive regulators of cell proliferation to control size and shape of leaf organ in a functionally redundant manner (Kim and Kende, 2004).

In addition, *GRF1/2/3* genes appear to be involved in cotyledon development, with evidence that the *grf1/2/3* triple mutants produce partially fused cotyledons, mostly heart-shaped, although only about 10% of the mutant population displays that morphological defect (Kim et al., 2003). This fusion phenotype is reminiscent of *cup-shaped cotyledon* (*cuc*) mutants (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). Briefly, any double homozygous combinations of the *cuc1*, *cuc2*, and *cuc3* mutations produce completely fused, cup-shaped cotyledons, while none of the single mutations cause a significant defect in their development. Unlike those *cuc* double mutants, however, the *grf1/2/3* triple mutants never produce cup-shaped cotyledons (Kim et al., 2003).

Because the phenotypes of the *grf* triple mutant are weak and partial in both their leaf and cotyledon development, and because the *GRF* gene family consists of nine members, we have hypothesized that some other members of that family might be involved in these processes in a functionally redundant manner. In this current study, we investigated the function of *GRF4* because it is most closely related to *GRF1/2/3* with respect to its expression pattern and deduced amino acid sequence (Kim et al., 2003). We isolated a T-DNA insertional mutant of *GRF4* and constructed a quadruple mutant by crossing it to the *grf1/2/3* triple mutant. The resulting quadruple mutants had much smaller leaves with reduced numbers of cells than those of their parental mutants. Furthermore, they showed not only severe fusion of cotyledons, such as cup-shaped ones, but also produced seedlings that lack a shoot apical meristem (SAM). Furthermore, we observed that the fusion was due to a defect in embryonic development of cotyledon primodia.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana plants were grown in soil in a growth chamber at 23°C and under a 16-h light/8-h dark photoperiod. The *grf1/2/3* triple mutant was constructed in the Wassilewskija (Ws) accession (Kim et al., 2003), while the *grf4-1* mutant was identified from the Syngenta T-DNA insertional mutants in Columbia (Col) (Sessions et al., 2002).

Identification of T-DNA Insertional Mutant and Construction of Quadruple Mutant

Gene-specific primers used to identify the homozygous *grf4-1* mutant were HK966 (5′-CTGTCTTGTCTCATAGTCTAAACAC-3′) and HK967 (5′-CCAGAGGAGAAGAAGTGGTTGTT-3′). The left-border primer sequence for T-DNA was HK989 (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′). DNA fragments amplified with the gene-specific and left-border primers were sequenced to confirm the insertion site. This *grf4-1* mutant was crossed to the *grf1/2/3* triple mutant, and F_2 progeny with fused cotyledons were

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†This paper is dedicated to the memory of Prof. Hans Kende, who was devoted to plant biology and who passed away on Sep 26, 2006.

selected and genotyped to identify homozygous *grf1/2/3/4-1* mutants by PCR.

RT-PCR and RNA Gel Blot Analysis

Total RNA was extracted with Tri Reagent (Sigma, USA) from 10-d-old seedlings of *grf4-1* mutants. RT-PCR was performed with the following primer sets: HK1148 (5′-GCTCTGCTGCAACTAAGATATCA-′3) and HK967 (5′-CCAGAGGAGAAGAAGTGGTTGTT-3′) for *GRF4*, and HK1136 (5′-ATGAAGATTAAGGTCGTGGCA-3′) and HK1137 (5′-TCCGAGTTTGAAGAGGCTAC-3′) for the control gene *ACT8* (Kim and Kende, 2004).

Dimensional Parameters of Leaves and Palisade Cells

Digital images of detached leaves from 20-d-old plants were acquired using a scanner. Area, length, and width of leaf blades as well as petiole length were determined with the image-analyzing program SCIONIMAGE (http://www.scioncorp.com). Leaf tissues were cleared in a chloral-hydrate solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of distilled water), as described by Tsuge et al. (1996). The number of subepidermal palisade cells that were aligned along a transverse axis in the maximum-width region was then counted. To determine cell areas, 20 palisade cells grouped in the halfway from the midvein to the leaf margin at the widest point were analyzed with the SCIONIMAGE software.

