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Genetic interaction between GROWTH-REGULATING FACTOR and CUP-SHAPED COTYLEDON in organ separation

Byung Ha Lee[1,3], Jae Og Jeon[1,3], Myeong Min Lee[2], and Jeong Hoe Kim[1,*]

[1]Department of Biology; Kyungpook National University; Daegu, Korea; [2]Department of Systems Biology; Yonsei University; Seoul, Korea

[3]These authors contributed equally to this work.

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The *Arabidopsis thaliana GROWTH-REGULATING FACTOR* (*GRF*) gene family comprises 9 members and encodes a class of transcription factors. We previously demonstrated that *GRF* genes played an essential role in formation of the boundary region between cotyledons, since their loss-of-function mutants developed fused cotyledons. Our present study shows that the *grf* mutants display fused floral organs as well. Such fusion phenotypes of embryonic and post-embryonic floral organs are highly reminiscent of the *cup-shaped cotyledon* (*cuc*) mutants. In order to test a genetic interaction between *GRF*s and *CUC*s, we constructed *cuc1 grf1/2/3, cuc2 grf1/2/3,* and *cuc3 grf1/2/3* quadruple mutants, and found that the mutants showed dramatic increases in cotyledon fusion as well as floral organ fusion. The results suggest that the signaling pathway of *GRF*s may be genetically associated with that of *CUC*s in the organ separation process.

Dicot plants develop 2 separated cotyledons, whose primordia arise bilaterally from the upper region of a globular embryo. The shoot apical meristem (SAM), the origin of all above-ground organs, is formed between the 2 cotyledon primordia.[1-4] Because the bilateral separation of cotyledons is spatiotemporally interconnected with SAM formation, failure in separation of cotyledon primordia frequently leads to abortion of SAM formation in diverse dicots, consequently resulting in no production of post-embryonic organs.[5-7] Therefore, the precise formation of the boundary region between cotyledons is critical for establishment of the plant body.

In *Arabidopsis thaliana* (Arabidopsis), the *CUP-SHAPED COTYLEDON* (*CUC*) genes play an essential role in both cotyledon separation and SAM formation.[6,8-10] The *CUC* genes, *CUC1* to *CUC3,* encode transcription factors with an NAC domain in the N terminus.[11,12] Although single mutations of *CUC*s did not cause any significant defects, double or triple mutations resulted in severe cotyledon fusions, indicating that the *CUC* genes are required for the cotyledon separation process in a functionally redundant manner.[6,9,10] In brief, approximately 25% of a segregating progeny of the *cuc1–1/cuc1–1 cuc2–1*/+ mutant (+ for wild-type allele; hereafter, simply *cuc1 cuc2*/+) developed cup-shaped cotyledons due to complete fusion of 2 cotyledons, and thus had no SAM tissue, indicating that *cuc1 cuc2* double homozygous mutants failed to form the organ boundary and SAM. In addition, a small fraction of the segregating progeny developed partially fused cotyledons, resulting in heart-shaped cotyledons. Importantly, *CUC* genes are also required for separation of post-embryonic floral organs.[6,10] For instance, *cuc1 cuc2* double homozygous mutants developed fused sepals alongside their margins as well as fused stamens.

We have previously identified and characterized the *GROWTH-REGULATING FACTOR* (*GRF*) family in Arabidopsis, which consists of 9 members and encodes a class of transcription factors.[13] Compared with the wild-type plant, the *grf1 grf2 grf3* (*grf1/2/3*) and *grf1/2/3/4–1* mutants developed small leaves, whereas each single mutant had no visible phenotype at all.[13,14] Analysis of cellular parameters showed that *GRF1* to *GRF4* act as positive regulators of cell proliferation in lateral organs, such as leaves and flowers.[14,15] We have also shown that *GRF1* to *GRF4* are required for formation of the cotyledon boundary and SAM tissues in a functionally redundant manner. Briefly, approximately 10% of *grf1/2/3* triple mutants produced partially fused cotyledons, and addition of the *grf4–1* mutation into the triple mutant dramatically enhanced the cotyledon fusion phenotype, up to approximately 60%, resulting in development of heart-shaped, single-cotyledon-like (shortly, single-like), and cup-shaped cotyledons.[14] The majority of single-like and cup-shaped mutant plants also lacked a functional SAM, consequently producing no shoot system. All of these defects stem from a failure in separation of

