Overexpression of *Brassica rapa* *GROWTH-REGULATING FACTOR* genes in *Arabidopsis thaliana* increases organ growth by enhancing cell proliferation

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Abstract *GROWTH-REGULATING FACTOR* (*GRF*) genes encode plant-specific transcription factors containing two conserved QLQ and WRC domains and play critical roles in regulating the growth and development of lateral organs, such as cotyledons, leaves, and flowers. To explore the agricultural potential of *Brassica rapa* *GRF* genes (*BrGRFs*), the researchers isolated seven *BrGRFs* (*BrGRF3-1*, 3-2, 5, 7, 8-1, 8-2, and 9) and constructed *BrGRF*-overexpressing *Arabidopsis thaliana* plants (*BrGRF*-OX). *BrGRF*-OX plants developed larger cotyledons, leaves, and flowers as well as longer roots than the wild type. The increase in size of these organs were due to increases in cell number, but not due to cell size. *BrGRF*-OX plants also had larger siliques and seeds. Furthermore, *BrGRF*-OX seeds produced more oil than the wild type. RT-PCR analysis revealed that *BrGRFs* regulated expression of a wide range of genes that are involved in gibberellin-, auxin-, cell division-related growth processes. Taken together, the data indicates that *BrGRFs* act as positive regulators of plant growth, thus raising the possibility that they may serve as a useful genetic source for crop improvement with respect to organ size and seed oil production.

Keywords Cell division, *GROWTH-REGULATING FACTOR*, Morphogenesis, Organ size, Seed oil, Transcription factor

Introduction

GROWTH-REGULATING FACTOR genes (*GRFs*) encode plant-specific transcription factors, which play a critical role in regulation of plant growth and development (Kim and Tsukaya 2015; Omidbakhshfard et al. 2015). The first *GRF* gene was identified in rice, being suggested to perform a regulatory role in stem elongation induced by the phytohormone gibberellin (Van der Knaap et al. 2000). *GRF* proteins contain two highly conserved QLQ (Gln, Leu, Gln) and WRC (Trp, Arg, Cys) domains in the N-terminal region. The QLQ domain serves as an interface for interaction with *GRF*-INTERACTING FACTORS, which act as transcription co-activators (Horiguchi et al. 2005; Kim and Kende 2004). The WRC domain has two distinctive structural features, namely a putative nuclear localization signal (NLS) and a zinc-finger motif (C₃H motif) for DNA binding (Van der Knaap et al. 2000). The C-terminal region of *GRFs* is involved in the transactivation activity.

GRFs have been isolated and characterized in a wide range of plant species, including *Arabidopsis thaliana*, *Brassica napus*, *Brassica rapa*, *Glycine max* (soybean), *Solanum tuberosum* (potato), *Oryza sativa* (rice), *Zea mays* (maize), *Physcomitrella patens* (moss), *Marchantia polymorpha* (liverwort), and other land plants (Kim and Tsukaya 2015; Omidbakhshfard et al. 2015). Many studies have reported that *GRFs* act as positive regulators of cell proliferation and thus determine the final size of lateral organs, such as cotyledons, leaves, and flowers (Horiguchi et al. 2005; Kim and Kende 2004; Kim et al. 2003; Liu et al. 2009; Wang et al. 2014). *GRFs* also play a role in development of female reproductive organs of *A. thaliana* (Liang et al. 2014), and are expressed in rice embryos and maize ears, suggesting a possible role in regulation of seed growth and development (Ye et al. 2004; Zhang et al. 2008). A *GRF* gene of *B. napus*, *BnGRF2*, was proposed to be responsible for increases in

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seed weight, probably by regulating cell number and photosynthesis; these, in turn, might be also responsible for the increased production of seed oil (Liu et al. 2012).

