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Title: Biological roles and an evolutionary sketch of the GRF-GIF transcriptional complex in plants

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ABSTRACT

GROWTH-REGULATING FACTORs (GRFs) are sequence-specific DNA-binding transcription factors that regulate various aspects of plant growth and development. GRF proteins interact with a transcription cofactor, GRF-INTERACTING FACTOR (GIF), to form a functional transcriptional complex. For its activities, the GRF-GIF duo requires the SWITCH2/SUCROSE NONFERMENTING2 chromatin remodeling complex. One of the most conspicuous roles of the duo is conferring the meristematic potential on the proliferative and formative cells during organogenesis. GRF expression is post-transcriptionally down-regulated by microRNA396 (miR396), thus constructing the GRF-GIF-miR396 module and fine-tuning the duo's action. Since the last comprehensive review articles were published over three years ago, many studies have added further insight into its action and elucidated new biological roles. The current review highlights recent advances in our understanding of how the GRF-GIF-miR396 module regulates plant growth and development. In addition, I revise the previous view on the evolutionary origin of the *GRF* gene family.

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Transcription factors control gene expression and thus regulate the patterns of plant growth and development. The number of transcription factors in *Arabidopsis thaliana* (Arabidopsis hereafter) has been estimated to be more than 2,000, which is comparable to that in humans (1-4). Besides the large numbers, a significant portion of them are present only in plants (4, 5). One class of plant-specific transcription factors, GROWTH-REGULATING FACTOR (GRF), was first identified in rice and Arabidopsis (the notion of 'plant-specific' needs revision, as described in the last section below), and found to exist in multiple homologous copies: Arabidopsis and rice have nine and twelve members, respectively (6-8). Later, GRF proteins were found to interact with GRF-INTERACTING FACTORs (GIFs) in Arabidopsis, which form a small family of three members: AtGIF1 (aka ANGUSTIFOLIA3, AN3), AtGIF2, and AtGIF3 (9, 10). Since then, many studies have demonstrated that GRFs and GIFs are *bona fide* interacting partner proteins that form a functional unit, and that the GRF-GIF complex plays essential roles in various aspects of plant growth and development (for review, see 11-13). It has also been well documented that microRNA396 (miR396) post-transcriptionally regulates the majority of *GRF* members and fine-tunes their expression, thus controlling GRF-GIF-dependent processes (14, 15).

It has been more than three years since the last comprehensive review articles were published on the GRF-GIF-miR396 module (11-13). During that time, many reports have been published, elucidating its new biological roles and identifying its downstream and upstream genes as well as target *cis*-elements. The current review highlights recent studies that have increased the understanding of the regulatory module. It also revises the previous view on the evolutionary origin of the *GRF* gene family.

What are GRF and GIF?

GRF proteins contain two highly conserved QLQ and WRC domains in the N-terminal

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half (6-8). The QLQ domain consists of highly conserved Gln-Leu-Gln (QX_3LX_2Q) and neighboring residues. The QLQ domain provides an interface for interacting with GIFs (9, 10). The WRC domain consists of the conserved spacing of three Cys and one His residues ($CX_9CX_{10}CX_2H$, simply the C_3H motif), which acts as a DNA-binding domain (DBD) (6, 7, 16-18). The C-terminal regions of GRF proteins are highly variable in length and composition of amino acid residues, and they function as a transactivation domain (7-10). AtGRFs with truncated C-termini have been shown to lose their transactivation activities, while OsGRF10 (rice) and ZmGRF10 (maize) with short C-termini have also exhibited no activities (9, 19, 20).

GIFs were identified by their capability interacting with GRFs and characterized as transcription cofactors with no DBD (9, 10). The interacting partnership between almost all members of the two protein families has been demonstrated in all plants tested (19-25). GIF proteins have the highly conserved SNH domain in the N-terminus that directly interacts with the GRF QLQ domain. The C-terminal regions of GIFs exert transactivation activities and are rich in Gln (Q) and Gly (G), and are thus called the QG domain. *GIF* genes are more ancient in terms of evolutionary origin than GRFs, and they exist in major lineages of eukaryotes, including humans, in which they are called SYT (synovial translocation protein), aka SS18 (synovial sarcoma associated protein) (26, 27).

Potential *cis*-elements bound by GRF and GIF

As transcription factors with the WRC DBD, GRFs are expected to regulate the expression of downstream target genes and bind to specific regulatory *cis*-elements in them. A GRF-targeting *cis*-element (GTE), TGTCAGG, was first identified in the promoter of *DEHYDRATION-RESPONSIVE ELEMENT BININDING PROTEIN2A* (*DREB2A*) in Arabidopsis: AtGRF7 bound to the GTE and led to the repression of *DREB2A* expression (Table 1) (17). AtGRF9 bound to the promoter region of a bZIP transcription factor gene,

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OBP3-RESPONSIVE GENE3 (*ORG3*), whose promoter contains a potential GTE, CTGACA (28); rice GRFs (OsGRF6 and/or OsGRF10), to a GTE (TGTGTTG) of *OsJMJ706* (a *JMJD2* family *jmjC* gene) and *OsCR4* (a gene for crinkly4 receptor-like kinase), upregulating their expression (19); OsGRF6, to CGSMR in the promoters of *AUXIN RESPONSE FACTOR2* (*ARF2*), *ARF7*, and an *YUCCA*-like gene, whose expression is up-regulated by *OsGRF6* overexpression (29).

Aside from GTEs, chromatin immunoprecipitation assays (ChIP) revealed that AtGIF1/AN3 proteins were strongly associated with the G-box and GAGA elements in the Arabidopsis genome, and that these elements were found to reside in the promoters of some target genes, including *CONSTANS-LIKE5* (*COL5*) and *HECATE1* (*HEC1*; Table 1) (23). Another ChIP assay using AtGIF1/AN3 as bait revealed the strong enrichment of a promoter region of *PLETHORA1* (*PLT1*), which contains a *cis*-element, TGTCAGA (30). Since AtGIF1/AN3 lacks a DBD, its association with *cis*-elements is made possible when it works in concert with transcription factors with DBDs, such as GRFs. Therefore, the high similarity between the elements found in *DREB2A* and *PLT1*, which were associated with AtGRF7 and AtGIF1/AN3, respectively, may not be the result of a coincidence. Nevertheless, the systematic inference and experimental verification of a canonical or consensus GTE still seems to be premature, or GTEs may be variable depending on the classes of GRFs and/or plant species. Indeed, the first GTE found in *DREB2A* was associated only with AtGRF7, and not with any other AtGRF members (17).