*Correspondence to: Jeong Hoe Kim; Email: kimjeon4@knu.ac.kr
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cotyledon primordia that form early in embryogenesis. It is therefore conceivable that the multiple *grf* mutants are highly similar to *cuc* mutants in regard to the cotyledon and SAM development, which thus suggests a possible association of *GRF*s with *CUC*s in the organ separation process.

Because *cuc1 cuc2* mutants displayed fused sepals and stamens,[6,10] we examined whether or not the *grf1/2/3* and *grf1/2/3/4–1* mutants developed similar floral defects. As a result, we found that a minute portion of those mutant flowers (11.5 and 15%, respectively) showed various fusion types between the same organs or between different organs, whereas only an ignorable portion of flowers from their constituent single mutants did so (**Table 1**). The result indicates that *GRF* genes are also involved in the separation process of floral organs.

Such fusion phenotypes of embryonic and post-embryonic floral organs of *grf* mutants are highly reminiscent of the *cuc* mutants. In order to test the possibility of a genetic interaction between *GRF*s and *CUC*s, we crossed the *grf1/2/3* triple mutant with *cuc* single mutants (*cuc1*, *cuc2*, *cuc3–1*, and *cuc3–2*), and isolated 4 different *cuc grf* quadruple mutants in the F_3 progeny. To our surprise, we found that all of the resulting *cuc grf* quadruple mutants showed a dramatic increase in cotyledon fusion, to the extent of approximately 50 to almost 100%, producing a variety of fusion types: petiolar, heart-shaped, single-like, cup-shaped, *shoot meristemless* (*stm*)-like, and butterfly-shaped cotyledons (**Fig. 1**). To the contrary, their parental lines, i.e, the *grf1/2/3* triple and *cuc* single mutants, showed a slight or almost no fusion phenotypes, respectively (**Fig. 1**). The result is clearly indicative of a genetic synergism between the *grf* and *cuc* mutations. In more detail, the majorities of *cuc1 grf1/2/3*, *cuc2 grf1/2/3*, and *cuc3–1 grf1/2/3* fusion types are heart-shaped and single-like cotyledons with a low proportion of cup-shaped cotyledons. It should be noted, however, that *cuc grf* quadruple mutants had