In the complete genome sequence of *B. rapa*, 17 non-redundant *GRFs* (*BrGRFs*) have been identified (Wang et al. 2014). *BrGRF* proteins are more closely related to those of *A. thaliana* (*AtGRFs*) than those of *O. sativa* (*OsGRFs*). *BrGRFs* were expressed in specific tissues and organs, and the transcription of most *BrGRFs* was induced by the phytohormone gibberellic acid (GA). The overexpression of *BrGRF8* in *A. thaliana* plants increased the size of leaves and other organs by regulating cell proliferation rather than cell volume, suggesting that *BrGRFs* might also control organ growth and development by regulating cell proliferation, as did some of *AtGRFs*.

In this study, the function of *BrGRFs* isolated from *B. rapa* L. (ssp. *pekinensis* cv. JangWon) were investigated using overexpression strategies. As a result, we demonstrate that *BrGRFs* play an important role in regulation of organ growth and propose that these genes could serve as a useful tool for genetic manipulation leading to improvement of agronomical traits of crops.

Material and Methods

Plant material and growth conditions

Brassica rapa L. (ssp. *pekinensis* cv. JangWon) seeds were sown on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.25% phytigel (pH 5.8) and stratified at 4°C for 5 days in darkness to induce synchronous germination. The plants were grown at 23°C for 2 weeks under long-day conditions (16-h light and 8-h dark), after which they were transplanted to soil and transferred to a growth chamber for further culture until use.

Arabidopsis thaliana Columbia seeds (Col-0) were surface-sterilized by vapor-phase sterilization (<http://www.plantpath.wisc.edu/fac/afb/vapster.html>; Desfeux et al. 2000) and plated on MS medium supplemented with vitamins, 1% sucrose, and 0.3% phytigel (Sigma, St. Louis, MO, USA). The seeds were stratified at 4°C for 2 days in the dark and then transferred to a growth chamber under a 16-h light/8-h dark photoperiod at 23°C.

Isolation of *BrGRF* cDNAs and construction of expression vectors

BrGRF cDNAs were synthesized using RNA extracted from shoot apical tissues of 3- to 4-week-old *B. rapa* plants. Three

micrograms of total RNA were reverse-transcribed in a 30- μ l reaction volume with 1 μ g of oligo (dT)₁₈ primer using MMLV reverse transcriptase (RNaseH free), according to the manufacturer's instructions (TOYOBO, Osaka, Japan). One microliter of the cDNA solution after dilution (1:3) was used for PCR amplification (95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec) in a volume of 25 μ l with gene-specific primer sets (Supplemental Table 1). All PCR products of accurate sizes were cloned into the pGEM-T-Easy vector (Promega, Madison, WI, USA), and confirmed by DNA sequencing.

In order to construct plant expression vectors, each *BrGRF* cDNA fragment was inserted into the sense orientation between the cauliflower mosaic virus (CaMV) 35S promoter (*35S-pro*) and the nopaline synthase terminator (*nos-ter*) of pCambia with *hpt* as a selectable marker gene. *BrGRF5* cDNA was inserted into the sense orientation between the *35S-pro* and *35S polyA* (*T35S*) of pB2GW7 with *bar* as a selectable marker gene (Supplemental Fig. 1A).

Generation and verification of transgenic plants

Flowers of *A. thaliana* were sprayed with *Agrobacterium tumefaciens* GV3101 cells with the recombinant binary vectors suspended in 5% sucrose and 0.05% Silwet-L77. The plants were then incubated in a growth chamber at 23°C and 100% humidity for 1 day, and allowed to grow in a growth chamber under a 16-h light/8-h dark photoperiod at 23°C. T₁ seeds and their progeny seeds were allowed to germinate on MS medium containing 50 mg/L hygromycin or 25 mg/L phosphinothricin for selection of transgenic plants. Transgenic plants with a single T-DNA insertion and a high level of *BrGRF* expression were chosen for further experimentation and were grown at 23°C during day and 21°C during night under a long-day photoperiod (16-h light). Phenotypes of transgenic plants were characterized at the developmental stages indicated.