Molecular function of the GRF-GIF duo in the transcriptional regulation

As mentioned above, ChIP assays revealed that AtGIF1/AN3 and maize GIF1/AN3 (ZmGIF1/ZmAN3) proteins were associated with the promoter regions of certain target genes (23, 25). The assays also showed that these associations were not limited to those known target

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genes, but detected widely over the whole genome of Arabidopsis, suggesting that AtGIF1/AN3 may be a key transcription cofactor acting together with GRFs and/or other transcription factors. Consistently with the notion, a series of tandem affinity purification (TAP) and co-immunoprecipitation (co-IP) approaches revealed that GIF1/AN3 proteins of Arabidopsis and maize were co-purified with the components of SWI2/SNF2 chromatin-remodeling complexes, including the core SWI2/SNF2 chromatin-remodeling ATPases, such as BRAHMA (BRM) and SPLAYED (SYD) (21, 23, 24). Upregulation of AtGIF1/AN3 target genes also required intact activities of BRM. These results give rise to the notion that GIF1/AN3 transcription cofactors may recruit both SWI2/SNF2 complexes and GRFs to GTEs, thus activating or repressing target genes (Fig. 1).

The results and notion are consistent with the fact that the human GIF homolog, SYT, directly interacts with the human BRM and its homolog (31, 32), as well as the fact that TAP experiments using the human SYT as bait also retrieved the components of human SWI2/SNF2 chromatin-remodeling complexes (33). The result suggests that the interaction between GIF1/AN3 and the SWI2/SNF2 complex may be mediated via direct interaction between GIF1/AN3 and plant BRM homologs, and that the interaction between GIF1/AN3 and the SWI2/SNF2 complex is not only evolutionarily conserved in metazoans and plants, but also essential for transcriptional regulation, despite the fact that metazoans lack GRFs.

Some additional interesting features of the GRF-GIF action are that they activate their own transcription through a positive feedback loop in Arabidopsis, rice, and maize, likely by forming the GRF-GIF-SWI2/SNF2 complex at the promoter sites of target GRFs and GIFs (Fig. 1; for detailed information, see 11, 25); and that Arabidopsis and rice GIF1 proteins translocate between different cell layers through plasmodesmata, thus coordinating the cell proliferation activities of different cell layers (34, 35).

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Regulatory roles of the GRF-GIF-miR396 module in leaf growth

Roles in cell proliferation of dicot leaf growth

GRF and *GIF* genes are highly expressed in almost all meristematic tissues, including leaf and floral organ primordia. Loss-of-function mutants of *AtGRF*s and *AtGIF*s had small and narrow leaves and petals, whereas their overexpressors developed larger ones (7, 9, 10, 36, 37). Determination of cellular parameters elucidated that AtGRFs and AtGIFs are positive regulators of cell proliferation in leaf primordia, providing cells with a meristematic potential or meristematicity.

In Arabidopsis, miR396 species, products of *AtMIR396a* and *AtMIR396b* genes, target and induce the cleavage of *AtGRF* mRNA species with the exceptions of *AtGRF5* and *AtGRF6* mRNAs, which lack the miR396 target site (14, 15). Therefore, the overexpression of *AtMIR396* (*35S:AtMIR396*) resulted in post-transcriptional down-regulation of target *AtGRF*s, resulting in small and narrow leaves along with a reduced number of cells. By contrast, the expression of *At-rGRF*s, which were manipulated to be resistant to miR396 by altering their target sites, induced an enhancement of cell proliferation, consequently resulting in large leaves (15, 21, 22, 38).

The function of the GRF-miR396 module holds up for other eudicot plants as well. The overexpression of Arabidopsis and *Populus trichocarpa MIR396*s in tobacco plants has been shown to result in small and narrow leaves (38, 39). Arabidopsis or *Brassica napus* plants overexpressing *B. napus* and *B. rapa GRF*s have also shown the development of enlarged leaves with more cells, along with increased expression of a set of cell cycle genes (41-43).

It has been shown that the AtGIF family controls both the rate and duration of cell division (37, 44). An increase in the *AtGIF1*/*AN3* activity not only enhanced, but also prolonged the expression of a marker gene for the G-to-M transition in cell cycle, *CYCB1;1*, in the leaf primordium (23), whereas decreases in *GIF* activities were accompanied by reductions in the

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expressions of cell cycle-related genes (37). Similarly, the overexpression of *AtMIR396* (*35S:AtMIR396*) and *At-rGRF3* resulted in reductions and increases in *CYCB1;1* expression (15, 21), respectively, indicating that the GRF-GIF duo plays a critical role in regulating cell cycle activities, consequently maintaining and supplying meristematic cells for cell proliferation in leaf primordia. Further study is needed as to what signaling pathway downstream of the GRF-GIF duo leads to the regulation of cell proliferation-mediating genes, including cell cycle-related genes.

The movement of the cell cycle arrest front (AF) from the distal to proximal regions of Arabidopsis leaf primordia during the early stages of leaf growth has been well documented (45). *35S:AtMIR396* induced the precocious AF movement toward the leaf proximal region, reducing cell proliferation activities and accelerating cell expansion (an indication of differentiation in plants) in the distal region behind AF, whereas an enhancement of the AtGRF5 activity exerted the opposite effects on those cellular processes, stimulating leaf growth (15, 46). Similarly, enhanced activities of AtGIF1/AN3 delayed the AF movement (23), and the distribution patterns of *AtGIF* expression were consistent with the AF movement (10, 47, 48), suggesting that the GRF-GIF-miR396 module is a crucial regulator of the AF movement. Gupta and Nath analyzed various types of leaf growth polarity present in 75 eudicot species, including the distal-proximal type of Arabidopsis, and found that the patterns of leaf growth polarity are tightly coupled with the abundance patterns of miR396 species and *GRF* mRNAs: regions of active cell proliferation are positively correlated with abundance of *GRF* mRNAs, whereas regions of cessation of cell proliferation and commencement of cell differentiation are positively correlated with miR396 abundance (49). The results suggest that, in eudicots, the patterns of cell proliferation and differentiation are controlled by the GRF-GIF-miR396 module.

