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The *Arabidopsis thaliana GRF-INTERACTING FACTOR* gene family plays an essential role in control of male and female reproductive development

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ABSTRACT

Reproductive success of angiosperms relies on the precise development of the gynoecium and the anther, because their primary function is to bear and to nurture the embryo sac/female gametophyte and pollen, in which the egg and sperm cells, respectively, are generated. It has been known that the GRF-INTERACTING FACTOR (GIF) transcription co-activator family of *Arabidopsis thaliana* (*Arabidopsis*) consists of three members and acts as a positive regulator of cell proliferation. Here, we demonstrate that GIF proteins also play an essential role in development of reproductive organs and generation of the gamete cells. The *gif*1 *gif*2 *gif*3 triple mutant, but not the single or double mutants, failed to establish normal carpel margin meristem (CMM) and its derivative tissues, such as the ovule and the septum, resulting in a split gynoecium and no observable embryo sac. The *gif* triple mutant also displayed severe structural and functional defects in the anther, producing neither microsporangium nor pollen grains. Therefore, we propose that the GIF family of *Arabidopsis* is a novel and essential component required for the cell specification maintenance during reproductive organ development and, ultimately, for the reproductive competence.

Introduction

Plant seeds are carriers of an embryo from which a plant starts a new life cycle. In angiosperms, flowers are pivotal reproductive organs for fertilization and setting seeds, allowing the generation-to-generation continuity. Reproductive success of angiosperms is contingent on the normal development of the gynoecium and the anther, which bear and house the female and male gametophytes, respectively.

The gynoecium of *Arabidopsis thaliana* (*Arabidopsis*) is composed of four distinctive regions at maturity: stigma, style, ovary, and gynophore (Ferrándiz et al., 1999, 2010). The ovary has discernible exterior portions along its longitudinal axis, i.e., two lateral carpels and two medial repla. Two carpels are congenitally fused together from their earliest emergence and are separated only by an intervening tissue, the septum, forming a bilocular chamber. The replum is the external part of the septum and marks exterior boundaries between carpel valves. Apically the ovary meets the style that is topped with the stigma, and while basally it meets the gynophores. Internally, the gynoecium consists of placenta, ovule, septum, and transmitting tract (Ferrándiz et al., 1999, 2010). Importantly, all of those internal tissues, as well as medial portions of the style and the stigma, originate from the carpel margin meristems (CMMs), meristematic structures that arise from the medial portions of the gynoecium primordium (Bowman et al., 1999; Azhakanandam et al., 2008).

Ovule development is completed through four main events: (1) primordium initiation and elongation from the placenta; (2) regionalization of primordium into three zones, i.e., funiculus, chalaza, and nucellus; (3) initiation and growth of integuments from the chalazal region; and (4) development of the embryo sac from the megaspore mother cell (MMC) (Bouman, 1984; Reiser and Fischer, 1993). The MMC, which is initially specified as the archesporial cell in the nucellus, performs the sporogenesis and gametogenesis consecutively to generate the embryo sac, thus giving rise to an egg cell and other gametophytic cells (Webb and Gunning, 1990; Yadegari and Drews, 2004).

Pollen grains are male gametophytes developing within microsporangia that reside within four lobes of the anther (Sanders et al., 1999). Each microsporangium consists of three outer concentric parietal layers – endothecium, middle layer, and tapetum – and

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harbors pollen mother cells (PMCs) in the center. All those parietal layers and PMCs are mitotic progeny of the archesporial cells that are earlier specified in the L2 layer of the stamen primordium (Sanders et al., 1999). PMCs, like MMC, perform the sporogenesis and gametogenesis consecutively and generate pollen grains containing two sperm cells and a vegetative cell. Therefore, establishment of the female and male gametophytes, together with that of the gynoecium and the anther in which they develop, is of pivotal importance for reproductive competence of angiosperms, including *Arabidopsis*.

We have previously uncovered a small family of transcriptional co-activators, GRF-INTERACTING FACTOR (GIF), in *Arabidopsis*. The GIF protein family comprises three members that form a functional complex with the GROWTH-REGULATING FACTOR (GRF) transcriptional factors (Kim et al., 2003; Kim and Kende, 2004; Horiguchi et al., 2005; Lee et al., 2009). GIF1 (also called *ANGUSTIFOLIA*3, *AN*3), GIF2 and GIF3 are all required for lateral organ growth, and act as positive regulators of cell proliferation in a functionally redundant manner. In short, the loss-of-function mutations in the *GIF*1/*AN*3 gene, *gif*1 and *an*3, resulted in small, narrow leaves and petals with a small number of cells. Although the *gif*2 and *gif*3 loss-of-function mutants displayed no obvious developmental phenotypes, double and triple combinations between *gif*1, *gif*2, and *gif*3 displayed a remarkably synergistic decrease in the sizes and cell numbers of lateral organs.