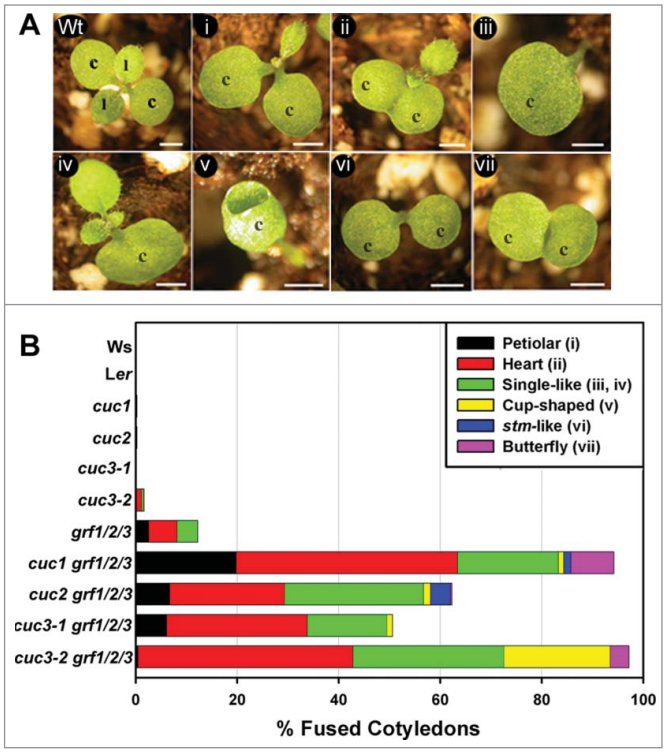

Figure 1. Cotyledon phenotypes of *cuc* and *grf* mutants. (**A**) Fusion types. Wt, Wild-type or wild-type-like mutant seedling with 2 separated cotyledons and young leaves; i, petiolar fusion; ii, heart-shaped; iii, single-cotyledon-like with no SAM; iv, single-cotyledon-like with a functional SAM; v, cup-shaped; vi, *stm*-like; vii, butterfly-shaped. The last 3 types rarely develop leaves, which is indicative of no SAM tissue. The marks c and l indicate cotyledons and leaves, respectively. Bars = 3mm. (**B**) Frequencies of cotyledon fusions. More than 500 seedlings from each genotype were examined.

Table 1. Percentages of floral fusions of *grf* and *cuc* mutants[a]

Genotype	Intra-Sepals	Intra-Petals	Intra-Stamens	Sepal-Petal	Petal-Stamen	Stamen- Pistil	Total[b]
Ws	—[d]	—	—	—	—	—	—
Col	—	—	—	—	—	—	—
Ler	—	—	—	—	—	—	—
grf1 (Ws)[c]	0.5	—	2.5	—	—	0.5	3.5
grf2 (Ws)	—	—	—	—	0.5	—	0.5
grf3 (Ws)	—	—	—	0.5	0.5	—	1.0
grf4-1 (Col)	—	—	0.5	—	—	—	0.5
grf1/2/3	1.0	0.5	4.0	2.0	3.5	1.5	11.5
grf1/2/3/4-1	1.0	1.0	3.0	1.5	8.0	0.5	15.0
cuc1 (Ler)	19.0	—	2.0	—	—	—	20.0
cuc2 (Ler)	3.5	—	0.5	—	—	—	4.0
cuc3-1 (Ler)	3.5	—	—	0.5	1.0	—	5.0
cuc3-2 (Ws)	0.5	—	0.5	—	1.5	1.0	3.5
cuc1 grf/1/2/3	91.0	—	0.5	—	1.5	1.0	91.5
cuc2 grf/1/2/3	12.5	—	2.0	3.0	2.0	1.5	19.5
cuc3-1 grf1/2/3	18.0	1.0	4.0	—	0.5	3.5	25.0
cuc3-2 grf1/2/3	6.0	1.0	0.5	—	13.0	81.0	83.5

[a]Two hundreds of flowers from 10 individual plants of each genotype were examined.
[b]Total percent is a sum percent after subtraction of numbers of plants with overlapping intra- or inter-fusions.
[c]The parenthesis denotes accessions.
[d]The dash indicates 0.0 percent.

a mixed genetic background, because *cuc1*, *cuc2*, and *cuc3–1* mutant alleles were in Landsberg *erecta* (L*er*) and the *grf1/2/3* mutant in Wassilewskija (Ws)(**Table 1**).[6,9,13] Therefore, it cannot be ruled out that the genetic synergism may, at least in part, be associated with such genetic promiscuity. To test the possibility, we constructed another quadruple mutant with pure Ws background, *cuc3–2 grf1/2/3*, in which all mutant alleles originated from Ws accession. We found that almost all of the *cuc3–2 grf1/2/3* quadruple mutants developed fused cotyledons (**Fig. 1**), indicating that the remarkable synergism was, indeed, due to mutations in *GRF* and *CUC* genes, but not due to the genetic promiscuity. Notably, the *cuc3–2 grf* quadruple mutants displayed a much higher proportion of cup-shaped cotyledons (the strongest fusion phenotype), by 20%, than other quadruple mutants did, and a much less proportion of the petiolar fusion type (the weakest phenotype), indicating that *cuc3–2 grf1/2/3* is the strongest mutant among other *cuc grf* quadruple mutants. The reason for this is that *CUC3* may contribute more to establishment of the cotyledon boundary.[9] Alternatively, it is possible that Ws accession may be more vulnerable to loss of *GRF* and *CUC* function. Differences in the phenotypic strength between *cuc3–1 grf1/2/3* and *cuc3*–2 g*rf1/2/3* mutants are likely because *cuc3–2* is a null allele while *cuc3–1* is a hypomorphic one.[9] Besides, *stm*-like plants, whose cotyledons were not distinctively fused but completely lacked a functional SAM (**Fig. 1A**),[14] were observed with a relatively high frequency in the *cuc2 grf*