A pea transcriptional complex consisting of BIGGER ORGANS (BIO) and ELEPHANT

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EAR LIKE LEAF1 (ELE1) negatively regulates leaf growth and interacts with a WUSCHEL-related transcription factor, LATHYDROIDES (LATH) (50). LATH has been shown to directly bind to a promoter region of a pea *GRF*, indicating that the negative regulator complex of leaf growth exerts it function through the repression of *GRF* expression. Arabidopsis *PEAPOD* (*PPD*) genes, orthologs of pea *ELE1*, are negative regulators of cell proliferation in leaves (51), giving rise to the possibility that PPDs may directly repress *AtGRF* expression in order to exert their negative role in the regulation of cell meristematicity.

CINCINNATA-like TCP (CIN-TCPs) transcription factors control the transition from cell proliferation to expansion during leaf morphogenesis and act as growth repressors (for review, see 52). The overexpression of Arabidopsis *CIN-TCP*s directly enhances *AtMIR396* expression, leading to precocious declines in *GRF-GIF* expression and leaf growth (15, 53). On the other hand, multiple loss-of-function mutations, *tcp2 tcp4* (*tcp2/4*) and *tcp2/4/10*, promoted leaf growth by increasing cell proliferation (54). These results suggest that certain negative regulators of leaf cell proliferation, including PPDs and TCPs, may exert their function, directly or indirectly and at least partially, through the repression of *GRF-GIF* expression.

Roles in cell expansion of dicot leaves

It should be noted that not all Arabidopsis GRFs seem to act as positive regulators of leaf growth. The loss-of-function *Atgrf9* mutation enhanced cell cycle activities, resulting in large leaves, whereas *AtGRF9* overexpression reduced those activities, resulting in small leaves (28, 55). This indicates that AtGRF9 negatively regulates leaf growth via the suppression of cell proliferation in leaf primordia. The authors also showed that the negative effect of AtGRF9 on cell proliferation was, at least partially, mediated by the regulation of a target gene, which encodes *ORG3* (aka *bHLH039*). AtGRF9 directly activated *ORG3* expression, and loss-of-function *org3* mutants developed large leaves with more cells, whereas *ORG3* overexpressors

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had small leaves with fewer cells. No additive effect on leaf growth was found in the *org3 Atgrf9* double mutant, and the enhancement of leaf growth by *org3* was not negated by *AtGRF9* overexpression. It remains unclear how the specific GRF member exerts the opposite function. In contrast, Horiguchi et al observed a very slight increase in the leaf size of the *Atgrf9* mutant, but this increase was not statistically significant (10). Furthermore, it has been reported that *AtGRF9* overexpressors produced only slightly enlarged leaves and that the same *Atgrf9* mutant allele did not contribute to changes in leaf size (22). This incongruity should be reconciled in the future.

Roles in cell proliferation and expansion of monocot leaves

It appears that monocot GRFs and GIFs primarily act as positive regulators of cell proliferation in leaves. *makiba3* (*mkb3*), a loss-of-function mutant allele of rice *GIF1* (*OsGIF1*), caused a reduction in the number of leaf epidermal cells, producing small leaves, whereas *MKB3* overexpression resulted in the opposite phenotypes, indicating that OsGIF1/MKB3 acts as a positive regulator of cell proliferation in the leaf organ as well (35). Similarly, CRISPR/CAS9-induced loss-of-function mutations of *OsGIF1* (C/C-*Osgif1*) reduced leaf size, whereas *35S:OsGIF1* increased leaf size (56). *mkb3* and C/C-*Osgif1* mutants also shared other phenotypes, such as leaf rolling and reductions in the length of stem internodes. Based on the analysis of subepidermal cells in the internodes and main veins of leaf blades, the latter attributed the change in leaf size to the change in cell size, rather not in cell numbers. However, the latter also suggested a role of OsGIF1 in regulating cell proliferation of some tissues, such as specialized epidermal cells of the leaf blade, proposing that both the cell proliferation and expansion processes are under the control of OsGIF1, likely depending on the cell types. *35S:OsMIR396d* and *Osgrf6* rice plants showed reductions in cell length of the stem internode, leaf collar, and leaf sheath, indicating that lack of GRFs leads to defects in the cell elongation

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process in rice (57).

The overexpression of *ZmGRF10*, which lacks the C-terminal transactivation domain, led to a reduction in the size of maize leaves by decreasing cell proliferation, as it could execute a dominant negative effect by competing with other ZmGRFs with transactivation activities, suggesting that the other ZmGRFs may act as positive regulators of cell proliferation (20, 24).Consistently with the notion, the overexpression of *Zm-rGRF1*, a miR396-resitant version, increased the number of dividing cells in the leaf division zone, producing longer leaves (24). Intriguingly, however, *Zm-rGRF1* overexpression prolonged the duration of cell cycling of those dividing cells, and thus the increase in leaf length was not as large as expected based on the increase in the number of dividing cells. Inversely, loss-of-function *Zmgif1* mutants developed shorter and narrower vegetative and ear leaves than the wild type, and the size of their epidermal cells were larger, which is indicative of a reduction in cell numbers and thus a defect in the cell proliferation process. All things considered, therefore, the timing of the transition from the meristematic state to differentiating state in the leaf organ is governed by the GRF-GIF-miR396 module, in consequence determining the leaf size and shape in both eudicot and monocot plants.

It should be noted that, like loss-of-function mutant leaves of Arabidopsis *GRF*s and *GIF*s as well as *35S:AtMIR396*, the rice *mkb3* and *Zmgif1* leaves developed larger cells, partially compensating for a reduced leaf size (25, 35). The compensation syndrome, which here I do not elaborate on, has been well documented (58).