To confirm the presence of transgenes in the selected plants, we performed PCR using genomic DNA with *hpt*-, *bar*-, and *BrGRF*-specific primer sets (Supplemental Table 1). PCR reactions were performed using 25 cycles of 30 sec at 95°C, 30 sec at 55°C, and 45 sec at 72°C, followed by a final extension at 72°C for 10 min.

For quantitative RT-PCR analysis of *BrGRF* transgenes, total RNA was extracted from leaves. Two micrograms of total RNA were converted to cDNA. One microliter of the cDNA solution after dilution (1:3) was used for PCR amplification with *BrGRF*-specific primer sets. The expression level of *Actin* (*At3g18780*) was used as a quantitative control.

Phenotypic characterization of transgenic plants

Digital images of detached leaves, cotyledons, and petals were acquired using a scanner, and their dimensional parameters (area, length, and width) were determined with the image-analyzing program SCIONIMAGE (Scion, Frederick, MD, USA).

In order to analyze cell number and size, plant tissues were cleared in Visikol VSKP-100 for 1 day. All microscopic images were obtained using an Axio imager M1 microscope installed with Axiovision4 microscopy software (Carl Zeiss Jena GmbH, Germany). The numbers of palisade cells in the subepidermal layer aligned along a longitudinal axis just beside the midvein or along a transverse axis in the maximum width region were counted. To determine cell area, 20 cells grouped halfway from the midvein to the leaf margin at the widest point were analyzed with SCIONIMAGE. Each sample was replicated three times. Areas of organs and cells were subsequently used to calculate total numbers of cells.

Measurement of lipid production in seeds

Total contents of seed lipids represent the sum values of 15 different fatty acids identified by gas chromatography (GC) analysis: decanoate (10:0), laurate (12:0), tridecanoate (13:0), miristate (14:0), palmitate (16:0), palmitoleate (16:01), heptadecanoate (17:0), stearate (18:0), oleate (18:1), linoleate (18:2), linolenate (18:3), arachidate (20:0), eicosenoate (20:1), behenate (22:0), and erucate (22:1). Individual fatty acids were determined according to the AOCS method Ce 1-62 (AOCS 1997) by using a GC 7890B (Agilent Technology, America). Transgenic *A. thaliana* seeds were crushed with chrome beads (2.3 mm dia. Cat No. 11079123c) by Tissue

Analyser II (Qiagen, Japan). Fatty acids were extracted using the CHCl₃: MeOH (2:1) solution. The pentadecanoic acid solution (1000 ppm) was used as an internal standard. Quantities of individual fatty acids were calculated from their chromatographic peaks based on a regression equation of the internal standard.

Expression profiles of growth-related genes

To analyze the transcript levels of cell division-, GA-, and auxin-related genes, total RNA was extracted from shoot apical tissues and leaves using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). We performed semi-quantitative RT-PCR using primer sets corresponding to following genes: 15 GA-related (Supplemental Table 2, Fleet and Sun 2005), 12 auxin-related (Supplemental Table 3, Li et al. 2007), and 39 cell division-related (Supplemental Table 4, Gonzalez et al. 2009; Krizek 2009). Relative expression levels were determined by semi-quantitative RT-PCR amplification using Quantity One (Bio-Rad, Hercules, CA, USA; Liang et al. 2011) and (<http://biochemlabsolutions.com/GelQuantNET.html>).