Histological Analysis

Clearing of the seedlings and embryos was carried out as described by Aida et al. (1997). Briefly, seedlings or siliques were fixed overnight in 9:1 ethanol:acetic acid at room temperature. After rehydration in a graded ethanol series (90, 70, 50, and 30%) for 20 min each, the tissues were cleared in the chloral-hydrate solution.

RESULTS

Isolation and Characterization of *grf4* Mutant

To investigate the biological function of *A. thaliana GRF4*, we used PCR-assisted genotyping to isolate a homozygous T-DNA insertional mutant, *grf4-1*, from the Syngenta collection (Sessions et al., 2002). Sequencing of the PCR products confirmed that the *grf4-1* mutant in the Col background had T-DNA in the first intron (Fig. 1A). RT-PCR analysis confirmed the absence of intact mRNA in the mutant (Fig. 1A). However, the *grf4-1* mutant showed no visible alteration in leaf size and cotyledons (see below). To test whether this *grf4* mutation exerted any additive effect on the phenotype of the *grf1/2/3* triple mutant, we crossed the *grf4-1* mutant to the triple mutant, and obtained homozygous quadruple mutants of *grf1 grf2 grf3 grf4-1* (*grf1/2/3/4-1*) in the F_2 progeny.

Effect of *grf* Mutations on Leaf Size

We assessed the effect of *grf* mutations on leaf size in 20-d-old plants. Because a portion of the population for the *grf* triple and quadruple mutants develops fused cotyledons,