Leaf senescence in Arabidopsis

It has been reported that increases in the *GRF* activities of Arabidopsis, *B. napus*, and *B. rapa* stimulate photosynthetic activities, resulting in an abundance of photosynthetic assimilates or seed oil (21, 41, 42, 59). The increases were concomitant with increases in total

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chlorophyll content and the rate of chloroplast division (41, 46). Activation of the *GRF-GIF* duo delayed leaf senescence, whereas their down-regulation accelerated it (21, 46). During dark-induced leaf senescence, the expression of specific marker genes for leaf senescence was markedly suppressed by *35S:At-rGRF3* but enhanced by *35S:AtMIR396* (21). The suppressive role of *GRF*s in leaf senescence may be explicable in light of the *GRF*-cytokinin interplay, as *35S:AtGRF5* increases the sensitivity of leaves to cytokinins (46). Cytokinins are well known to act not only as potent stimulators of cell proliferation but also as specific suppressors of leaf senescence (for review, see 60). The nature of the *GRF*-cytokinin interplay requires further investigation, although AtGIF1/AN3 directly activated *CYTOKININ RESPONSE FACTOR2* (*CRF2*) and repressed *ARABIDOPSIS RESPONSE REGULATOR4* (*ARR4*) (23).

Recently, an Arabidopsis gain-of-function mutant, *oresara15-1D* (*ore15-1D*), was shown to delay leaf senescence and promote organ growth (61). *ORE15* encodes a transcription factor belonging to the PLATS family (PLANT A/T-RICH SEQUENCE- AND ZINC-BINDING PROTEIN). The suppression of leaf senescence by *ore15-1D* was accompanied by reductions in the expression of senescence marker genes, while the promotion of leaf growth by *ore15-1D* was coupled with the upregulation of *AtGRF5* and *AtGIF1/AN3* expression as well as cell cycle genes. The ORE15 transcription factor directly bound to the promoters of *AtGRF1* and *AtGRF4*. In contrast to *ore15-1D, ore15* null mutations functioned inversely in most of those physiological and molecular phenotypes. The findings demonstrate that ORE15 is not only a negative regulator of leaf senescence, but also a positive regulator of leaf cell proliferation. Therefore, ORE15 provides a genetic link mediating both of the processes, and the dual function of ORE15 is likely manifested, in part, through control of the GRF-GIF-miR396 module. Indeed, the *an3* mutation promoted leaf senescence in the presence of *ore15*, but nullified the effect of *ore15-1D*. Both the leaf cell proliferation and senescence events occur temporally separated in Arabidopsis, i.e., at the primordial and mature stages, respectively.

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Therefore, it remains to be addressed in the future how the ORE15-GRF-GIF pathway regulates both of the cellular processes.

Regulatory roles in root growth and development

It was recently demonstrated that AtGRFs are required for the transition of stem cells into transit-amplifying cells in the root meristem (62). Briefly, the abolishment of *AtGRF* activities by *35S:MIR396* suppressed the activities of cell cycle markers in root tips, reducing the root elongation rate and root length, whereas the overexpression of *At-rGRF3* exhibited opposite effects on the marker activities. Unexpectedly, however, the final length of the *35S:At-rGRF3* root was short. The contradiction may be comprehensible in light of an additional function of AtGRFs in the root: ectopic *AtGRF* expression interferes with the normal patterning of cell divisions in the stem cell niche and organization of the quiescent center (QC). Nevertheless, it is intriguing that the heterologous overexpression of *At-rGRF3* in *B. oleracea* resulted in longer roots than the wild type (63).

AtGIF1/AN3 also plays crucial roles in QC organization, which are, interestingly, independent of *GRF* activities (30). The AtGIF1/AN3 action was shown to be mediated, at least in part, by regulating the expression patterns of *PLETHORA1* (*PLT1*), as *PLT1* was ectopically expressed in the *an3* mutant. As mentioned above, *PLT1* is one of the direct targets of AtGIF1/AN3. The report suggests that, as AtGIF1/AN3 lacks a DBD and its role in QC organization is independent of GRF, its targeting to *PLT1* should be associated with another transcription factor, likely in concert with the SWI2/SNF2 chromatin-remodeling complex. The result and its implication are consistent with the roles of AtGIF1/AN3 in suppressing ectopic *PLT1* expression during the development of embryonic polarity: if *PLT1* not suppressed, the apical regions of embryos, which are presumed to develop into cotyledons, are converted to ectopic roots, as observed in the *an3 han* double mutant (64). HAN (HANABA

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TARANU) encodes a GATA transcription factor (65). In the legume plant *Medicago truncatula*, deactivation of *MtGRF*s by *MtMIR396a* and RNA interference (RNAi) inhibited root growth, due to reductions in both the cell cycling activity and the numbers of transit-amplifying cells in the meristematic zone of the root, though it did not affect the organization of the root apical meristem (66).

Regarding monocot root growth, the roots of *35S:OsMIR396d* rice plants possessed fewer cells in the G2/M phase, suggesting that a lack of *OsGRF* activities may lead to reductions in cell cycling activities in rice roots (57). It should be noted, however, that the reduced cell cycling activity did not affect the root length, although *35S:OsMIR396d* boosted the brassinosteroid (BR)-induced inhibition of root growth. By contrast, a loss-of-function rice mutant, *Osgrf6*, had shorter roots than the wild type (57). Taken together, the GRF-GIF duo generally seems to be required for root growth in both eudicot and monocot plants. However, this notion may not hold up straightforwardly, since one needs to separately analyze the duo's effects on cell cycling activities in the transition zone of the root meristem as well as the organization of the stem cell niche.