Results and Discussion

Establishment of *BrGRF*-overexpressing *A. thaliana* plants

As mentioned above, an earlier study identified and characterized all 17 non-redundant *BrGRFs* (Wang et al. 2014). To investigate their biological function in detail, we isolated 10 *BrGRF* cDNAs and renamed them *BrGRF1*, 2-1, 2-2, 3-1, 3-2, 5, 7, 8-1, 8-2, and 9 on the basis of the similarity to the *AtGRF* orthologues according to the phylogenetic relationship (Table 1, Supplemental Fig. 2). Comparison of

Table 1 *AtGRF* family members and accession numbers of BAC clones from the *B. rapa* genome database

<i>Arabidopsis</i> gene name	AGI	BAC clone ^a	BAC Acc. No.	<i>B. rapa</i> gene name	CDS Acc. No.	CDS (bp)	Amino acid
<i>AtGRF1</i>	At2g22840	KBrH110M01	AC237306	<i>BrGRF1</i>	JN698686	1557	518
<i>AtGRF2</i>	At4g37740	KBrB006J12	AC189204	<i>BrGRF2-1</i>	JN698687	1356	451
		KBrH005P10	AC189552	<i>BrGRF2-2</i>	JN698688	1344	446
<i>AtGRF3</i>	At2g36400	KBrB031G07	AC189304	<i>BrGRF3-1</i>	Bra023066	1239	368
				<i>BrGRF3-2</i>	Bra005268	1167	388
<i>AtGRF4</i>	At3g52910	-	-	-	-	-	-
<i>AtGRF5</i>	At3g13960	-	-	<i>BrGRF5</i>	Bra027384	1164	387
<i>AtGRF6</i>	At2g06200	-	-	-	-	-	-
<i>AtGRF7</i>	At5g53660	KBrB078H21	AC189470	<i>BrGRF7</i>	Bra022667	1047	348
<i>AtGRF8</i>	At4g24150	KBrH123I10	AC241192	<i>BrGRF8-1</i>	Bra019244	1260	419
		KBrH003F06	AC172868	<i>BrGRF8-2</i>	Bra013767	1209	402
<i>AtGRF9</i>	At2g45480	KBrB073H13	AC232521	<i>BrGRF9</i>	Bra039334	1317	438

^aBAC clones were selected from the BrGP database.

Fig. 1 Phenotypes of *BrGRF*-OX plants. (A) Eight-day-old *BrGRF*-OX seedlings. The bar indicates 1 mm. (B) Primary root elongation. Bar, 1 mm. (C) and (D) Soil-grown plants at 15 and 20 days, respectively. Bar, 1 cm. (E) and (F) Leaf size and morphology at 15 and 22 days, respectively. Bar, 1 cm. Wt, untransformed wild-type plants; the numbers indicate line numbers of *BrGRF*-OX

cDNA and BAC clone sequences indicated that all *BrGRF* proteins consisted of 348 to 518 amino acid residues and contained the conserved QLQ and WRC domains in the N-terminal region (Table 1; Wang et al. 2014). Seven *BrGRFs* (*BrGRF3-1*, *3-2*, *5*, *7*, *8-1*, *8-2*, and *9*) under the control of the CaMV 35S promoter were introduced into *A. thaliana* plants by *A. tumefaciens*-mediated transformation. We selected 30 independent T₁ plants on selection media containing hygromycin or phosphinothricin. Genomic PCR analyses confirmed the presence of *BrGRF* in each line (data not shown). Transgenic lines displaying the typical 3-to-1 segregation ratio were selected, and thus all 21 lines for seven *BrGRFs* (3 lines per each gene) were established to contain a single T-DNA insertion (Supplemental Fig. 1B). RT-PCR analysis of T₃ lines confirmed that the established transgenic plants overexpress *BrGRFs*, thus named *BrGRF*-OX (Supplemental Fig. 1C).

Pleiotropic phenotypes of *BrGRF*-OX plants with respect to growth and development

We analyzed dimensional parameters of cotyledons and leaves of all *BrGRF*-OX plants at different developmental stages. Cotyledons of 8-day-old *BrGRF*-OX seedlings were larger than those of the wild type (Fig. 1A). Quantitative measurements revealed that the surface area and petiole length

of *BrGRF*-OX cotyledons increased by 32 to 51% and 55 to 100%, respectively, over those of the wild type (Fig. 2A). *BrGRF*-OX plants also had longer primary roots compared with the wild type at the same stage (Fig. 1B, 2E).