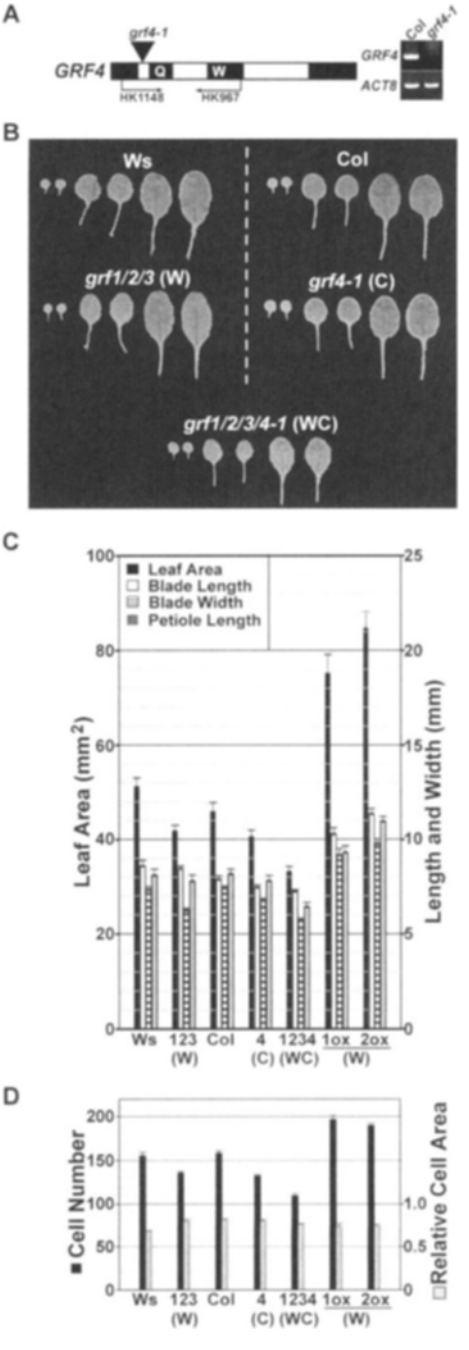

Figure 1. Leaf phenotype of *grf* mutants. (**A**) Schematic structure of *GRF4* gene showing T-DNA insertion site. Inverse triangle indicates T-DNA integration site; black and white boxes, exons and introns, respectively; Q and W, the conserved QLQ and WRC domains of GRF proteins, respectively. RT-PCR analysis of *GRF4* mRNA is shown to the right of drawing. HK1148 and HK967 are forward and reverse primers for PCR amplification. (**B**) Cotyledons and first to fourth rosette leaves were detached and aligned. *grf1/2/3* triple mutant is in Ws background (W); *grf4-1*, Col (C); *grf1/2/3/4-1*, a mixed background (WC). (**C**) Dimensional parameters of first two leaves (n=10). The mark 123 below vertical bars indicates *grf1/2/3*; 4, *grf4-1*; 1234, *grf1/2/3/4-1*; 1ox and 2ox, overexpressors of *GRF1* and *GRF2*, respectively. (**D**) Numbers and areas of palisade cells from first two leaves. Numbers of cells aligned in a transverse line at maximum width of leaves were determined (n=10). Areas of 20 cells grouped in middle between midvein and leaf edge at maximum width were measured and presented at arbitrary scale, where cell area of Ws corresponded to 4086 ± 40 μm^2. Error bars indicate standard error.

causing the delayed emergence of true leaves (Kim et al., 2003), we selected mutant individuals whose cotyledons were developmentally normal. The *grf1/2/3* triple mutant displayed smaller and narrower leaves than its corresponding wild-type Ws (Fig. 1B, left), whereas the *grf4-1* single mutant was almost indistinguishable from its corresponding wild-type Col (Fig. 1B, right). In contrast, the *grf1/2/3/4-1* quadruple mutant had much smaller leaves than those of the parental mutants (Fig. 1B, below). The number of rosette leaves and inflorescence heights did not differ significantly from those of their parental mutants (data not shown).

For quantitative analysis, we measured dimensional parameters of the first two true leaves from the wild types and mutants (Fig. 1C). This quantification revealed that, compared with Col, leaf area of *grf4-1* mutant was reduced by 11%, as a result of slight decreases in leaf width and length. In contrast, both the leaf area and petiole length of *grf1/2/3/4-1* were further reduced, by about 18% over *grf1/2/3* and by about 30% over *grf4-1*. This remarkable decline in leaf area was due to reductions in both leaf width and length. Compared with their corresponding wild types, leaves of the *grf1/2/3* and *grf4-1* mutants had significantly fewer palisade cells along a transverse axis at the maximum width, and the *grf1/2/3/4-1* quadruple mutant had much smaller numbers of palisade cells than did the parental mutants (Fig. 1D). However, cell areas generally did not differ from each other except that those from the *grf1/2/3* triple mutant and wild-type Col were slightly larger than from Ws (Fig. 1D).

We also re-estimated the effect of *GRF1* and *GRF2* overexpression on the numbers of palisade cells in the first two leaves, because we previously (Kim et al., 2003) had used calculations rather than direct counting to determine the numbers of petiolar epidermal cells but not palisade cells. Here, the leaves of both *GRF1* and *GRF2* overexpressors were larger and contained more palisade cells than those of their corresponding wild-type Ws (Fig. 1C, D).

Effect of *grf* Mutations on Development of Cotyledon and Shoot Apical Meristem

We examined cotyledon development in 10-d-old seedlings with various combinations of *grf* mutations. By 10 d after germination, the wild-type plants had formed two separate cotyledons, the first two larger leaves, and the third and fourth smaller ones (Fig. 2A). In contrast, none of the single and double mutants showed any significant alterations in their cotyledon development (Fig. 3). However, 14% of the *grf1/2/3* triple mutants produced partially fused cotyledons -- petiolar, heart-type, or single-type fusions -- that were similar to those shown in Figure 2B through D, while the remainder of the triple mutant population had normal-type cotyledons (Fig. 2, 3). Finally, the *grf1/2/3/4-1* quadruple mutants displayed not only those partially fused cotyledons but also much more severely fused cotyledons than in the triple mutants, including spoon- and cup-shaped cotyle-