Regulatory roles in the development of floral organs in Arabidopsis and monocots

The Arabidopsis *gif1/2/3* triple mutant displayed severe defects in the growth and development of all floral organs (67). Most conspicuously, the mutant gynoecium was split into two carpels along the medial regions, because the primordial replum cells of carpel margin meristems (CMMs) failed to maintain their meristematicity, precociously differentiating into papillar cells and thus not completely accomplishing the carpel fusion process. The mutant gynoecium either completely lacked or showed poor development in all internal tissues of the ovary (ovules, the septum, and the transmitting tract), which are all derived from CMMs. The *gif* triple mutant also had malformed anthers with no development of microsporangia bearing

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pollen grains, because the archesporial cells and their progeny lost meristematicity. Taken together, these results indicate that AtGIFs are essential factors for the establishment of the reproductive competency.

Since GRFs and GIFs form a functional unit for transcriptional regulation, the deactivation of *AtGRFs* is expected to cause similar floral aberrancies. Indeed, some strong 35S:*MIR396* lines frequently developed single-carpel gynoecia, instead of two, and, on rare occasions, split gynoecia (22, 69). Recently, the strong deactivation of both *AtGIF* and *AtGRF* by *gif 35S:MIR396* and *grf* multiple mutations allowed for further insight into the roles of *AtGRFs* in floral organ development. Those mutants completely aborted the pluripotent CMMs and archesporial cells of the anther (69). Strikingly, the mutant gynoecium developed no ovary at all, forming a rod-shaped gynoecium only with the stigma, style, and replum: the interior and exterior tissues of the gynoecial body were entirely filled in and covered with replum tissues. It is therefore obvious that AtGRFs are essential factors for the meristematic competency of formative cells in floral tissues, as are AtGIFs. Furthermore, the results showed that the lack of CMM development allows for the replum cells to infiltrate the whole gynoecial body, suggesting a developmental antagonism between the ovary and replum. GRF and GIF proteins are abundantly localized in the formative tissues of gynoecium and anther primordia, and the localization patterns of both proteins match exactly (67, 69). It has been shown that *HEC1* is a direct target gene of AtGIF1/AN3 (23), thus giving rise to the possibility that it may mediate the duo's action in floral organ growth and development.

The rod-shaped gynoecium phenotypes of those mutants were exacerbated by the *pinoid-3* mutation and 1-N-naphthylphthalamic acid, which is indicative of interplay between the GRF-GIF duo and polar auxin transport (69). It is noteworthy that, although the floral organ phenotypes of *gif1/2/3* and *gif 35S:MIR396* overlap on a broad scale, some of the details differ: the former predominantly displayed split gynoecia and yet developed the ovary, though poor,

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whereas the latter completely failed to form the ovary. In addition, the *grf1/2/3/5* quadruple mutant, the strongest among the *grf* mutants obtained, mostly lost their ovary, but hardly developed split gynoecia. The results suggest that, in addition to their common pathway, AtGRFs and AtGIFs may have their own specific roles in the regulation of cell proliferation and differentiation during gynoecium development. The down-regulation of tobacco *GRFs* by *AtMIR396* and *PtMIR396* caused aberrant floral organs, which were reminiscent of the *grf* phenotypes, suggesting that the functionality of the GRF-GIF duo is conserved in eudicot floral organs (39, 40).

35S:OsMIR396 and *Osgrf6/10* double mutants frequently produced aberrant floral organs: open husks, long sterile lemmas, and/or anomalous numbers of the stigma and anther (19). *OsCR4* and *Osjmj706* were shown to be directly activated by OsGRF10, thus mediating, at least in part, the roles of OsGRFs in floral organ development: the open-husk phenotype was also induced by the deactivation of *OsCR4* and *Osjmj706* (70, 71); *35S:OsJMJ706* partly rescued the floral defects of *35S:OsMIR396* (19).

Rice GIF1/MKB3 has been shown to be involved in floral organ development, as spikelets of the *mkb3* mutant exhibited morphological abnormalities: the shapes of the lemma and palea were distorted, and the width of the palea was significantly reduced (35). The *mkb3* mutant was not able to complete the ovule formation and integument elongation processes, and also produced no pollen or abnormal pollen, similar to the Arabidopsis *gif* mutants.

The maize *gif1* mutant also showed many defects in floral organs: it is male and female sterile; it produced multiple silks, or pistils, per floret; and its nucellus protruded out of the carpel, as seen in Arabidopsis *gif* mutants (25). Interestingly, in the *Zmgif1* mutants, extra numbers of spikelet meristems (SMs) were initiated from spikelet pair meristems (SPMs) in both ears and tassels, indicating that the axillary meristems lose their determinate nature, and thus ZmGIF1 is involved in promoting determinacy of the inflorescence. The situation seems

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to be contrary to that of leaf growth, in which *Zmgif1* leaf cells are less meristematic, producing small leaves with fewer cells, as mentioned previously. Rice and maize *GIF1* mRNAs are highly expressed in floral organ primordia, SMs, and SPMs (25, 35). In conclusion, the GRF-GIF duo of both eudicot and monocot plants plays essential roles in the growth and development of floral organs, thus warranting successful reproduction. Additionally, depending on different evolutionary pathways, it may have co-opted a switch function balancing the determinacy and indeterminacy of spikelet meristems in monocots.

Regulatory roles in sculpting plant architecture of monocot plants

Roles in regulation of stem elongation and plant height

The deactivation of *OsGRF*s by loss-of-function mutations, *35S:OsMIR396*, and RNAi resulted in semi-dwarf rice plants (18, 19, 29, 57). On the other hand, a rice dominant quantitative trait locus (QTL), *GRAIN SIZE ON CHROMOSOME2* in the Baodali line (*GS2-BDL*) caused a slight increase in height with significantly longer leaves (72). The *GS2-BDL* locus corresponds to *OsGRF4*, whose transcripts lost its miR396 target site by a mutation in it, thus increasing its transcripts level but not affecting the amino acid sequence of the OsGRF4 protein and thus its function (Os*grf4-1D*$^{GS2\text{-}BDL}$ hereafter for simplicity). Of note, however, no significant changes in those phenotypes were detected in Os*grf4-1D* $^{GS2\text{-}JDL}$, which contained the same kind of a gain-of-function mutation from the *GRAIN SIZE AND WEIGHT2* QTL (*GS2*) in Judali as Os*grf4-1D*$^{GS2\text{-}BDL}$ (73). The reduced height of *35S:OsMIR396d* rice was due to the short internodes with compromised cell elongation. *35S:OsMIR396d* also increased the degree of the leaf angles, because the cell elongation of the adaxial side of the leaf collar was less affected than that of the abaxial side. Taken together, this indicates that the rice height is controlled by the GRF-miR396 module. The compromised and differential elongation of stem intermodal and leaf collar cells of *35S:OsMIR396d* was shown to be intimately linked with the

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signaling and biosynthetic pathways of the phytohormones BR and gibberellin (GA): OsBZR1, a key transcription activator of BR signaling, directly activated *OsMIR396d* expression, while OsGRF6 promoted GA biosynthesis and signaling. Inversely, Os*grf4-1D*^{GL2} stimulated seedling growth and reduced leaf angles, and the central negative regulator in BR signaling, OsGSK2, physically interacted with OsGRF4, inhibiting *OsGRF4* expression (74).