The leaf sizes of *BrGRF*-OX lines were also larger than those of the wild type throughout the vegetative growth stages (Fig. 1C-F). The second leaves of 15-day-old plants and the fourth leaves of 20-day-old plants were used for the determination of surface areas. As a result, surface areas of 15-day-old and 20-day-old *BrGRF*-OX leaves increased by 18 to 130% and 56 to 69%, respectively, compared with those of the wild type (Fig. 2B, 2C). The increases in the surface area resulted from increases in both length and width of leaf blades. Petiole lengths of *BrGRF*-OX leaves also increased. These results are similar to those of *AtGRF*-OX plants (Horiguchi et al. 2005; Kim et al. 2003; Wang et al. 2014). Bolting and flowering times of *BrGRF*-OX plants were not different from those of the wild type (data not shown). *BrGRF*-OX plants also had larger flowers than the wild type, developing normal stamens and gynoecia (Fig. 2D, 3A). The surface area of *BrGRF*-OX petals increased by 13 to 30% over that of the wild type (Fig. 2D). The increases in the surface area of petals were due to increases in both length and width. These results demonstrate that *BrGRF* overexpression increases plant organ size: influences of *BrGRF8-1*-OX and *BrGRF8-2*-OX are greater than those of the others. Wang et al. (2014) also reported similar data, in which *BrGRF8* overexpression in *A. thaliana* increased sizes of leaves and other organs by regulating cell proliferation. Interestingly, *BrGRF*-OX plants had larger siliques and seeds than the wild type (Fig. 3B, 3C). Morphological observation revealed that both the length and width of seeds increased significantly in *BrGRF*-OX lines compared with the wild type (Table 2), but seed number per silique of transgenic plants was similar to the wild type (data not shown). Weight and oil contents of *BrGRF*-OX seeds increased by 14 to 34% and 15 to 61%, respectively, compared with wild-type seeds. In the transgenic plant seeds, the amount of most fatty acids increased compared with the wild-type, however the composition of various fatty acids in the transgenic plant seeds are not significantly changed compared with the wild-type (Supplemental Table 5). Similarly, it has been reported that up-regulation of *OsGRF4* in rice and overexpression of *BnGRF2* in *A. thaliana* greatly enhanced the size, weight, and yield of seeds (Che et al. 2016; Duan et al. 2016; Hu et al. 2015; Li et al. 2016; Liu et al. 2012). In addition, *Arabidopsis* seeds overexpressing *BnGRF2* contained 10% more oil than wild-type seeds (Liu et al. 2012).

Fig. 2 Quantification of dimensional parameters of *BrGRF*-OX organs. (A) Cotyledons at 8 days. (B) and (C) The 2nd and 4th leaves at 15 and 20 days, respectively. (D) Mature petals at 35 days. (E) Length of primary roots of 8-day-old seedlings. Error bars represent the standard error (SE). Wt, untransformed wild-type plants; the numbers at X axes indicate line numbers of *BrGRF*-OX

Table 2 Seed characters of *BrGRF*-OX plants

Transgenic Plant	Seed length (mm)	Seed width (mm)	Seed weight (mg/100 seeds)	Seed lipids (mg/100 seeds)
Wt	0.465 ± 0.003	0.265 ± 0.002	1.64 ± 0.02	0.66 ± 0.070
<i>BrGRF3-1</i>	0.552 ± 0.002	0.308 ± 0.003	2.01 ± 0.01	0.87 ± 0.078
<i>BrGRF3-2</i>	0.518 ± 0.003	0.313 ± 0.003	2.14 ± 0.02	1.06 ± 0.073
<i>BrGRF5</i>	0.553 ± 0.002	0.321 ± 0.002	1.87 ± 0.02	0.76 ± 0.049
<i>BrGRF7</i>	0.547 ± 0.003	0.313 ± 0.003	2.19 ± 0.01	0.98 ± 0.054
<i>BrGRF8-1</i>	0.522 ± 0.003	0.314 ± 0.002	2.06 ± 0.02	1.03 ± 0.084
<i>BrGRF8-2</i>	0.567 ± 0.002	0.318 ± 0.003	2.20 ± 0.05	1.02 ± 0.016
<i>BrGRF9</i>	0.528 ± 0.003	0.314 ± 0.003	2.09 ± 0.02	1.00 ± 0.074