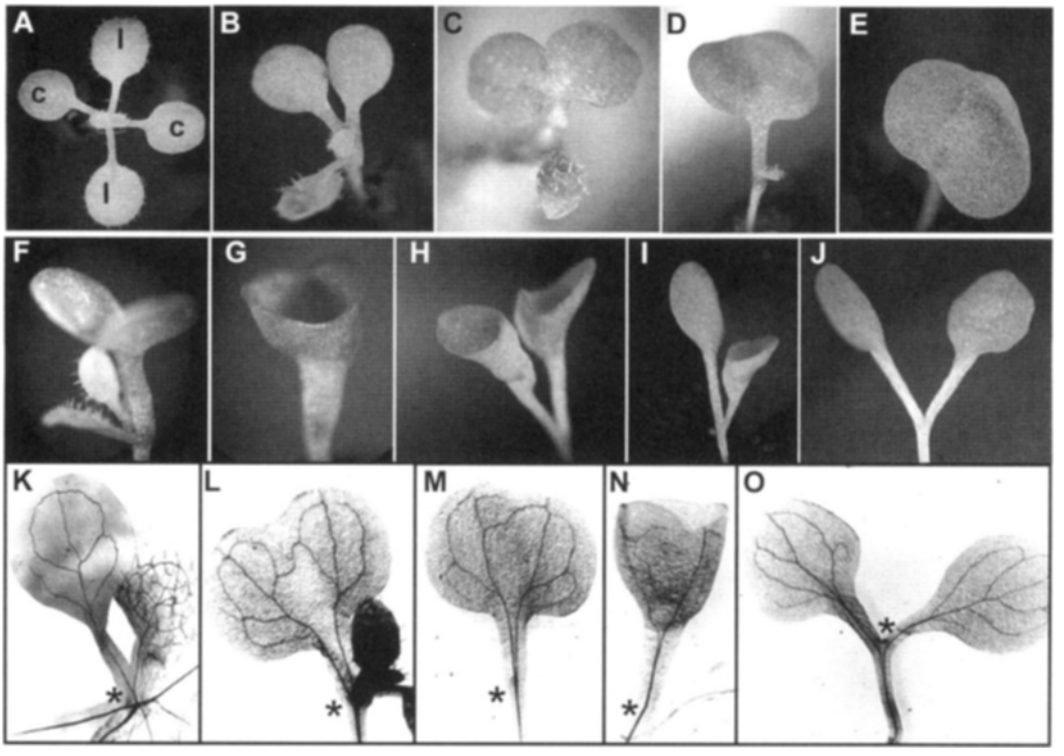

Figure 2. Seedling phenotypes and vasculature structures 10 d after germination. (**A**) Wild-type. (**B-J**) *grf1/2/3/4-1* mutants: (**B**) petiolar fusion of cotyledons, (**C**) heart-type, (**D**) single-type, (**E**) spoon-shaped, (**F**) true leaves jutting from fused cotyledons, (**G**) cup-shaped, (**H**) two-cup *stm*-type, (**I**) one-cup *stm*-type, (**J**) *stm*-type without fusion. The marks c and l indicate cotyledon and leaf, respectively. (**K-O**) Vasculature phenotypes. Digital images of stereoscopic pictures were contrasted inversely to highlight vascular strands, using Adobe Photoshop software. Asterisks indicate SAM site. (**K**) One cotyledon from wild type is in view. (**L-O**) *grf1/2/3/4-1* mutants: (**L**) heart-type cotyledon, (**M**) single-type, (**N**) cup-shaped, (**O**) *stm* type.