Both the rice *mkb3* and *Zmgif1* mutants exhibited dwarf phenotypes due to shortened internodes, indicating that, like GRFs, GIFs are also involved in the regulation of stem elongation, and thus plant height (25, 35). Unexpectedly, however, the overexpression of *Zm-rGRF1* resulted in dwarfism (24), likely due to a perturbation in the stem elongation process due to its nature of a strong ectopic expression.

Based on results derived from the deactivation of monocot *GRF* and *GIF* genes, it is clear that the compromised cell elongation process is a primary cause of short internodes, suggesting that the GRF-GIF-miR396 module is involved in the regulation of cell elongation in stem growth, rather than cell proliferation. It is noteworthy, however, that those studies have focused on cell elongation of internode regions. Given that the first *GRF* member, *OsGRF1*, was identified in the intercalary meristem of rice plants, which supplies internode tissues with new cells (6), examining the cell cycling activities in the intercalary meristem of the rice and maize mutants could provide further insight into the role of the GRF-GIF-miR396 module in the regulation of monocot stem elongation.

The Arabidopsis inflorescence stem showed a bi-phasic growth pattern in response to different dosages of *gif* mutations: the *gif1* single mutant developed longer stems than the wild type, whereas the *gif* triple mutant had much shorter ones (37), although its nature was not investigated in detail. Interestingly, Arabidopsis roots also showed a bi-phasic pattern: *gif1* roots, longer; *gif1/2/3*, shorter (30).

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Roles in regulation of grain size and panicle development of monocots

In monocot plants, the activities of the GRF-GIF-miR396 module affected the grain size and architecture of panicles, such as the length and number of branches as well as spikelet numbers. The up-regulation of *OsGRF*s by *35S:OsGRF6*, *Os-rGRF6*, and a target mimicry of miR396 (*35S:MIM396*) increased the numbers of panicle branches and spikelets, resulting in high yield, whereas the down-regulation of *OsGRF*s by *35S:OsMIR396d* and RNAi caused the opposite phenotypes (29). The report suggested that regulation of the axillary branches and spikelet numbers by *OsGRF*s appeared to be mediated by stimulated auxin biosynthesis and signaling. The dominant *Osgrf4-1D* mutations and *OsGRF* overexpression also markedly increased grain size and panicle length, consequently producing more grains with increased weight (72, 73, 75, 76). The deactivation of *OsGRF*s by loss-of-function approaches impaired those yield traits (19, 76). As expected, the OsGIF1 function in the panicle traits parallels that of OsGRFs. The *mkb3* and C/C-*Osgif1* mutants had shorter branches and/or reduced size and weight of grains, whereas the overexpression of *OsGIF1* increased both grain size and weight (35, 56, 73, 75). Therefore, it is clear that the GRF-GIF duo acts as a positive regulator of grain size and panicle development in rice. Maize *gif1* mutants also displayed severe defects in the inflorescence architecture: reduced lengths of tassels and ears as well as reduced numbers of tassel branches, but increased numbers of short branches in the ear (25).

Evidence indicates a role of eudicot GRFs in determining seed size. *35S:AtGRF1* and *35S:AtGRF5* have been shown to increase seed size, albeit not always accompanied by increases in seed weight (77). Arabidopsis plants overexpressing *BnGRF2a* and *BrGRF*s as well as *B. napus* plants overexpressing *BrGRF*s all developed large seeds with increased weight (41, 42, 43). The promotive effect on seed growth may be closely associated with the increases in photosynthetic activities and senescence retardation by GRFs.

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Regulatory roles in plant-pathogen interaction and in responses to UV-B light

Syncytium formation occurring in Arabidopsis roots by an infective cyst nematode (*Heterodera schachtii*) was deterred by *35S:AtMIR396* and the *grf1/2/3* triple mutation, indicating that AtGRFs are required for the reprogramming processes of root cells, such as changes in cell fate, re-differentiation, and cell proliferation (78). This leads to an interesting, evolutionary question of how the parasite wired up the GRF-miR396 module in order to induce the nourishment source tissue. Similarly, in *M. truncatula*, *35S:MtMIR396* reduced the frequency of colonization by arbuscular mycorrhizal fungi, whereas *35S:MIM396* frequently reversed it (66). This indicates that the GRF-miR396 module promotes (sym)biotic associations with microbes in the rhizosphere.

Genes involved in the regulation of defense responses and disease resistance were found to be enriched in the potential target candidates of AtGRF1 and AtGRF3 (79). In support of that, Arabidopsis plants expressing *35S:MIR396* enhanced the susceptibility to infection, thus increasing fungal biomass, whereas *35S:MIM396* plants displayed broad resistance to fungal pathogens with concomitant activation of defense responses, indicating that GRFs help deter pathogenic organisms (80).

In Arabidopsis leaves, UV-B light induced the accumulation of miR396 and thus reduced the abundance of *AtGRF* mRNA, resulting in repressed cell proliferation (81). Therefore, the GRF-miR396 module mediates, at least in part, the UV-B-repression of leaf growth, and also likely provides a protective mechanism against UV-B light, as plant cells with UV-B-damaged DNA are not to proliferate. Arabidopsis E2Fc transcription factor acts upstream of *AtMIR396*, probably activating, directly or not, the expression of *AtMIR396* or of genes that encode proteins involved in the processing of mir396 precursors (82). UV-B light induced the accumulation of miR396 in maize leaves as well, and caused a reduction in cell proliferation and a shortened growth zone (83). These results suggest that both dicot and monocot plants

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may have adopted the parallel molecular apparatus in order to cope with the detrimental effect of UV-B light.