When performing genetic analyses in regard to lateral organ growth, we noticed that the *gif*1 *gif*2 *gif*3 triple mutant, but no single and double mutants, developed split gynoecia. Here, we demonstrate that the *gif* triple mutant displays severe structural and functional defects in the CMM and its derivatives (collectively, CMM tissues) as well as in the anther. Histological analyses revealed that, in the *gif* triple mutant, meristematic cells of the CMM tissues and the functional megaspore fail to develop properly, resulting in split gynoecia and a disruption of embryo sac development. Furthermore, the archesporial lineage cells in the mutant anther failed to produce the microsporangium, PMCs, and pollen. Therefore, we propose that the *GIF* family of *Arabidopsis* is a novel and essential component required for the development of the male and female reproductive structures and gametes.

Materials and methods

Plant materials

Wild-type *A. thaliana* (L.) Heynh plants were used, and all of the *gif* mutants are in the same accession (Lee et al., 2009). Growth conditions were described in Lee et al. (2009).

Scanning electron microscopy (SEM)

Flower clusters were harvested into a FAA solution (5 ml of ethanol, 0.5 ml of acetic acid, 1 ml of 37% formaldehyde, 3.5 ml of distilled water). The samples were incubated under vacuum (550 mmHg) and were transferred to a fresh FAA solution at 4 °C. Next day, the fixative was replaced with an OsO_4 solution (1 g of OsO_4 in 100 ml of 25 mM sodium phosphate buffer, pH 7.2; Heraeus, South Africa). The samples were incubated at 4 °C overnight, then rinsed with sodium phosphate buffer three times, dehydrated through an ethanol series at room temperature and stored in 100% ethanol before use. After the critical point dry (HCP-2 critical point dryer, Hitachi, Japan), the samples were mounted on stubs, coated with gold particles, and subjected to SEM (S-4300 & EDX-350, Hitachi, Japan).

Histological analysis

Flower clusters were fixed as mentioned above, washed with 1 × PBS (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH7.0) for 30 min twice, and dehydrated in a graded ethanol series, after which ethanol was replaced with a mixture of an equal volume of ethanol and Histoclear (National diagnostics, USA), then with Histoclear alone three times, and finally with a mixture of a third of Histoclear and two thirds of solid paraplast chips (Merck, Germany). The samples were stored at room temperature overnight and incubated further at 60 °C for 2 h. The last step was repeated three times with freshly melted paraplast. The samples were transferred to a plastic mold (Simport, Canada). Tissue blocks were sectioned 8 μm in thickness by a microtome (Leica RM2125RT, Germany). Tissue sections were stained with 0.1% toluidine blue O, except the *GUS* transgenic flowers (Sigma-Aldrich, USA).

Differential interference contrast microscopy (DIC)

Flower clusters were fixed with ethanol: acetic acid (6:1) and were washed with 100% ethanol three times and then with 70% ethanol once. The flower samples were cleared in a chloral hydrate solution (8 g of chloral hydrate, 1 ml of glycerol, and 2 ml of distilled water), and their images were obtained using a light microscope (Eclipse NI-U, Nikon, Japan).

Construction of GIFpro::GIF::GUS transgenic plants

Genomic DNA of the wild-type plant was amplified by PCR using primer pairs (see Supplementary Table 1). Amplified DNAs of *GIF*1, *GIF*2, and *GIF*3 included the promoter, introns, and exons except the stop codon, and were approximately 2.9, 1.8, and 1.7 kbp in length, respectively. It should be noticed that the promoters of *GIF*2 and *GIF*3, including 5′ untranslated region, are extremely short (396 and 291 base pairs, respectively), because they are closely adjoined, in a head-to-head manner, to neighboring genes (At1g01150 and At4g00840, respectively; Supplementary Fig. 1). After digestion with *Sal*I and *Xba*I, PCR products were put in frame to the β-*glucuronidase* (*GUS*) gene of the *pBI*101.1 vector according to In-Fusion™ Advantage PCR Cloning Kit (Clontech, USA), resulting in the *GIF-GUS* translational fusion constructs. Those constructs were introduced into *Arabidopsis* plants by the *Agrobacterium tumefaciens*-mediated transformation (Clough and Bent, 1998). Dozens of independent T_1 plants for each construct were selected on MS agar plates (0.5 × Murashige-Skoogs salts, 1% sucrose, 0.8% phytoagar, 50 μg/ml kanamycin; all from Duchefa Biochemie, the Netherlands, except sucrose, which was from Amresco, USA). Single-insertion lines were subjected to the GUS staining procedure. All of the lines for each construct showed an identical staining pattern and, therefore, a typical pattern was presented.

Procedures for in situ hybridization and GUS staining

Gene-specific DNA region was amplified by PCR using primer pairs (see Supplementary Table 1) and ligated into the *pGEM-T* (for GIF1) or *pGEM-Teasy* (GIF2 and GIF3) vector (Promega, USA). Production of anti-sense probes was achieved by in vitro transcription from SP6 (GIF1 and GIF3) or T7 (GIF2) promoter. The in situ hybridizations were carried out as reported previously (Wynn et al., 2011). The GUS staining procedure was performed according to Rodrigues-Pousada et al. (1993) with a slight modification.