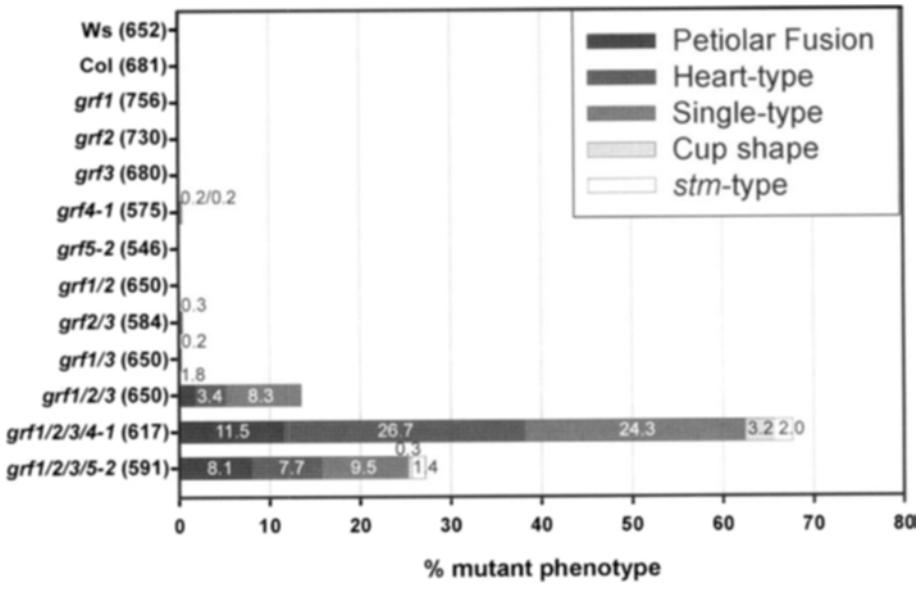

Figure 3. Frequencies of cotyledon fusion in multiple mutants 10 d after germination. Percentages of various fusion types were scored in progeny of normal-type mutants. Values in or over bars indicate percentage of specific fusion type; numbers in parentheses, numbers of seedlings examined.

dons (Fig. 2B-G). In addition to the two-cotyledon fusion, some of the quadruple mutants, albeit rarely, had two separate cotyledons, at least one of which having developed into an independent cup shape without the involvement of the other (Fig. 2H, I). Interestingly, a significant portion of the quadruple mutant population showed the typical phenotype of the *shoot meristemless* (*stm*) mutant, i.e., lacking a shoot apical meristem (SAM; Fig. 2J) (Barton and Poethig, 1993). In fact, the mutant seedlings shown in Figure 2E, G, H, and I also lacked the activity of a SAM function, as demonstrated by the absence of any developing true leaves. Although mutant plants with petiolar, heart-type, and, often, single-type fusions did produce true leaves, their formation was delayed and aberrant (Fig. 2B-D). These true leaves were sometimes trapped in the petioles, protruding out from the fused cotyledons (Fig. 2F).