The evolutionary genesis of GRFs

It has been described in the last review articles that GRFs are land plant-specific genes, since genomic and transcriptomic resources available then revealed their presence only in plants (land plants or embryophytes) but not in metazoans, fungi, and protists, including 'green algae' (11, 12). The 'green algae' are members of chlorophytes and charophytes that are paraphyletic to land plants (Fig. 2). However, a recent paper reported the presence of a single GRF gene in the genome sequence of a charophyte, *Klebsormidium nitens* (previously known as *K. flaccidum*) (84). This prompted me to scrutinize other charophycean sequences that have been recently deposited in public databases and I found single GRF genes in the genomes or transcriptomes of almost all orders of charophycean algae, but not in coleochaetales (*Chaetosphaeridium globosum* and *Coleochaete scutata*), probably because of insufficient coverages of their transcriptomic sequences (Fig. 2). However, I have still not been able to detect the presence of GRF in any chlorophytes. This sequence profile calls for a revision of the previous notion regarding the evolutionary origin of GRFs: GRFs are not land plant-specific transcription factors but streptophyte-specific. Streptophytes comprise both charophytes and land plants, and are paraphyletic to chlorophytes (Fig. 2) (85, 86). Therefore, it is likely that a GRF gene may have evolutionarily emerged in an ancestral charophyte after its divergence from chlorophytes, and that an ancestral land plant inherited and duplicated it, thus diversifying its function to meet the biological complexity of or to give rise to the complexity in ensuing lineages of land plants.

How could the GRF gene have been invented in an ancestral charophyte? This question may remain unanswered for years. One may speculate that an ancient QLQ domain derived

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from the N-terminus of the SWI2/SNF2 chromatin-remodeling ATPases (BRM and its homologs) have acquired the WRC domain, resulting in an ancestral GRF gene (9, 11). SWI2/SNF2 ATPases are universally present in eukaryotes, including viridiplantae, and they play essential roles in the chromatin remodeling process (11, 87, 88). According to the Pfam profile, QLQ domains exist in 66 different architectures with 2303 entries (http://pfam.xfam.org, PF08880). Half of the entry proteins have the QLQ domain together with WRC as GRF proteins; roughly the other half together with the SNF2_N domain of the SWI2/SNF2 ATPases; and only a few entries are together with other kinds of domains. These combinatorial structures with QLQ are compatible with the notion that the SWI2/SNF2 ATPase QLQ domain might be co-opted into an ancient GRF gene.

The Pfam profile also reveals that the WRC domain, which contains the DNA-binding C_3H motif, is present in streptophytes and Mamiellophyceae, but is not present in Chlorophyceae and Trebouxiophyceae or any other organisms (Fig. 2; http://pfam.xfam.org, PF08879). WRC domains exist in 26 different architectures with 1984 entries: more than half of the entry proteins have the WRC domain together with the QLQ domain as GRFs, a quarter are mostly uncharacterized proteins with a single WRC domain but with no associated known domains, and the rest have single or multiple WRC domains associated with other kinds of known domains. Interestingly, the GRFs of *Chara globularis* and *Chara braunii* belonging to Charales have a QLQ domain followed by three consecutive WRC domains (Fig. 2). This profiling suggests that the evolutionary swapping of the WRC domain might have frequently occurred in virideplantae (“green plants”). Therefore, it is tempting to speculate that QLQ and WRC domains might have been co-opted into a GRF protein in an ancestral charophyte.

The origin of GIF genes is much more ancient than that of GRFs, as they exist in most eukaryotes, such as virideplantae, and metazoans, and not in fungi and protists other than ‘green algae’ (11, 12). GIFs are present in the genomes of a charophyte (*K. nitens*) and chlorophytes

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(*Chlamydomonas reinhardtii, Ostreococcus lucimarinus,* and *Ostreococcus tauri*) (84). Additionally, this profiling identified GIFs in the genomes or transcriptomic sequences of chlorophytes (*Botryococcus braunii, Chlorella variabilis NC64,* and *Prototheca wickerhamii*) and charophytes (*Chaetosphaeridium globosum* and *Chlorokybus atmophyticus*; Fig. 2).

Both genomic and cDNA sequences were available for some of those algal GIFs (*C. reinhardtii, V. cateri,* and *K. nitens*), allowing for the construction of their exon-intron structures. I found that the SNH domains of those three algae and land plants are encoded in the first three exons with conserved intron positions and phases (data not shown). The analysis suggests that the structure of GIF genes has been highly conserved during the evolutionary path of chlorophytes, charophytes, and land plants. Therefore, it is a plausible hypothesis that the GRF-GIF partnership was established in an ancestral charophyte. It would be interesting to explore whether charophycean GRFs and GIFs interact together; then if so, what the biological role and molecular function of the duo are, especially in terms of evolution, and what chlorophytic GIFs do in the absence of the canonical partner protein GRF.

In summary, the GRF-GIF-miR396 module plays essential roles in the growth and development of angiosperms. It regulates the meristematic potential of primordial cells during leaf growth, determining the final size and shape of the leaf organ. The GRF-GIF duo is a prerequisite for floral organ development, and thus enables the production of the formative cells, such as CMMs and egg cells as well as microsporangia and sperm cells. It is also involved in the regulation of leaf longevity and photosynthetic efficiency in mature leaves. Importantly, the monocot GRF-GIF duo also promoted the yield traits, such as grain size and panicle architecture, warranting crop productivity. Finally, the GRF gene has a charophycean origin, so studies on GRFs of basalmost land plants and charophytes could shed light on their significance in the evolution-developmental history of a main lineage of life, the streptophyte.

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CONFLICTS OF INTEREST

The author declares no conflict of interest.

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Table 1. Potential *cis*-elements bound by GRFs and AtGIF1/AN3.

