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RESEARCH PAPER

The *BnGRF2* gene (*GRF2-like* gene from *Brassica napus*) enhances seed oil production through regulating cell number and plant photosynthesis

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Abstract

Seed yield and oil content are two important agricultural characteristics in oil crop breeding, and a lot of functional gene research is being concentrated on increasing these factors. In this study, by differential gene expression analyses between rapeseed lines (zy036 and 51070) which exhibit different levels of seed oil production, *BnGRF2* (*Brassica napus* growth-regulating factor 2-like gene) was identified in the high oil-producing line zy036. To elucidate the possible roles of BnGRF2 in seed oil production, the cDNA sequences of the rapeseed *GRF2* gene were isolated. The Blastn result showed that rapeseed contained *BnGRF2a/2b* which were located in the A genome (A1 and A3) and C genome (C1 and C6), respectively, and the dominantly expressed gene *BnGRF2a* was chosen for transgenic research. Analysis of 35S-*BnGRF2a* transgenic *Arabidopsis* showed that overexpressed *BnGRF2a* resulted in an increase in seed oil production of >50%. Moreover, *BnGRF2a* also induced a >20% enlargement in extended leaves and >40% improvement in photosynthetic efficiency because of an increase in the chlorophyll content. Furthermore, transcriptome analyses indicated that some genes associated with cell proliferation, photosynthesis, and oil synthesis were up-regulated, which revealed that cell number and plant photosynthesis contributed to the increased seed weight and oil content. Because of less efficient self-fertilization induced by the longer pistil in the 35S-*BnGRF2a* transgenic line, Napin-*BnGRF2a* transgenic lines were further used to identify the function of *BnGRF2*, and the results showed that seed oil production also could increase >40% compared with the wild-type control. The results suggest that improvement to economically important characteristics in oil crops may be achieved by manipulation of the *GRF2* expression level.

Key words: *BnGRF2*, chlorophyll, leaf morphology, oil production, seed size.

Introduction

Vegetable oil is an important edible product and its use as an industrial resource is increasing, particularly as a source for biodiesel (Graef *et al.*, 2009; Lu *et al.*, 2011; Rogalski and Carrer, 2011). With the recent population growth and the developing global economy, the demand for vegetable oil has risen sharply (Lu *et al.*, 2011). Over 22 Mt of rapeseed (*Brassica napus*) oil was produced globally during 2009–2010 and it represented the third largest source of vegetable oil (Tan *et al.*, 2010). Therefore, increasing rapeseed oil production is of paramount importance for the supply of vegetable oil for food and non-food applications (Weselake *et al.*, 2009). The amount of oil production per seed is determined by seed weight and oil content. Therefore, most genetic studies and breeding programmes

on oilseed crops focus on elevating seed weight and/or oil content.

Seed weight, also characterized as seed mass or seed size generally, has been widely accepted as a complex trait controlled by polygenes. In plants, organ morphogenesis and size are the result of coordinated patterns of cell proliferation, expansion, and differentiation (Mizukami and Fischer, 2000; Horiguchi *et al.*, 2005; Kozuka *et al.*, 2005; Dupuy *et al.*, 2010). After extensive studies over a long time, many genes which influence cell proliferation and expansion in seed development have been identified. In *Arabidopsis*, knowledge of most genes controlling seed mass development came from analyses of mutants. Several factors regulating seed size have been identified (Berger *et al.*, 2006). Seed size is increased in *ap2* or *arf2/mnt* mutants (Jofuku *et al.*, 2005; Ohto *et al.*, 2005; Schruff *et al.*, 2006) and reduced in *ttg2* mutants (Johnson *et al.*, 2002; Garcia *et al.*, 2005), and this effect appears to take place in the integument surrounding the seed. In addition to the integument, endosperm size has been found to play a major role in regulation of seed size (Berger *et al.*, 2006). Loss-of-function of the WRKY transcription factor gene *MINI3* and the leucine-rich repeat (LRR) kinase gene *IKU2* (Garcia *et al.*, 2003; Luo *et al.*, 2005), which reduce seed mass, stop the nuclear divisions associated with cellularization. Some genes from quantitative trait loci (QTL) that influenced seed weight have been cloned in a number of crop plants, for example *GS3* (Fan *et al.*, 2006; Takano-Kai *et al.*, 2009; Mao *et al.*, 2010), *GW2* (Song *et al.*, 2007), *qSW5* or *GW5* (Shomura *et al.*, 2008; Weng *et al.*, 2008), and *GIF1* (Wang *et al.*, 2008) in rice, and *SW4.1* in tomato (Orsi *et al.*, 2009). In oilseed crops, although many QTLs that influence seed weight have been mapped, the specific genes associated with these QTLs have not been identified so far (Teng *et al.*, 2009; Chen *et al.*, 2011).

In oilseed plants, seed storage oils are composed primarily of triacylglycerol (TAG), which is synthesized from glycerol-3-phosphate and fatty acids (FAs) (Ohlrogge and Browse, 1995; Voelker and Kinney, 2001). The pathways of biosynthesis of FAs and TAG have been well characterized (Ohlrogge and Browse, 1995; Harwood, 1996; Beisson *et al.*, 2003). Over the past 20 years, the model plant *Arabidopsis thaliana* and the oil crop rapeseed have been used as part of a concerted research effort centred on genes related directly to seed oil biosynthesis, including those involved in energy metabolism (*PDHK* and *G6PDH*), FA biosynthesis (*ACCase* and *G3PDH*), and the TAG synthesis pathway (*GPAT*, *LPAAT*, and *DGAT*) (Baud and Lepiniec, 2009; Weselake *et al.*, 2009). However, since oil synthesis is a highly coordinated process that involves carbon metabolism, FA synthesis, and TAG synthesis pathways, manipulation of a single gene may not be an efficient approach for elevating seed oil content significantly (Thelen and Ohlrogge, 2002; Cahoon *et al.*, 2007; Weselake *et al.*, 2009). In recent years, candidate genes encoding seed-specific transcription factors such as *GL2*, *WRI1*, *LEC1*, and *LEC2* in *Arabidopsis* and *BnWRI1*, *BnLEC1*, and *BnL1L* in rapeseed have been shown to play important roles in regulation of FA biosynthesis (Shen *et al.*, 2006; Baud *et al.*, 2007; Mu *et al.*, 2008; Santos-Mendoza *et al.*, 2008; Liu *et al.*, 2010; Tan *et al.*, 2010). Therefore, oil synthesis-related transcription factors might represent promising targets for producing a higher oil yield in oil crops through genetic engineering (Ekman *et al.*, 2008).

GRF (growth-regulating factor) was first reported to perform a regulatory role in rice stem growth, and sequence analyses indicated that it might function as a transcription factor (Van der Knaap *et al.*, 2000). Investigation of GRF-interacting factors (GIFs) suggests that GRFs may act as transcriptional activators (Kim and Kende, 2004). The conspicuous function of *Arabidopsis* GRFs was proved in development of leaves, cotyledons, and floral organs (Kim *et al.*, 2003; Kim and Kende, 2004; Horiguchi *et al.*, 2005; Liu *et al.*, 2009). Some researchers showed that GRFs functioned in *Arabidopsis* female reproductive development and ovule formation (Wynn *et al.*, 2011) and were also expressed in rice embryo (Ye *et al.*, 2004) and maize ear (Zhang *et al.*, 2008), which suggested the possible roles of GRFs in seed development. In the present study, from microarray analyses of 20 d ovules in rapeseed lines with high and low levels of oil production (zy036 and 51070, respectively), a high expression level of a GRF2-like gene was observed in zy036, which suggested that this gene might have effects in controlling seed oil production. By transgenic work, it was proved that overexpression of *BnGRF2* could increase oil production by inceasing the seed mass and oil content. Furthermore, by analysing its downstream genes, it was possible to explore how the gene acts on seed oil production.

Materials and methods

Plant growth conditions

Wild-type (WT) Col-2 and *BnGRF2* transgenic *Arabidopsis* plants were grown in pots with compost soil. Seeds were pre-incubated in the dark for 3 d at 4 °C before transferring to a growth room with a continuous artificial light period of 16 h (24 °C) and a dark period of 8 h (22 °C) at a photon flux density of 100–120 μmol m^{-2} s^{-1}. WT *Arabidopsis* and *BnGRF2* transgenic plants were grown side by side in the same container to minimize variables that arise from differences in the microenvironment of the growth room.