Overexpression of *BrGRFs* enhances organ growth by promoting cell proliferation

The final size of plant organs is a result of the coordinated action of two processes, i.e., cell division and cell expansion (Gonzalez et al 2010). To examine the cellular basis for the alterations in organ size, we observed adaxial palisade cells of cotyledons and leaves as wells as epidermal cells

of petals, and found that there was no significant differences in cell sizes between the *BrGRF*-OX and the wild-type organs (Fig. 4). This observation was confirmed by quantitative analysis as well (Fig. 5). In contrast, total numbers of cells in cotyledons, leaves, and petals of *BrGRF*-OX plants significantly increased by 33.7 to 55.8%, 15 to 130%, and 19 to 45.1%, respectively, in comparison with the wild type (Fig. 5). These results indicate therefore that the large size

Fig. 3 Phenotypic characters of reproductive organs and seeds of *BrGRF*-OX. (A) Flowers, (B) Siliques, and (C) Seeds. Wt, untransformed wild-type plants; the numbers indicate line numbers of *BrGRF*-OX. Bars, 1 mm

of *BrGRF*-OX organs is due to increases in cell numbers, but not due to increases in cell size. Our data are well in agreement with previous studies in which overexpression of *AtGRFs*, *BnGRF2*, and *BrGRF8* promoted cell proliferation activities and thus increased organ size (Kim and Lee 2006; Liu et al. 2012; Wang et al. 2014). In conclusion, the seven *BrGRFs* examined in this study seem to act as positive regulators of the cell proliferation process, consequently determining the final size of organs.

Fig. 4 Microscopic views of adaxial palisade cells in cotyledons and leaves, and epidermal cells of petals of *BrGRF*-OX. Wt, untransformed wild-type plants; the numbers indicate line numbers of *BrGRF*-OX. Bar, 10 μ m

Expression patterns of cell division- and hormone-related genes in *BrGRF*-OX plants

Since *BrGRF*-OX lines resulted in increases in cell number and organ size, it is conceivable that *BrGRFs* may regulate the expression of GA-, auxin- and cell division-related genes involved in the control of organ growth. The notion prompted us to analyze expression patterns of 15 GA-, 12 auxin-, and 39 cell division/expansion-related genes using semi-quantitative RT-PCR in shoot apical tissues and leaves. In shoot apical tissues, expression levels of GA-related *GLI*, auxin-related *AXR1*, and cell division-related *ATHB16* were increased by *BrGRF* overexpression, whereas those of cell expansion-related genes, *EBP1* and *EXP3*, were reduced (Fig. 6A). It has been shown that *ATHB16* functions as a negative regulator of leaf

Fig. 5 Numbers and sizes of adaxial palisade cells in cotyledons and leaves, and epidermal cells of petals of *BrGRF*-OX. (A) Cotyledons at 8 days. (B) and (C) The 2nd and 4th leaves at 15 and 20 days, respectively. (D) Mature petals at 35 days. Error bars represent the standard error (SE). Wt, untransformed wild-type plants; the numbers at X axes indicate line numbers of *BrGRF*-OX