Proteins	*cis*-elements	Target genes	Transcriptional regulation
AtGRF7	TGTCAGG[a]	*DREB2A*	-[b]
AtGRF9	CTGACA	*ORG3*	+
OsGRF6	TGTGTTG	*OsJMJ706*	+
OsGRF9		*OsCR4*	+
OsGRF6	CGSMR[c]	*ARF2*	+
		ARF7	+
		YUCCA-like	+
AtGIF1/AN3	CACGTG	*COL5*	+
	GAGAGAGA	*COL5*	+
		HEC1	+
	TGTCAGA	*PLT1*	-

[a] Nucleotide sequences read from the 5' to 3' direction.

[b] Minus and plus symbolize up- and down-regulation of target gene expression, respectively.

[c]S indicates G and C; M, A, and C; R, A, and G.

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Figure legends

Figure 1. Schematic summary of molecular and biological functions of the GRF-GIF duo. The core and common molecular features of the duo are depicted in the circle, in which the GRF-GIF duo associated with the SWI2/SNF2 complex performs transcriptional regulation of target genes, including its own (auto-activation), and miR396 post-transcriptionally represses *GRF* expression. The biological functions common to eudicots and monocots are shown in red, i.e., the promotion of leaf growth via the regulation of cell cycling and promotion of cell cycling in root meristematic zones; the biological functions confirmed in Arabidopsis and other eudicots are shown in green and black, respectively; the biological functions validated in rice and maize are shown in brown and blue, respectively. The solid arrows and block bars indicate experimentally verified promotive and inhibitory actions, respectively, while the dotted ones indicate speculated possibilities.

Figure 2. Phylogenetic relationships among 'green plants' and the presence of *GRF-GIF* genes. Depicted are the relationships among the three lineages of 'green plants': chlorophytes, charophytes, and land plants. The numbers of *GRF* and *GIF* are indicated in black boxes, while diamond bullets indicate the presence of the WRC domain. Species depicted in gray tone only have whole transcriptomic resources, but no whole genome sequenced. n designates 'not present'; asterisks indicate that the *GRF* genes are predicted to encode three consecutive WRC domains after the QLQ domain.

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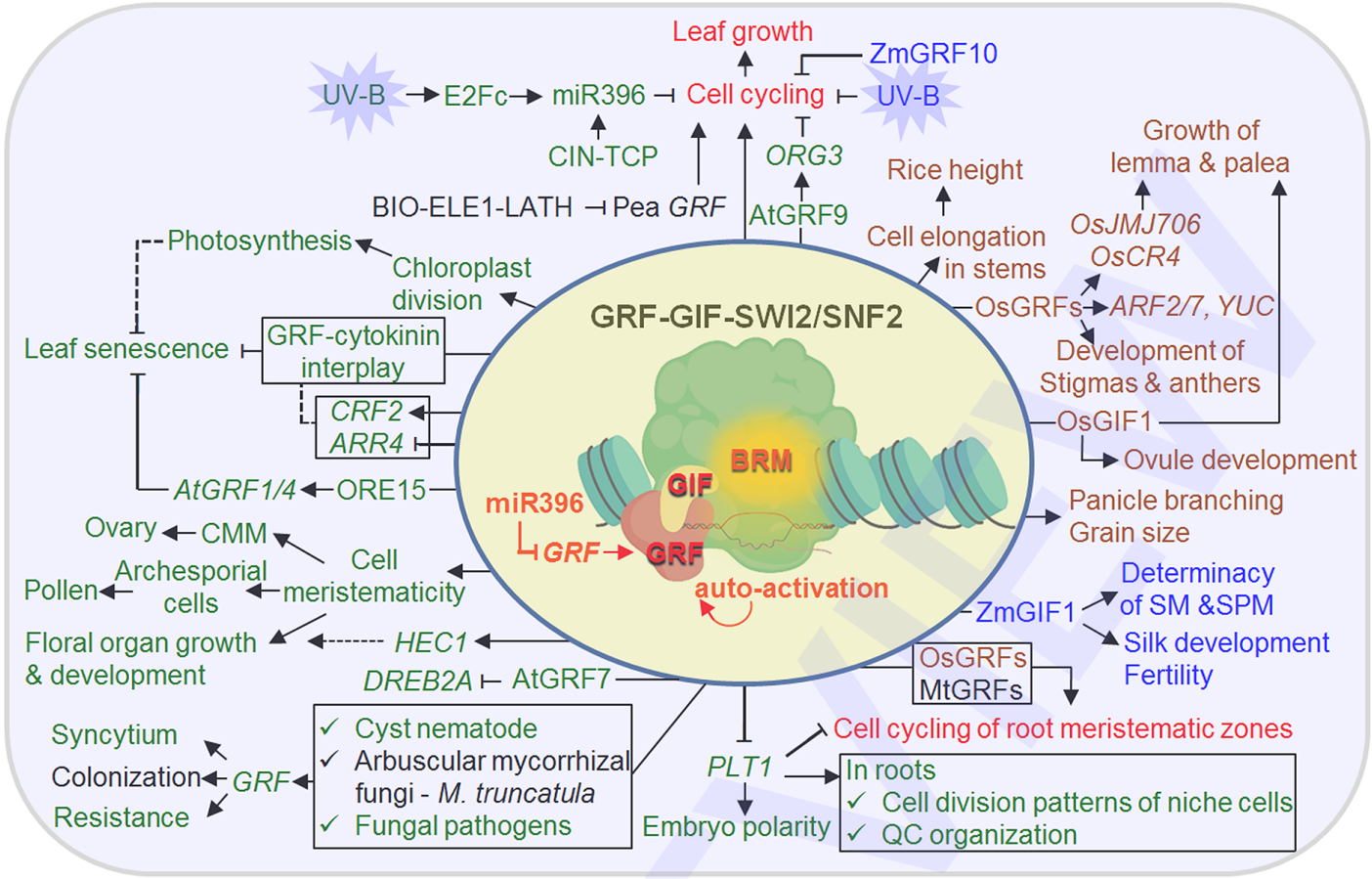

Fig. 1.

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