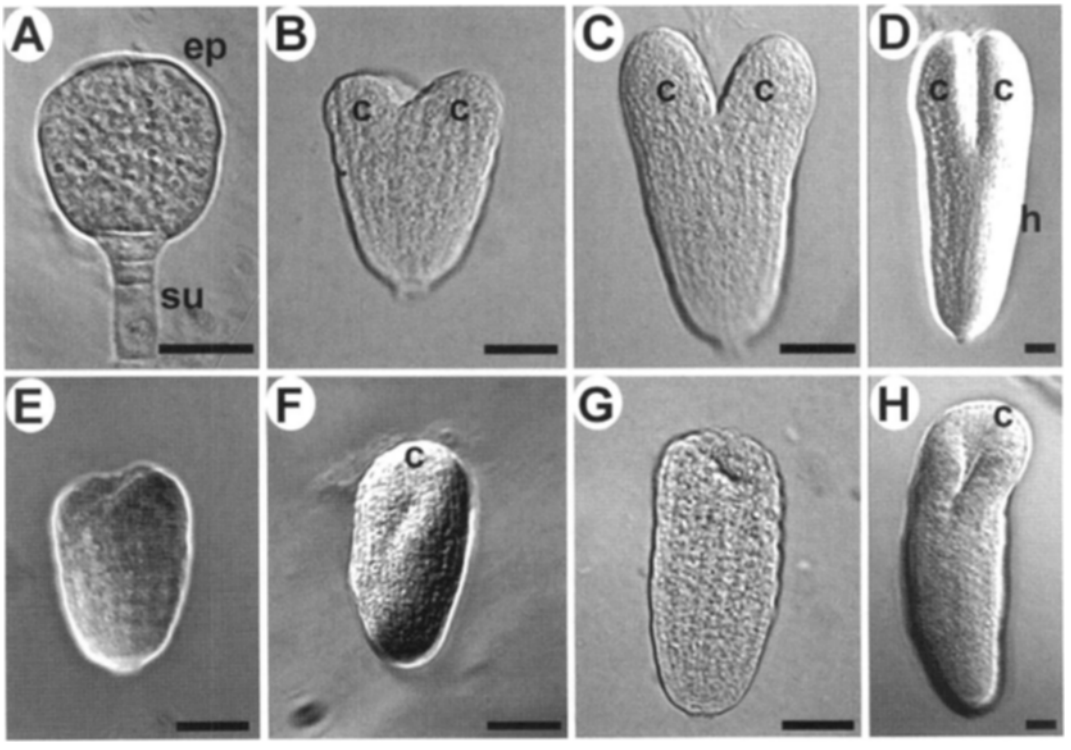

Figure 4. Embryonic defects of *grf1/2/3/4-1* mutant. Developing embryos were cleared, separated from ovules, and observed with Normarski optics. (**A-D**) Wild-type embryos: (**A**) globular, (**B**) early heart, (**C**) early torpedo, (**D**) torpedo stages. (**E-H**) *grf1/2/3/4-1* embryos: (**E, F**) early heart, (**G**) early torpedo, (**H**) torpedo stages. c, cotyledon primordium; ep, embryo proper; h, hypocotyls; su, suspensor. Scale bars, 20 μm.

To examine whether those abnormal cotyledons resulted from two cotyledons that had been fused together, we compared their vascular structure with normal cotyledons. The typical vasculature strands of a wild-type cotyledon had one midvein running along the longitudinal axis, with two symmetric lateral loops (Fig. 2K). In contrast, the heart- and single-type cotyledons had two midveins with adjacent lateral veins connected together (Fig. 2L, M), while the cup-shaped cotyledons also displayed two midveins, front and back (Fig. 2N). In addition, we determined that the cup-shaped and *stm*-type seedlings, as well as most single-cotyledon types, revealed no trace of a SAM (Fig. 2M-O) whereas the wild-type plants showed well-developed vasculature strands of true leaves (Fig. 2K).

The effect of *grf* mutations was quantified in detail (Fig. 3). Introducing the *grf4-1* mutation into the *grf1/2/3* mutant greatly enhanced the frequency of the fusion phenotype, to 67%. The majority (51%) of the quadruple mutants had heart- and single-type cotyledons. Cup-shaped and *stm*-type seedlings were present at a low but significant frequency in the quadruple mutants -- 3.2% and 2.0%, respectively -- but never in the triple mutants.

Embryogenesis of *grf1/2/3/4-1* Mutants

At the globular stage, a wild-type embryo consisted of a spherical embryo proper and a filamentous suspensor (Fig. 4A). Because of prominent cell divisions at the bilateral ends of the upper region, the embryo proper subsequently formed two bulges that developed into cotyledon primordia, taking a heart shape (Fig. 4B). Afterward, the cotyledon primordia and hypocotyl region elongated to become a torpedo-shaped embryo (Fig. 4C, D). Up to the globular stage, no differences were visible between our wild-type and *grf1/2/3/4-1* embryos (data not shown). However, we were able to observe that the quadruple mutant embryos at that early heart stage formed an all-round or only a one-bulge shape, lacking the bilateral symmetry (Fig. 4E, F). At later stages, the mutant phenotype became more apparent, mostly resulting in a single, spatula-shaped cotyledon by the torpedo stage (Fig. 4G, H). These results demonstrate that aberrant cotyledons of the quadruple mutants were caused by a fusion event that occurred at the early heart stage in embryogenesis.