Vector construction and plant transformation

BLAST was used to compare the *Arabidopsis GRF2* sequence against *Brassica rapa* and *Brassica oleracea* genome databases, and two homologues of *GRF2* were identified in each species. Fragments encoding *BnGRF2a* and *BnGRF2b* were amplified from zy036 with the primers designed against the *Brassica* coding sequences using an RT-PCR kit (Qiagen, Düsseldorf, Germany). PCR amplifications were carried out with 35 cycles of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 90 s. The amplicons were cloned into the Gateway entry vector pCR/GW/TOPO (Invitrogen, Carlsbad, CA, USA) using TA overhangs. Proper orientation and integrity were confirmed by sequencing with the *BnGRF2a*R and M13 primers.

To construct the expression plasmid pEarleyGate100-*BnGRF2a*, the *BnGRF2a* coding region was transferred from pCR/GW/TOPO

to the pEarleyGate100 (Invitrogen) using the Gateway™ LR Clonase™ Enzyme Mix (Invitrogen). The construct was confirmed by PCR with the *Cauliflower mosaic virus* (CaMV) 35S promoter primer pCaMVp and *BnGRF2a*R.

For construction of the napin promoter vector, the *BnGRF2a* coding region was obtained with the primers BnGRF2aSmaI and BnGRF2aBamHI. PCR amplification was carried out with 30 cycles of 94 °C for 30 s, 60 °C for 90 s, and 72 °C for 90 s, followed by digestion with *Sma*I and *Bam*HI, and ligation into the pBI vector (reconstructed with the napin promoter). The constructed vector was confirmed by PCR with primers NapinP and *BnGRF2a*R. *Arabidopsis* was transformed with *Agrobacterium tumefaciens* strain GV3101 using the floral dip method (Clough and Bent, 1998). Transformants expressing the *bar* resistance gene were selected and confirmed by PCR with pCaMV, NapinP, and *BnGRF2*R primers. Homozygous lines overexpressing *BnGRF2a* were selected for phenotypic and microarray analyses. Primers used for gene isolation and expression vector confirmation are listed in Supplementary Table S1 available at *JXB* online (the underlined bases indicate additional restriction sites for *Sma*I and *Bam*HI).

Real-time RT-PCR

Total RNA was extracted from rapeseed tissues and *Arabidopsis* tissues (young leaf of 20 d after germinating, fully expanded adult leaf and silique of 10 d after flowering) using the Plant Mini RNeasy kit (Qiagen). The reverse transcription reaction was performed using the First Strand cDNA Synthesis Kit for RT-PCR (Takara, Dalian, China). Primers (*BnGRF2a*RTf, *BnGRF2a*RTr, *BnGRF2b*RTf, and *BnGRF2b*RTr) were designed to detect expression of *BnGRF2a* and *BnGRF2b* in rapeseed. Rapeseed *β-actin2* and *β-actin3* (Gao *et al.*, 2004; Weng *et al.*, 2005) served as endogenous reference genes and were amplified using the primers Bnactin2-F, Bnactin2-R, and Bnactin3F, Bnactin3R. Primers for *BnGRF2a* (*BnGRF2a*RTf and *BnGRF2a*RTr) and *AtGRF2* (*AtGRF2*RTf and *AtGRF2*RTr) were used to detect *BnGRF2a* expression levels in transgenic *Arabidopsis*. *Arabidopsis β-actin1* (At2g37620) and *UBQ10* (At4g05320) served as an endogenous reference genes and were amplified using the primers AtactinF1, AtactinR, and AtUBQ10F, AtUBQ10R (Czechowski *et al.*, 2005; Rus *et al.*, 2006). The real-time PCR contained 1 μl of 10-fold diluted first-strand cDNA, 1 μl of SYBR Green (Applied Biosystems, Carlsbad, CA, USA), 0.2 mM dNTP, 1× LA buffer, 0.5 U of LA Taq (Takara) and 10 μM of each primer. Initial denaturion time was 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. A melting curve was performed after PCR cycles to verify that only one PCR product was amplified. Experiments were performed in triplicate and data were obtained from three independent experiments with similar results. The absolute slope values of the curve of log cDNA dilution versus ΔC(T) were assessed and the efficiencies of primers for target and reference genes were considered equal if the absolute slopes were <0.1. Primers used for real-time PCR are listed in Supplementary Table S1 at *JXB* online.

Insertion site analyses of transgenic lines

Leaf DNA from two transgenic lines, 35S-Bn*GRF2a*-2 and 35S-Bn*GRF2a*-10, was extracted using the Plant DNA extraction kit (Qiagen). Random primers AD1 and AD2 as well as vector primers LBR1, LBR2, and LBR3 were used for TAIL (thermal asymmetric interlaced) PCR. Cycle settings in PCR amplifications were carried out according to the descriptions in Liu *et al.* (1995).

Pleiotropic phenotypes of transgenic Arabidopsis *leaves and seeds*

Leaves were excised from WT Col-2, 35S*BnGRF2a*-2, and 35S*BnGRF2a*-10 plants using a razor blade and scanned to acquire digital images for determination of the surface area. As described previously (Tsuge *et al.*, 1996), the leaves were fixed with FAA and cleared with chloral hydrate solution (200 g of chloral hydrate, 20 g of glycerol, and 50 ml of dH_2O). Leaf cells were observed by differential interference contrast (DIC) microscopy (DM6000B; Leica Microsystems, Japan). Seeds from the WT and the Napin-*BnGRF2a*-3 line were photographed under a dissection microscope, weighed, and measured. Mature embryos were imbibed for 1 h and dissected under a microscope according to the description in Ohto *et al.* (2005) with slight modification, and photographed with an Olympus compound microscope. Seed oil contents were detected by NMR PQ001 (Niumag, China).

Chlorophyll content and photosynthesis detection

Tissue samples (1.0 g) were obtained from fully expanded leaves and 15 d siliques after flowering. At least three replicates were taken from different plants of each line. After freezing in liquid nitrogen, the samples were ground to a power in 50 ml of extraction solution (2:1 acetone:95% alcohol) and incubated in the dark at 4 °C overnight to ensure complete extraction of chlorophyll. The cell debris was pelleted by centrifugation for 1 min at 15 000 *g*, and absorbance of the supernatant was measured at 646.6, 663.6, and 750 nm, as described previously (Porra *et al.*, 1989). Chlorophyll content was expressed in micrograms. The photosynthetic parameters were measured with a portable photosynthesis system LI-6400XT (LI-COR Biosciences, Lincoln, NE, USA) in a growth room. The measurement conditions were as follows: 24 °C, a photon flux density of 100–120 μmol m^{-2} s^{-1}, and 40–60% humidity. All data were determined as the means of five plants.

Microarray analyses

Microarray analyses were performed with WT and 35S-*BnGRF2*-overexpressing plants by the Agilent *Arabidopsis* Oligo DNA microarray. Young leaves were harvested from 20-day-old *Arabidopsis* plants after germination and frozen immediately in liquid nitrogen. Total RNA was extracted using the RNeasy plant mini kit (Qiagen). Spectrophotometric analyses of the isolated total RNA were carried out at 260 nm and 280 nm to determine sample concentration and purity. Regulated genes were identified with a stringent significance threshold, namely a mean >1.5-fold change (transgenic relative to WT control samples) and a P value ≤0.01, based on at least three out four replicates. Primers used in real-time PCR experiments of six genes are listed in Supplementary Table S1 at *JXB* online.

Results

Sequence analyses of BnGRF2 from the rapeseed line zy036

The amount of oil per seed from rapeseed lines zy036 and 51070 (2.14 mg and 1.38 mg per seed, respectively) has been reported (Hua *et al.*, 2012). Gene expression analyses of 20 d ovules from zy036 and 51070 showed differential expression of *BnGRF2* (homologous to *Arabidopsis AtGRF2*, At4g37740) (Supplementary Table S2 at *JXB* online). To isolate the gene for functional analyses, the *Arabidopsis GRF2* cDNA sequence was compared with the *Brassica* genomic databases: *B. rapa* (Wang *et al.*, 2011) and *B. oleracea* (scaffold sequences, unpublished) using BLASTN. The results indicated that rapeseed contained two *GRF2* homologues named *GRF2a*/*2b* which are located in the A genome (A1 and A3 chromosome) and the

C genome (C1 and C6 chromosome), respectively. With primers designed against Brassica *GRF2a/2b*, the coding region sequences of *BnGRF2a* (GenBank accession nos JN831660 and JN831661) and *BnGRF2b* (GenBank accession nos JN831662 and JN831663) were isolated from 20 d ovules of zy036. As shown in Fig. 1, the predicted polypeptides encoded by *BnGRF2a/2b* were homologous to *AtGRF2*, with 65% and 69% identity, respectively. Similar to the other members of the AtGRF family, BnGRF2 contains highly conserved QLQ and WRC domains, with the cysteine (C) and histidine (H) residues of the C3H motif, as well as the TQL and GGPL motifs (Kim *et al.*, 2003).