cell expansion (Wang et al. 2003); that the ectopic expression of cell expansion-related *EXP10*, *EXP3*, and *EBP1* genes leads to larger leaves and longer petioles by enlarging cell size (Cho and Cosgrove 2000; Horváth et al. 2006; Gonzalez et al. 2009; Kwon et al. 2008). Therefore, our results suggest that, in shoot apical tissues, *BrGRFs* may up-regulate GA-, auxin-, and cell division-related genes, thus promoting organ growth via enhancement of cell division, whereas they may act to restrict expression of cell expansion-related genes. In *BrGRF*-OX leaves, expression levels of GA-related *GMYB65/SOC*, auxin-related *IAMT1/IAA3/IAA6/IAA16/SUR1/SUR2/AXR1*, and cell division-related *CYCB1;1/CYCB 2;1/DWF4/EXOR/AVP1/DA1* all increased, although their expression levels varied depending on *BrGRF*-OX lines (Fig. 6B). Previous studies have shown that GA- and auxin-related genes involved in their biosynthesis, transport, or signaling responses play critical roles in organ morphogenesis (Fleet and Sun 2005; Li et al. 2007). For example, overexpression of GA-related genes resulted in increases in leaf lobe formation and plant growth (Fleet and Sun 2005). The auxin-related genes exerted

Fig. 6 Expression patterns of GA-, auxin-, and cell division-related genes. (A) Shoot apical tissues. (B) Leaves. Error bars represent the standard error (SE). Wt, untransformed wild-type plants; the numbers at X axes indicate line numbers of *BrGRF*-OX. *ATHB17*, *ARABIDOPSIS THALIANA* HOMEBOX-LEUCINE ZIPPER PROTEIN 17; *AVP1*, PYROPHOSPHATE-ENERGIZED VACUOLAR MEMBRANE PROTON PUMP 1; *AXR1*, AUXIN RESISTANT 1; *CYCB1;1*, *CYCLIN-B1-1*; *CYCB2;1*, *CYCLIN-B2-1*; *DA1*, Protein *DA1*; *DWF4*, *DWARF 4*; *EBP1*, *ERBB-3 BINDING PROTEIN 1*; *EXOR*, *EXORDIUM PROTEIN 65*; *IAA3*, 6, and 17, *INDOLE-3-ACETIC ACID INDUCIBLE 3*, 6, and 17; *IAMT1*, *IAA CARBOXYLMETHYLTRANSFERASE 1*; *SUR1* and 2, *SUPERROOT 1* and 2

major effects on plant growth and development by regulating cell division and cell expansion (Li et al. 2007; Perrot-Rechenmann 2010). The fact that the expression of *CYCB1;1* and *CYCB2;1* was increased by *BrGRF* overexpression indicates that *BrGRFs* may control the cell proliferation activities by positively regulating expression of cell cycle genes in leaves, as described by Lee et al. (2009). It has been shown that the overexpression of *DWF4* and *EXOR*

results in larger leaves with longer petioles and enhances organ growth (Choe et al 2001; Coll-Garcia et al. 2004). *AVP1* is involved in the control of auxin transport, and its overexpression dramatically enhanced organ growth by increasing cell number (Li et al. 2005). *DA1* also controls the final size of seeds and organs in *A. thaliana* by regulating the period of cell proliferation (Li et al. 2008). Taken together, we suggest that transcriptional regulation of those genes by *BrGRF-OX* may result in bigger organ size by increasing cell numbers.

Conclusion

We have investigated the biological function of seven *BrGRFs* by overexpressing them in *A. thaliana*, and found that *BrGRF-OX* plants produced larger cotyledons, leaves, flowers, and seeds. The results indicate that *BrGRFs* act as positive regulators in regulation of growth and development of plant organs. The positive effect resulted from enhancement of cell proliferation activities. We also found that *BrGRFs* regulate expression of several GA-, auxin-, and cell division-related genes involved in the control of organ growth. Therefore, we propose that *BrGRFs* can be utilized for improvement of agronomically important traits of crop plants.

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