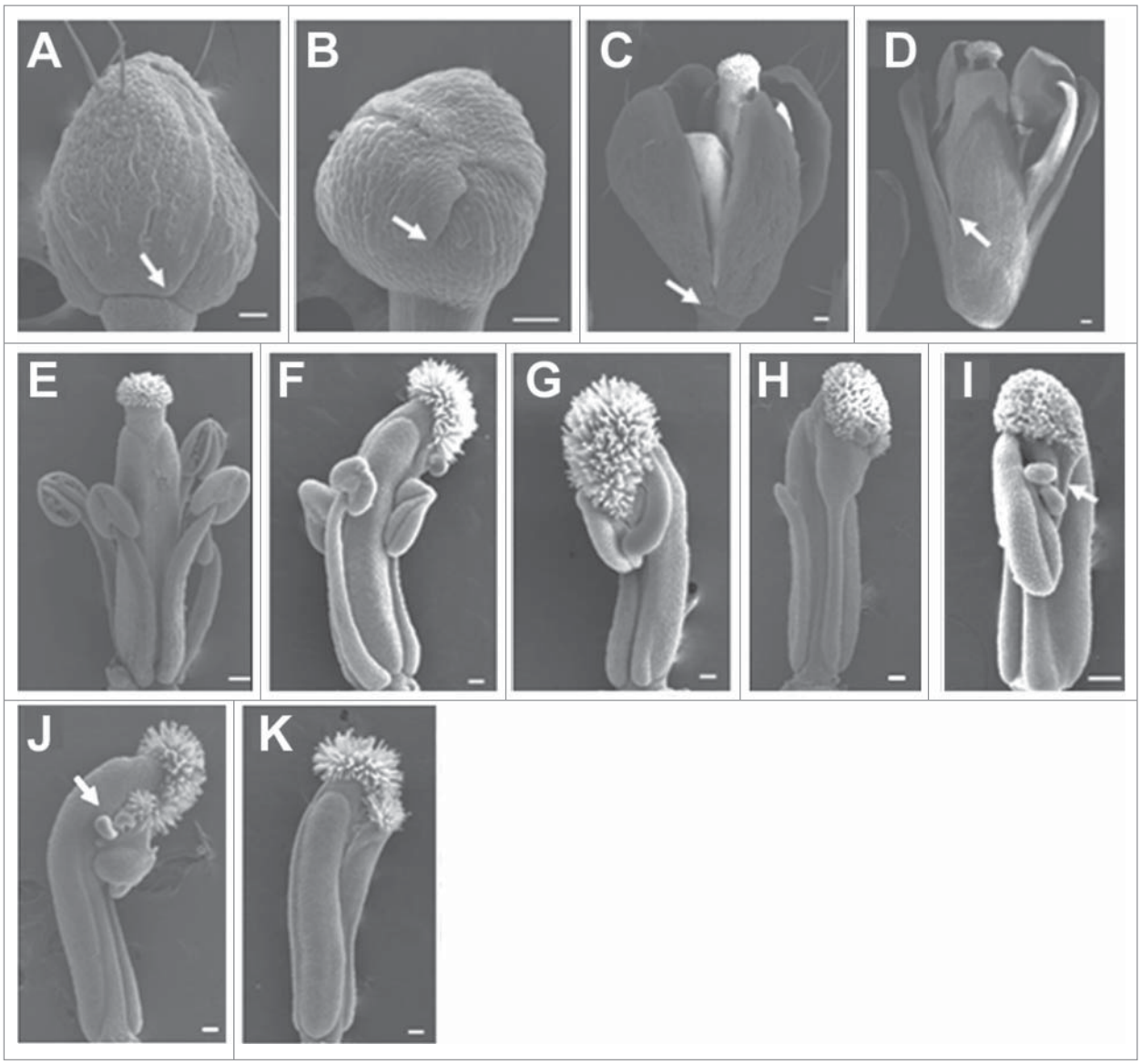

Figure 2. Scanning electron micrographs of floral organs. (**A**) and (**C**) Wild-type flowers. Sepals are separated from the receptacle (arrows). Bars = 50 μm. (**B**) and (**D**) Fused sepals of *cuc1 grf1/2/3* flowers. Arrows indicate the uppermost point of the fused parts. Bars = 100 μm. (**E**) Wild-type flower. Sepals and petals were removed for exposure of the pistil and stamens. Bar = 100 μm. (**F–K**) The *cuc3–2 grf1/2/3* flowers. Stamens are fused to the pistil. Sepals, petals, and unfused stamens were removed. Arrows indicate ovule-like protrusions. Bars = 100 μm.

quadruple mutant, probably because the *CUC2* gene played a pivotal role especially in SAM formation.[6,9]

In addition to cotyledon fusion, we found that floral organ structures are also severely affected by *cuc grf* quadruple mutations (**Fig. 2**; **Table 1**). For example, almost all sepals of *cuc1 grf1/2/3* flowers were fused to each other along their margins from the middle part to the basal end, whereas only 19% of *cuc1* sepals and almost no *grf1/2/3* sepals showed the fusion phenotype (**Fig. 2A-D**; **Table 1**). The other *cuc grf* quadruple mutants also showed a synergistic effect on sepal fusion, but with a low frequency (6 to 18% of flowers). Another distinctive fusion type was found in the *cuc3–2 grf* quadruple mutant. That is, while all 6 wild-type stamens were normally separated from the pistil (**Fig. 2E**), some stamens of most *cuc3–2 grf* quadruple mutants were fused to the pistil along the filament (**Fig. 2F**) or along the whole stamen (**Fig. 2G-K**). The fusion boundary sometimes bore ovule-like structures (**Fig. 2I and J**), and fused anthers often developed papillar tissues at the tip (**Fig. 2H, J, and K**). Quantitatively, 81% of *cuc3–2 grf* quadruple mutant flowers showed the stamen-to-pistil fusion, and, additionally, 13% of them showed petal-to-stamen fusion (**Table 1**).