To investigate the *BnGRF2* expression pattern in detail, *BnGRF2a/2b* transcript levels were analysed in different rapeseed tissues (root, stem, leaf, flower bud, silique wall, and ovule) in zy036 and 51070. Real-time PCR results confirmed differential expression of the *BnGRF2* gene in the ovule, with the expression level of *BnGRF2a/2b* >8-fold higher in zy036 than in 51070. Differential *BnGRF2a/2b* expression levels were also observed in the other five tissues, with the highest and lowest levels being found in the silique wall and flower bud, respectively (Fig. 2; Supplementary Fig. S1 at *JXB* online). Moreover, there were 10- to 30-fold higher expression levels of *BnGRF2a* than *BnGRF2b* in different tissues, which suggested that *BnGRF2a* was the dominant expressed gene compared with *BnGRF2b* and should be chosen for further research.

Transformation of Arabidopsis *with 35S*-BnGRF2a *and identification of transgenic lines*

With the aim of studying the possible function of BnGRF2, *Arabidopsis* plants were transfected with *BnGRF2a* under the control of the CaMV 35S promoter. After three rounds

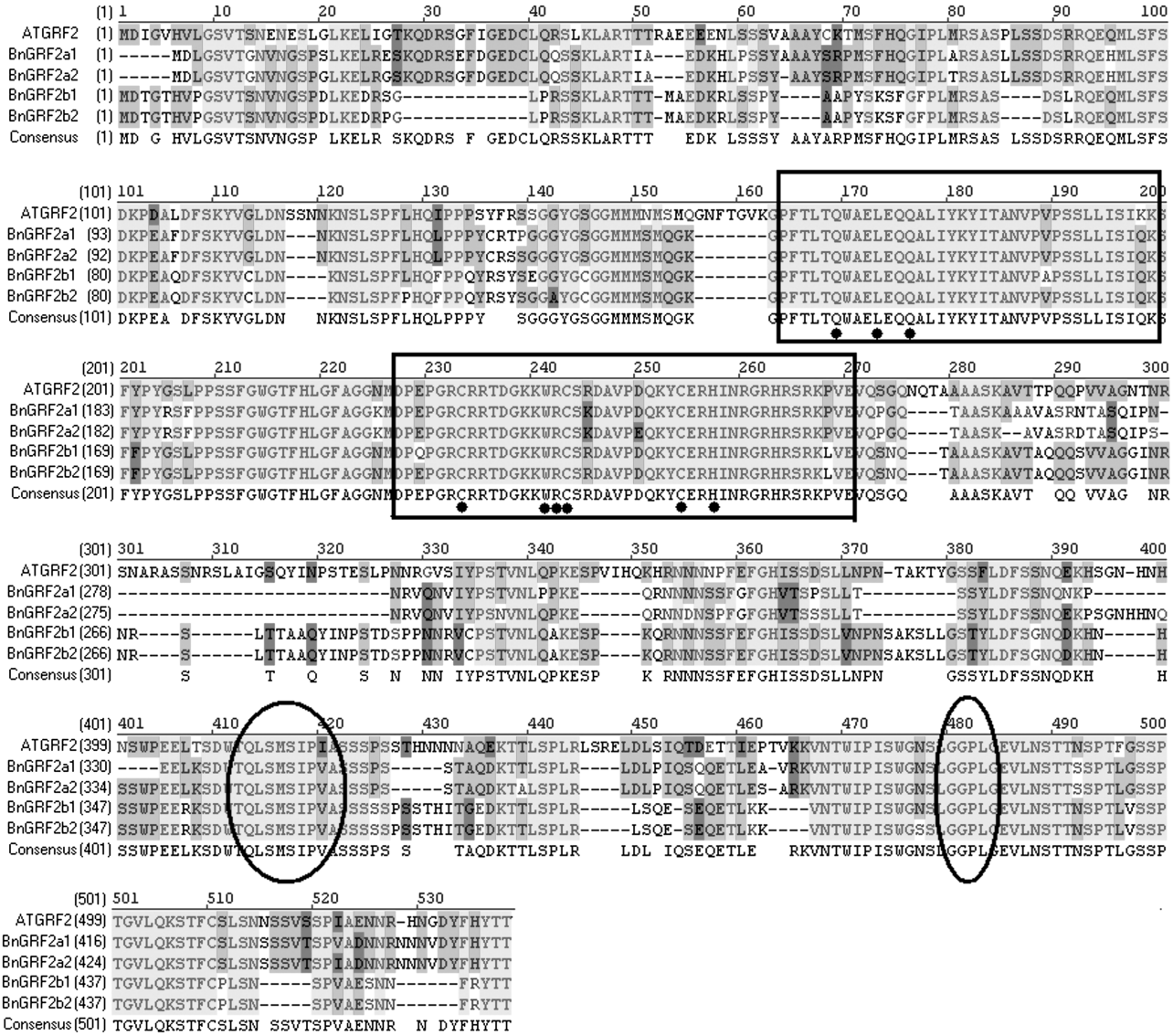

Fig. 1. Sequence alignment of AtGRF2 and BnGRF2. Black boxes indicate the QLQ and WRC domains. The cysteine (C) and histidine (H) residues of the C3H motif in the WRC domain are shown with a black dot. The TQL and GGPL motifs are highlighted with ellipses. (This figure is available in colour at *JXB* online.)

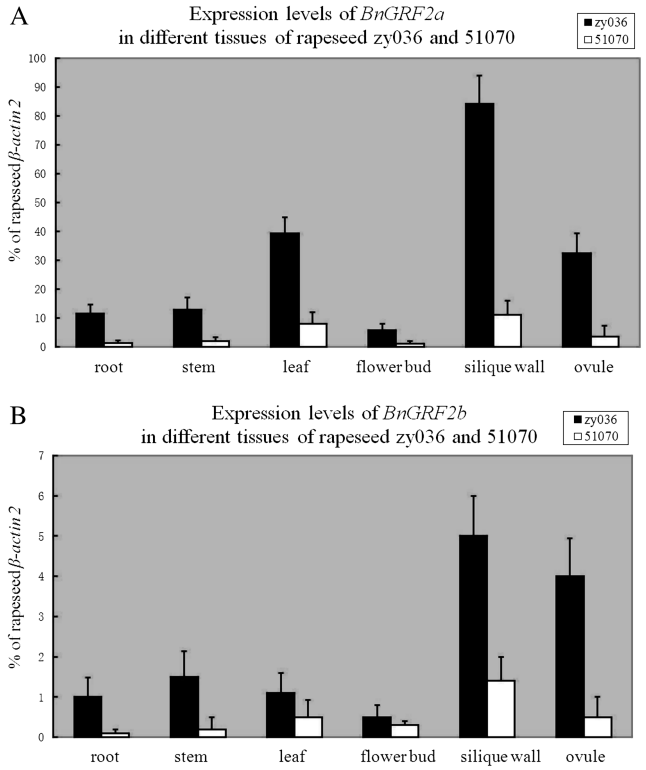

Fig. 2. Real-time PCR analyses of the *BnGRF2* gene in different tissues (root, stem, leaf, flower bud, silique wall, and ovule) between rapeseed lines zy036 and 51070. (A) Expression analyses of *BnGRF2a*. (B) Expression analyses of *BnGRF2b*. Data expression was normalized to rapeseed *β-actin2*, and expression levels of *BnGRF2* were compared with that of *β-actin2*. Data presented are mean values of three biological replicates, and error bars represent standard deviations.

of Basta selection, 21 independent homogenous T_3 transgenic lines were obtained. Total RNA from young leaves of independent transgenic *Arabidopsis* lines was extracted for quantitative RT-PCR. The results showed that *BnGRF2a* was highly expressed in all the selected lines and the transgenic plants with phenotypes different from that of the WT exhibited higher expression levels of *BnGRF2* (Fig. 3A). Insertion sites of the transgenic lines were identified to confirm the successful transformation of *BnGRF2a*. Sequence analyses of insertion sites indicated that the vector sequence inserted into different *Arabidopsis* chromosomes. For example, the *BnGRF2a* gene was inserted in chromosomes 1 and 2 for 35S-*BnGRF2a*-2 and 35S-*BnGRF2a*-10, respectively (Supplementary Fig. S2 at *JXB* online).