DISCUSSION

We have shown here that the *grf4-1* mutation, when combined with the *grf* triple mutations, results in a great reduction in cell numbers, and, thus, small leaves (Fig. 1). This suggests not only that *GRF4* is involved in regulating cell numbers in the leaf organ but also that *GRF1* through *GRF4* act in a highly redundant manner. Consistent with this notion of functional redundancy, the structures of the four gene products and our previous phylogenetic analysis indicate that, among the GRF members, these four are most closely related to each other, and that the tissue-specific expression pattern of *GRF1* through *GRF4* also overlaps, with comparable levels of mRNAs (Kim et al., 2003). One might argue that the smaller leaf size and cell numbers from the quadruple mutant may simply be due to the effect of their mixed genetic background of different accessions, i.e., Ws and Col. That possibility, however, can be ruled out because the *grf4-1* mutation itself causes a slight but significant reduction in cell numbers and leaf size. Horiguchi et al. (2005) also have reported that an *Arabidopsis* mutant allele of *GRF5*, *grf5-1*, has slightly smaller leaves and that overexpression of *GRF5* induces bigger leaves, both phenomena resulting from changes in cell numbers. This further implicates a general role for the *GRF* gene family in regulating cell proliferation in the leaf organ.

Functional redundancy among the *GRF1* through *GRF4* genes also seems to hold true for the developmental process of cotyledon separation. This is supported by the fact that more mutations of those genes cause more frequent and much stronger fusion of cotyledons, which result from developmental defects during embryogenesis (Fig. 2, 3, and 4). It seems that functioning of the *GRF* genes somehow should be associated with that of the *CUC* gene family because the *grf1/2/3/4* mutants produce a significant portion of cup-shaped cotyledons, which is the typical phenotype of *cuc* mutants (Fig. 2, 3) (Aida et al., 1997; Vroemen et al., 2003). We have also found that the fusion phenotype of the *grf1/2/3* triple mutation is synergistically enhanced by *cuc2* mutation (JHK, unpublished data), further supporting the notion of a functional relationship between the two gene families.

STM is a homeobox gene required for SAM maintenance; its loss-of-function mutants lack a SAM (Barton and Poethig, 1993; Long et al., 1996). Here, we discovered that a small but significant proportion of the *grf* quadruple mutants displayed the typical *stm* phenotype without cotyledon fusion (Fig. 2, 3). Even a majority of the single-type cotyledons, in addition to the cup-shaped cotyledons, lacked a SAM. These results suggest that *GRF* genes are also involved in SAM development.

CUC and *STM* genes are expressed in an overlapping region that has presumptive SAM fate early in embryogenesis (Long et al., 1996; Aida et al., 1999; Takada et al., 2001). *CUC* has been proven to be required for *STM* expression because *STM* mRNAs are not detected in the *cuc1 cuc2* double mutants, thereby accounting for the lack of a SAM in the double mutant (Aida et al., 1999). In the *stm* mutant background, the expression patterns of *CUC1* and *CUC2* are perturbed, providing an explanation for the slight fusion phenotype of *stm* cotyledons (Aida et al., 1999; Aida and Tasaka, 2006). Therefore, it is very interesting that our *grf* quadruple mutant displayed both *cuc* and *stm* characteristics. Molecular studies on the developmental phenotype of the *grf* mutants may provide important insight into explaining how the *GRF* genes are involved in the current context of *CUC-STM* function while also adding to our understanding of cotyledon and SAM development.

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