Conclusion and Perspective

In conclusion, the *cuc* and *grf* mutations acted synergistically to enhance not only cotyledon fusion but also intra- (sepal-to-sepal) or inter-floral organ fusion (petal-to-stamen and stamen-to-pistil), indicating that the signaling pathways of *GRF* and *CUC* genes may be associated in the organ separation process during embryogenesis and floral organ formation.

Based on the synergism between the *grf* and *cuc* mutations, it is probable that the *GRF* and *CUC* genes may act in a common pathway leading to organ separation. One possibility is that *GRF* and *CUC* genes might act in a downstream/upstream manner. However, overexpression of *GRF1* and *GRF2* did not ameliorate the fusion phenotypes of *cuc1 cuc2* at all, and neither did overexpression of *CUC1* and *CUC2* affect the fusion phenotypes of the *grf* mutant, thus nullifying the possibility (data not shown). Alternatively, their signal transduction pathways may act in parallel to each other, although, in this scenario, it is hard to explain the dramatic synergism displayed by *cuc* and *grf* mutations. Lastly, it is tempting to speculate that GRF and CUC proteins may form a functional complex to establish the organ boundary. In fact, we have observed that GRF1 proteins bind CUC1 and CUC2 in an in vitro assay (data not shown), supporting the last notion. Yet, it should be mentioned that more experimental supports are required for the notion to be confirmed; the supporting data should include evidence for *in planta* interaction between GRF and CUC proteins and for compatible expression patterns of *CUC*s and *GRF*s. With all things considered, we believe that, in the light of importance of the *CUC* and *GRF* genes in plant development, this notion is worthy of further pursuit in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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