*Pleiotropic phenotypes in 35S-*BnGRF2a *transgenic* Arabidopsis

The 35S-*BnGRF2a* transgenic lines showed clear morphological differentiation enabled them to be distinguished from the WT at ~2 weeks after germination, with transgenic cotyledons exhibiting a longer petiole (Fig. 3B, C). At the flowering stage, the leaf area of transgenic plants was 20–30% larger than that of the WT control (Fig. 3D, Table 1). Leaf width and tooth morphology were also affected, while the lengths of blades and petioles similar to those of the control (Fig. 3D). A DIC microscope was used to compare palisade cells in the subepidermal layers of mature leaves of WT control and transgenic plants. Since no significant difference in epidermal cell size was observed, it was likely that the enlarged leaf area of *BnGRF2a*-overexpressing plants was mediated directly by an increase in cell number (Fig. 3E). In addition, the fully expanded leaves of transgenic lines contained more chloroplasts and higher chlorophyll contents than those of the WT control (Fig. 4A, B). Consistent with this result, it was not surprising to find that the photosynthetic rate of transgenic leaves was >40% higher than that of the WT control (Fig. 4C).

The bolting and flowering times of transgenic lines were delayed by ~5 d (Fig. 3E, Table 1). The flower buds of transgenic *Arabidopsis* had a longer pistil than the WT, but developed a normal stamen, which resulted in less efficient self-fertilization and thus they were nearly sterile (Fig. 3F). Transgenic plants developed greater seed mass (obtained by artificial pollination) with weight increases of >40% and oil production increases of >50% compared with the WT control (Fig. 3G, Table 1). However, reduced fertility in 35S-*BnGRF2* transgenic lines led to the question of whether the large seed mass phenotype could result simply from allocation of extra resources to the few seeds produced. To determine whether the larger seed size is caused by reduced siliques or a higher *BnGRF2a* expression level, all flowers on a WT plant were removed, with the exception of three flowers on each secondary inflorescence. Seeds from 35S-*BnGRF2* transgenic plants were heavier (2.9±0.21 mg and 3.0±0.30 mg, respectively) than those from the deflowered WT control (2.3±0.29 mg), even though the seed number in the silique was comparable. Together, these results suggest that the effect of BnGRF2a on seed mass is not primarily due to its effect on fertility in 35S-*BnGRF2* transgenic lines.

*Seed analyses in Napin-*BnGRF2a *transgenic lines*

To confirm further the effect of BnGRF2a on seed mass, an overexpression vector driven by the napin promoter was constructed. Following transformation and basta selection of *Arabidopsis*, 31 independent homogenous transgenic lines were obtained. Among four lines, seed sizes were increased to different degrees, and quantitative RT-PCR analyses of the transgenic siliques (10 d after flowering) indicated that these lines exhibited high levels of *BnGRF2a* expression (Fig. 5A).

Phenotypic analyses showed the obviously increased widths of the cotyledons, and the hypocotyl generated larger seeds in the Napin-*BnGRF2a*-3 line, which increased the cotyledon area resulting in it being ~1.4-fold larger than that of the WT (Fig. 5B, C, Table 2). Comparison of the epidermal cell layer from the central region of the cotyledon showed no significant difference in cell size between transgenic *Arabidopsis* and the WT (Fig. 5C, Table 2). Compared

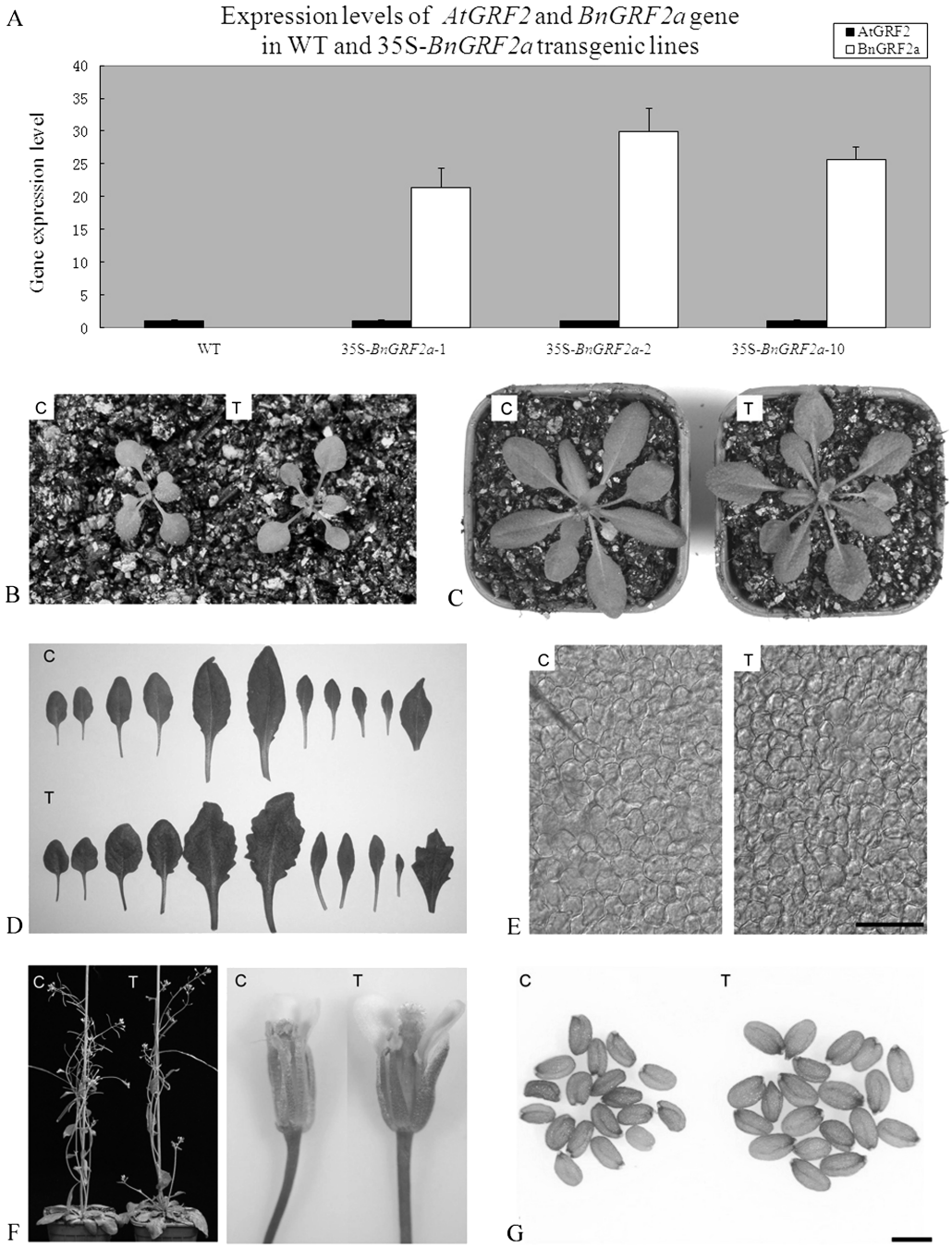

Fig. 3. Phenotype analyses induced by overexpression of *BnGRF2a* in *Arabidopsis*. (A) Real-time PCR analyses of *BnGRF2a* expression levels in the adult leaves of 35S-*BnGRF2a* transgenic *Arabidopsis* lines. Data expression was normalized to *Arabidopsis β-actin1* (At2g37620) and relative to the expression of *AtGRF2*. Data presented are mean values of three biological replicates and error bars represent standard deviations. (B–D) Leaf phenotypes at different developmental stages (B, 2 weeks after germination; C, bolting time; D, flowering time). (E) Paradermal view of palisade cells in the subepidermal layer of adult leaves in the wild type and the 35S-*BnGRF2a*-2 transgenic line. Bar, 100 μm. (F) The longer pistil induced near sterility in 35S-*BnGRF2a*-2 transgenic *Arabidopsis*. (G) A *BnGRF2a* transgenic plant produces large seeds (shown are mature dried seeds from the wild type and the 35S-*BnGRF2a*-2 transgenic line). C, wild-type control; T, transgenic plant. Bar, 500 μm. (This figure is available in colour at *JXB* online.)

with the control seeds, the transgenic seed showed a >30% increase in weight without change in seed number per silique, which means seed yield could increase >30%. The chlorophyll content of 15 d ovules was >20% higher in the Napin-*BnGRF2a*-3 line and seed oil production in mature seed was >40% higher (Table 2).

Microarray analyses of BnGRF2a-*regulated pathways*

To investigate the genome-wide effects on transcription resulting from *BnGRF2a* overexpression, microarray experiments were performed on three independent biological replicates of 35S-*BnGRF2a* and WT control plants. RNA

Table 1. Phenotypic parameters including bolting time, flowering time, leaf number, leaf area, and seed characters in wild-type control and 35S-*BnGRF2a* transgenic lines

	WT	**35S-*BnGRF2a*−2**	**35S-*BnGRF2a*−10**
Bolting time (d)	20.9±0.5	25.7±0.8	27.8±0.6
Flowering time (d)	25.1±0.3	30.3±0.5	32.8±0.9
No. of leaves before flowering	10.2±0.6	11.3±0.2	10.0±0.3
Adult leaf area (cm^2)	3.79±0.26	4.63±0.12	5.17±0.11
Seed weight (mg/100 seeds)	1.9±0.25	2.9±0.21	3.0±0.30
No. of cells in a leaf	134 100±9200	163 800±4200	182 900±3900
Seed oil production (mg/100 seeds)	0.59±0.07	0.91±0.09	1.01±0.12

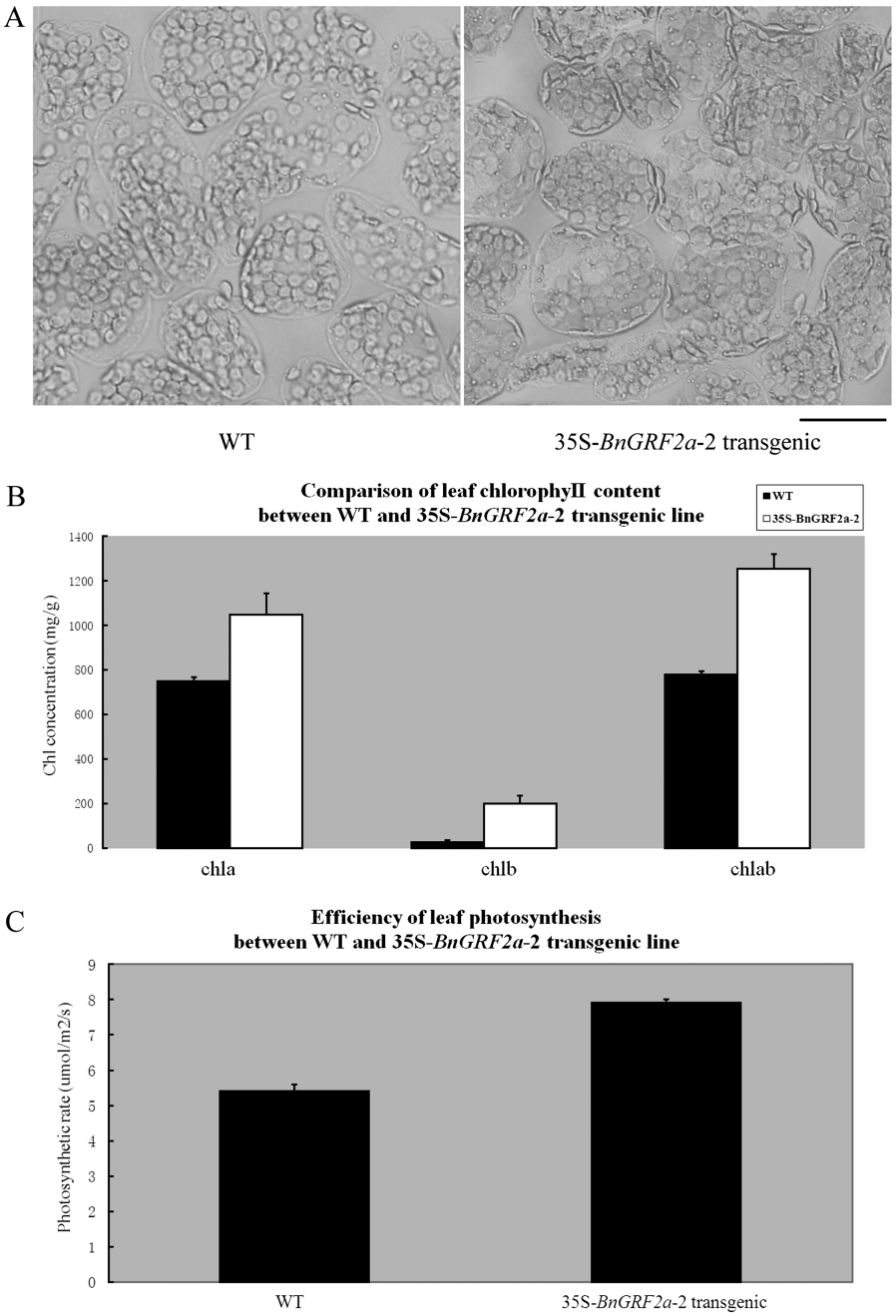

Fig. 4. The detection of chloroplast (A), chlorophyll content (B), and photosynthetic efficiency (C) in fully expanded leaves of 4-week-old plants of the wild-type (WT) control and the 35S-*BnGRF2a*-2 line. Bar, 20 μm. Data presented in B and C are mean values of three biological replicates and error bars represent standard deviations. (This figure is available in colour at *JXB* online.)

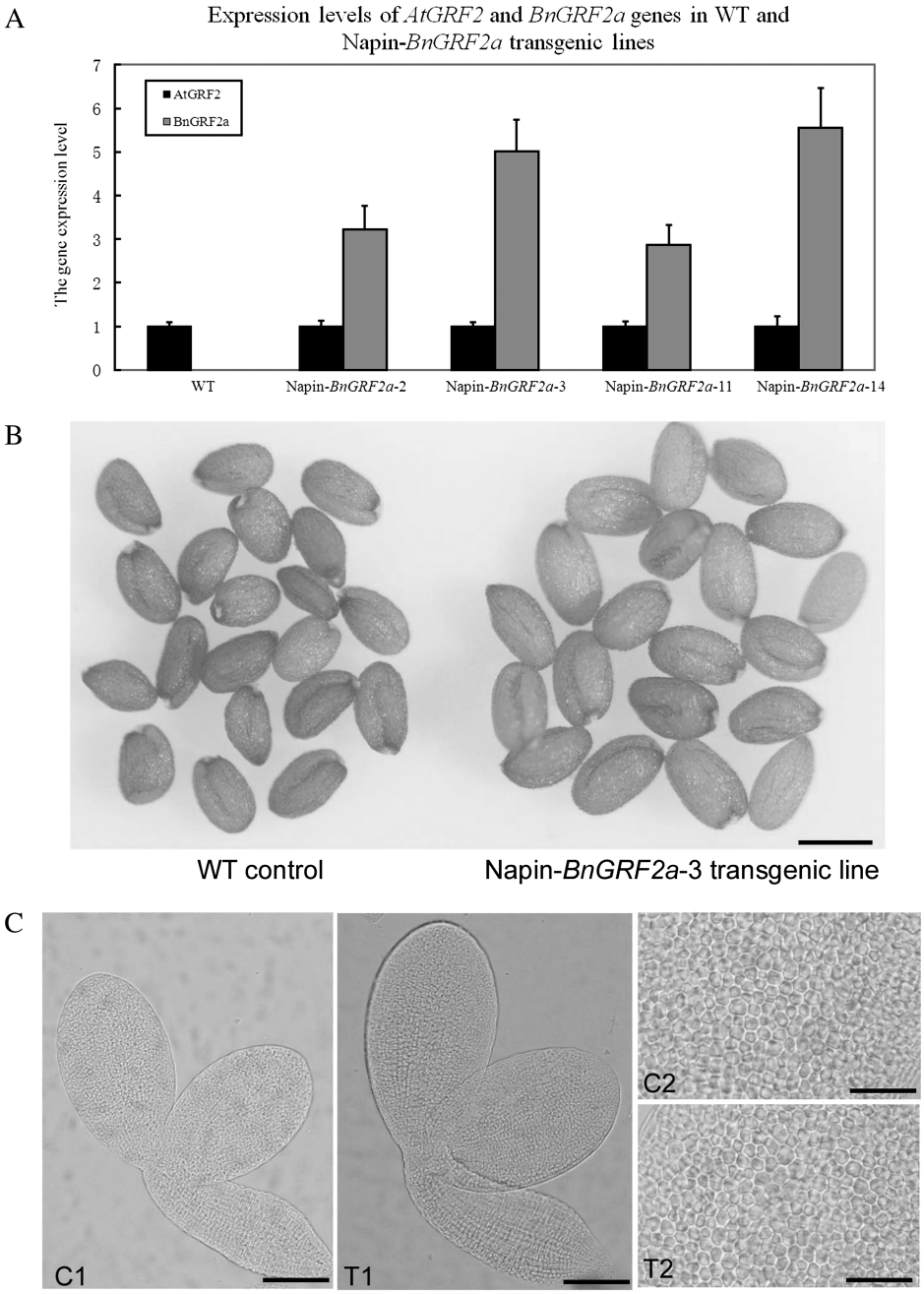

Fig. 5. Analyses to *BnGRF2a* expression levels and seed phenotypes in Napin-*BnGRF2a Arabidopsis* lines. (A) Relative gene expression of *BnGRF2a* in the silique (10 d after flowering) of the transgenic Napin-*BnGRF2a Arabidopsis* line was identified by qRT-PCR. Data presented are mean values of three biological replicates, and error bars represent standard deviations. Data expression was normalized to *β-actin1* (At2g37620) and relative to the expression of *AtGRF2*. (B) Mature seeds obtained from mature dried seeds of the wild type and the Napin-*BnGRF2a*-3 line. Bar, 500 μm. (C) Mature embryos obtained from mature dried seeds of the wild type (C1) and the Napin-*BnGRF2a*-3 line (T1). Bar, 240 μm. Epidermal cell layer in the central region of cotyledons from embryos of the wild type (C2) and the Napin-*BnGRF2a*-3 line (T2). Bar, 80 μm. (This figure is available in colour at *JXB* online.)

from young leaves of 4-week-old plants was chosen for gene expression analyses. An overview of the differential gene expression between the two genotypes is presented in Supplementary Table S3 at *JXB* online. Genetic up-regulation of the *BnGRF2a* expression level in *Arabidopsis* resulted in increased expression of 1601 genes and decreased expression of 1234 genes.

To classify and analyse the genes affected by *BnGRF2a*, the differentially expressed genes were submitted to an enrichment analyses (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi). When the genes were classified according to Gene Ontology (GO) terms relating to biological process, cellular compartment, and molecular function, transcription factor-related terms were highly

Table 2. Comparison of seed characteristics between wild-type *Arabidopsis* and Napin-*BnGRF2a* transgenic lines

Genotype	Weight (mg/100 seeds)	Oil production (mg/100 seeds)	Dimensions (length×width, mm)	Area per cotyledon (mm^2)	No. of cells in a cotyledon	Chlorophyll content (mg/g)
Col-2 WT	1.9±0.24	0.61±0.08	0.40±0.020×0.23±0.011	0.09±0.01	717±80	0.29±0.04
Napin-*BnGRF2a*−3	2.5±0.19	0.86±0.07	0.43±0.023×0.30±0.025	0.13±0.01	1035±80	0.35±0.06
Napin-*BnGRF2a*−14	2.4±0.27	0.92±0.10	0.42±0.018×0.29±0.017	0.12±0.02	955±160	0.39±0.01

represented and significantly enriched (Supplementary Table S4 at *JXB* online; Fig. 6A). When classified with MapMan, 313 of 345 differentially expressed transcription factor genes were classified into 49 gene families (Fig. 6B). Among the up-regulated transcription factors, 42.9% (84/196) belonged to five main families, namely the WRKY domain family, the MYB domain and related transcription factor family, the basic helix–loop–helix (bHLH) family, the AP2/EREBP family, and the C2H2 zinc finger family (Fig. 6C). In addition to transcription factors, the regulated genes were mainly involved in several different metabolic processes relating to hormones, secondary substances, carbohydrates, lipids, and amino acids (Fig. 6B).

Up-regulated genes in possible BnGRF2*-regulated pathways*

Corresponding to the pleiotropic phenotypes of the transgenic lines, there was up-regulation of a number of genes that were thought to be involved in regulatory pathways (Supplementary Table S5 at *JXB* online; Table 3). In 35S-*BnGRF2a* and Napin-*BnGRF2a* transgenic lines, overexpression of *BnGRF2a* resulted in an enlarged leaf and cotyledon area, respectively, which were induced by increasing cell numbers. According to microarray analyses, some genes related to cell division [*PHB3* (At5g40770), *RCC1* (At3g53830, At1g69710), and *MAPKKK16* (At4g26890)] and the cell cycle [*CYCH;1* (At5g27620), *CYCA2;1* (At5g25380), *CYCA2;3* (At1g15570), *CYCD4;2* (At5g10440), *CYCD6;1* (At4g03270), and *CYCP4;1* (At2g44740)] were up-regulated in the *BnGRF2a* transgenic lines (Table 3).

As might be expected from the increased chlorophyll content and photosynthetic efficiency caused by BnGRF2, some genes relating to light harvesting and chlorophyll biosynthesis were up-regulated. *GLK2* (golden2-like) (At5g44190), which was up-regulated in the transgenic lines, is known to stimulate expression of genes related to light harvesting and chlorophyll biosynthesis (Waters *et al.*, 2008, 2009). Partially up-regulated genes such as *lhcb2.4* (At3g27690), *CHI* (At1g44446), *lhcb3* (At5g54270), *lhcb6* (At1g15820), and *LTP* (At5g48490) were also found to be coincident with the up-regulated genes induced by GLK2. Also, some genes related to light harvesting and chlorophyll biosynthesis such as *PUB44* (At1g20780), *CPN60A* (At2g28000), *PORA* (At5g54190), *lhcb2.1* (At2g05100), and *lhca4* (At3g47470) were up-regulated in the transgenic lines (Table 3).

In addition, some up-regulated genes such as *KCS16* (At4g34250), *GPAT* (at4g01950), *F4I10.40* (at4g33110), *GDPD6* (at5g08030), *SAMT* (AT5G38020), *SMO1-3* (AT4G22753), and oleosin proteins (at5g56100 and at5g61610) were classified as being involved in lipid and FA biosynthetic and storage processes by GO category (Table 3).

According to the leaf phenotypes of the 35S-*BnGRF2a* transgenic line, although the expression level of most up-regulated genes in transgenic young leaves was <2-fold compared with the WT control (Table 3), they might be more highly expressed in the adult leaf. To confirm this, six genes, *MAPKKK16, CYCA2;1*, *lhcb2.1*, *PORA*, GPAT, and oleosin, were chosen to detect their expression levels in adult leaf of the 35S-*BnGRF2a* transgenic lines. The results showed that most genes were expressed more highly in adult leaf than in young leaf (Fig. 7). The up-regulation of these genes was further verified in seed of the Napin-*BnGRF2a*-3 line (Supplementary Fig. S3 at *JXB* online).

Discussion

The present study began with gene expression analyses of 20 d ovules from the zy036 and 51070 rapeseed lines, which exhibited different levels of seed oil production. The differentially expressed gene *BnGRF2* was chosen for further examination to elucidate its function in oil production. BLAST analyses of the *Brassica* sequence databases indicated that two *BnGRF2* homologues (*BnGRF2a* and *BnGRF2b*) are located in the rapeseed A or C genome. Real-time PCR showed that the two homologues were expressed differentially in six rapeseed tissues, and *BnGRF2a* is expressed dominantly compared with *BnGRF2b*. In *B. rapa* and *B. oleracea*, quite a lot of homologous genes showed differences in expression levels (unpublished), which is a ubiquitous phenomenon during polyploid genome evolution, and suggested the importance of dominantly expressed genes in plant development. Therefore, *BnGRF2a* was chosen for further functional research.

In a previous study, GRFs were believed to function as transcription activators (Kim and Kende 2004); therefore, it was not a surprise that BnGRF2 induced pleiotropic phenotypes in *Arabidopsis*. However, it must be noted that greater phenotypic differences were observed in transgenic overexpression of BnGRF2 (Supplementary Table S6 at *JXB* online), compared with previous reports regarding overexpression of AtGRF2 or other GRF genes (Kim *et al.*, 2003; Kim and Kende, 2004; Horiguchi *et al.*, 2005; Liu

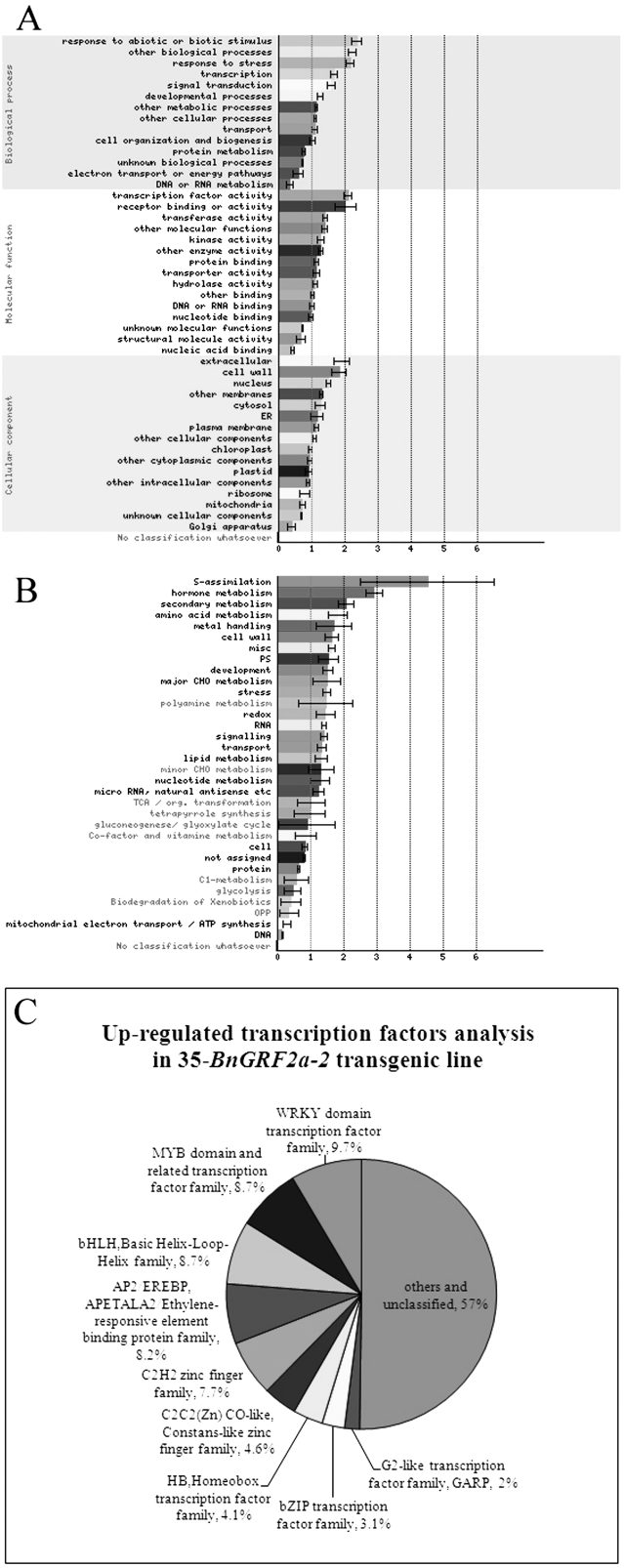

Fig. 6. Analyses of dfferentially expressed genes induced by the *BnGRF2a* gene in *Arabidopsis*. (A) Gene Ontology (GO) analyses of differentially expressed genes. (B) Differentially expressed genes classified with MapMan. (C) Analyses of transcription factors up-regulated in the *BnGRF2* transgenic line. (This figure is available in colour at *JXB* online.)

et al., 2009). First, the increased cell number enlarged the leaf area in *BnGRF2a* transgenic lines. However, in *Arabidopsis*, overexpression of *AtGRF2* resulted in larger leaves because of an increase in cell size. Secondly, the adult leaf colour of the transgenic line was dark green. Thirdly, because of the longer pistil and normal stamen, near sterility was induced compared with *AtGRF2* transgenic plants. Finally, the seed phenotype was not referred to in previous studies. It is thought that this discrepancy is due to the difference in gene sequences. Although *BnGRF2* and *AtGRF2* show good conservation of key domains, they only exhibit 69% identity overall, and these sequence differences might be responsible for the phenotypic differences. Research on *ZmGRF2* and *ZmGIF3* in *Arabidopsis* which only showed delayed bolting of the inflorescence stem and acceleration of the elongation of the stem seemed to confirm this view point (Zhang *et al.*, 2008).

To elucidate how the metabolic pathways participate in the phenotypic changes observed, genome-wide analyses of the *BnGRF2a*-responsive transcriptome were performed by a microarray method. Generally, genes regulate seed mass by changing the cell number (Song *et al.*, 2007; Weng *et al.*, 2008), cell size (Liu *et al.*, 2010), or both (Ohto *et al.*, 2005). In the present experiments, the cell number in the leaf and seed was found to be increased in *BnGRF2a*-overexpressing transgenic lines, which was consistent with the *AtGRF5* gene in the leaf (Horiguchi *et al.*, 2005), but different from *AtGRF1* and *AtGRF2* genes which increase the leaf cell size in *Arabidopsis* (Kim *et al.*, 2003). With respect to the increased cell number, it was found that some genes related to cell division and the cell cycle were up-regulated (Inze and De Veylder, 2006; Meyerowitz, 1997).

Improvement of the photosynthetic rate in transgenic lines was found, which was supported by some up-regulated genes related to light harvesting and chlorophyll biosynthesis. Just like the phenotype of the leaf in the 35S-*BnGRF2a* transgenic lines, the chlorophyll content of developing seeds was also higher in transgenic Napin-*BnGRF2a Arabidopsis*, which coincided with differences in the leaf and ovule between zy036 and 51070 (data not shown). Interestingly, the transgenic seeds contained 10% more oil than WT seeds, possibly as a result of improved photosynthetic efficiency.

Light reactions of photosynthesis play an important role in defining plant fitness and productivity by influencing a seed's carbon economy and how that carbon is stored (Goffman, 2005). Previous studies with developing rapeseed embryos had shown that lipid accumulation was stimulated by light (Aach and Heise, 1998; Ruuska *et al.*, 2004; Schwender *et al.*, 2004). This finding suggested that FA synthesis in the embryo might be dependent on the supply of reducing power (NADPH and NADH) and ATP provided by the light reactions of photosynthesis (Ruuska *et al.*, 2004; Goffman *et al.*, 2005; Schwender *et al.*, 2006). To a certain extent, photosynthetic efficiency increases with an increasing concentration of light and, thus, light stimulates lipid accumulation by increasing photosynthetic efficiency (Li *et al.*, 2006). Some genes relating to FA

Table 3. List of up-regulated genes related to pleiotropic phenotypes (cell number, photosynthesis, and lipid and fatty acid synthesis) in the 35S-*BnGRF2a-2* transgenic *Arabidopsis* line

Gene ID	Short discription	GO function	Subcellar localization	Transcript level
Cell division and cycle				
At1g15570	CYCA2;3, cyclin-dependent protein kinase regulator	Cell cycle regulation	Nucleus	1.68
At1g69710	Putative regulator of chromosome condensation (RCC1) family protein	Chromatin binding	Cellular component	2.03
At2g44740	CYCP4;1, cyclin-dependent protein kinase	Cell cycle regulation	Nucleus	1.82
At3g53830	Regulator of chromosome condensation (RCC1) family protein/UVB-resistance protein-related	Chromatin binding	Cellular component	1.61
At4g03270	CYCD6;1, cyclin-dependent protein kinase	Cell cycle regulation	Nucleus	1.74
At4g26890	MAPKKK16 (mitogen-activated protein kinase kinase kinase 16); kinase	Protein tyrosine kinase activity		1.87
At5g10440	CYCD4;2, cyclin-dependent protein kinase	Cell cycle regulation	Nucleus	1.75
At5g25380	CYCA2;1, cyclin-dependent protein kinase regulator	Cell cycle regulation	Nucleus	2.58
At5g27620	CYCH;1, cyclin-dependent protein kinase/protein binding/protein kinase	Cell cycle regulation	Nucleus	1.80
At5g40770	ATPHB3 (prohibitin 3)	Cell division		1.60
Photosynthesis				
At1g15820	LHCB6 (light-harvesting complex PSII)	Photosynthesis	Plastoglobule	1.61
At1g20780	armadillo/beta-catenin repeat protein-related/U-box domain-containing protein	Regulation of chlorophyll biosynthetic process	Plasma membrane	1.64
At1g27730	STZ (salt tolerance zinc finger); nucleic acid binding/transcription factor/zinc ion binding	Photosynthesis	Nucleus	5.50
At1g44446	CH1 (chlorophyll *b* biosynthesis); chlorophyllide *a* oxygenase	Chlorophyll biosynthetic process	Thylakoid membrane	1.68
At1g68190	Zinc finger (B-box type) family protein	Transcription regulation	Intracellular	1.64
At2g05100	LHCB2.1 (photosystem II light-harvesting complex gene)	Photosynthesis	Chloroplast thylakoid membrane	1.80
At2g28000	CPN60A (chloroplast/60 kDa chaperonin alpha subunit)	Chloroplast organization	Chloroplast envelope	1.59
At3g09490	Chloroplast lumen common family protein	Photosynthesis, light reaction	Chloroplast thylakoid lumen	1.67
At3g27690	LHCB2:4 (photosystem II light-harvesting complex gene)	Photosynthesis	Chloroplast envelope	1.59
At3g47470	LHCA4 (photosystem I light-harvesting complex gene)	Photosynthesis, light harvesting	Chloroplast thylakoid membrane	1.71
At4g01330	Protein kinase family protein	Protein amino acid phosphorylation	Plasma membrane	1.99
At5g44190	GLK2 (GOLDEN2-LIKE 2); DNA binding/transcription factor	Regulation of chlorophyll biosynthetic process	Nucleus	2.23
At5g48490	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	Lipid transport	Endomembrane system	2.53
At5g54190	PORA; oxidoreductase/protochlorophyllide reductase	Chlorophyll biosynthetic process	Chloroplast	2.50
At5g54270	LHCB3 (light-harvesting chlorophyll-binding protein)	Photosynthesis	Chloroplast thylakoid membrane	1.90
Lipid and fatty acid synthesis				
At1g01120	KCS1 (3-ketoacyl-CoA synthase 1); acyltransferase	Fatty acid elongase activity	Cytosolic ribosome	1.91
At2g15090	Fatty acid elongase, putative	Fatty acid elongation	Endoplasmic reticulum	3.48
At2g28630	beta-Ketoacyl-CoA synthase family protein	Acyltransferase activity	Endoplasmic reticulum	1.89
At2g46720	HIC (high carbon dioxide); acyltransferase	Fatty acid elongation	Endomembrane system	1.67
At3g10280	Fatty acid elongase 3-ketoacyl-CoA synthase, putative	Fatty acid elongation	Endomembrane system	1.55
At4g01950	ATGPAT3/GPAT3 (glycerol-3-phosphate acyltransferase 3)	Acyltransferase activity		2.81
At4g22753	SMO1-3 (sterol 4-alpha methyl oxidase); catalytic	Fatty acid biosynthetic process	Endoplasmic reticulum	1.57
At4g33110	Coclaurine *N*-methyltransferase, putative	Lipid biosynthetic process	Plasma membrane	1.54
At4g34250	Fatty acid elongase, putative	Transferase activity	Endomembrane system	2.62
At5g04530	beta-Ketoacyl-CoA synthase family protein	Transferase activity	Endomembrane system	3.81
At5g08030	Glycerophosphoryl diester phosphodiesterase family protein	Glycerol metabolic process	Endomembrane system	3.18
At5g38020	*S*-Adenosyl-L-methionine:carboxyl methyltransferase family protein	Fatty acid biosynthetic process	Cellular component	1.71
At5g56100	Glycine-rich protein/oleosin	Lipid storage	Membrane	2.01
At5g61610	Glycine-rich protein/oleosin	Lipid storage	Membrane	4.79

Transcript level, transcript level ratio between the transgenic sample and the WT control

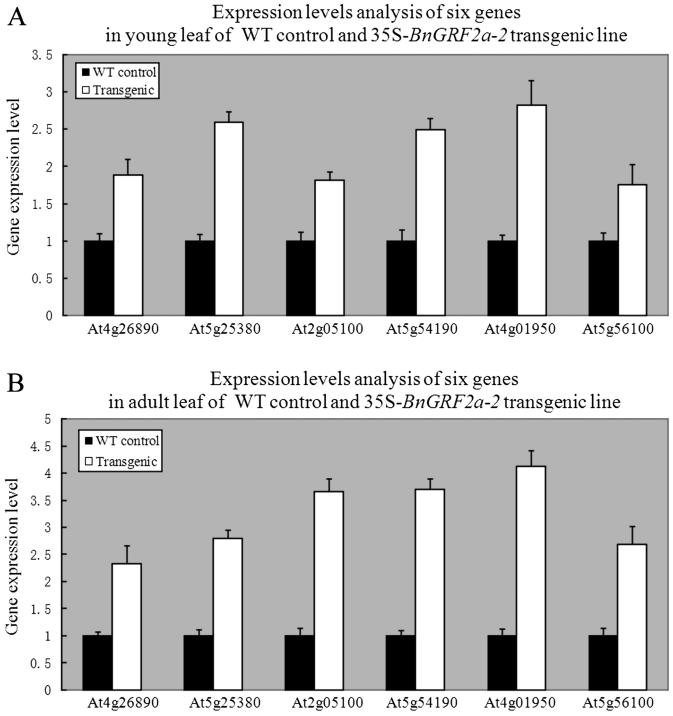

Fig. 7. Comparison of the expression level of up-regulated genes in young leaf and adult leaf between the wild-type (WT) control and the 35S-*BnGRF2a*-2 transgenic line. (A) Data from microarray analyses. (B) Data from real-time PCR analyses. Data expression was normalized to *β-actin1* (At2g37620) and relative to the expression of genes in the WT control. Data presented are mean values of three biological replicates, and error bars represent standard deviations.

synthesis and oil storage were up-regulated in the transgenic lines. For example, KCS16 is a member of the 3-ketoacyl-CoA synthase family, which is involved in the biosynthesis of very long chain FAs. In *Arabidopsis*, a *kcs16* mutant had a reduced eicosenoic acid content in seeds (Joubès *et al.*, 2008). Modest seed oil content increases were also observed in recombinant expression studies using modified safflower GPAT cDNA and GPAT from *Escherichia coli* (Jain *et al.*, 2000).

Of course, for oil crop breeding, what is of concern is the agricultural characteristics such as seed weight and oil content. At present, much research on improving oil production by transgenic technology is ongoing. However, previous studies focused on genes which changed only seed mass or oil content. Also, most of the genes encode the enzymes and transcription factors regulating carbon metabolism, FA synthesis, and TAG synthesis pathways (Baud and Lepiniec, 2009; Weselake *et al.*, 2009). In the present study, it was found that *BnGRF2a* increases oil production by regulating the capacity of both the source (changing photosynthesis efficiency) and the sink (changing cell number), which is more significant for elevating oil production in oil crop breeding. Although overexpression of *BnGRF2a* in flower buds induced less efficient self-fertilization, such a drawback could be avoided using a tissue-specific promoter. Thus, this gene may represent a suitable candidate for genetic manipulation to improve the economic characteristics of seed weight or oil production in crops.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Real-time PCR analyses of the *BnGRF2a* gene in different tissues of rapeseed lines zy036 and 51070.

Figure S2. Localization of the insertion point of the vector sequence in the 35S-*BnGRF2a*-2 and 35S-*BnGRF2a*-10 transgenic lines.

Figure S3. Expression level comparison of up-regulated genes between siliques (10 d after flowering) of WT control and the Napin-*BnGRF2a*-3 transgenic line.

Table S1. Primers used for gene isolation, expression vector confirmation, and real-time PCR in this study.

Table S2. Up-regulated genes of 20 d ovules in zy036 compared with 51070.

Table S3. An overview of differential gene expression between the leaf of 35S-*BnGRF2a*-2 and WT control plants.

Table S4. Transcription factors involved in differentially expressed genes between the leaf of 35S-*BnGRF2a*-2 and WT control plants.

Table S5. Up-regulated genes that were thought to be involved in possible BnGRF2-regulated pathways.

Table S6. Phenotype comparisons between transgenic *Arabidopsis* lines overexpressing *BnGRF2a* and *AtGRF*.

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