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RESEARCH PAPER

# A role for Arabidopsis growth-regulating factors 1 and 3 in growth–stress antagonism

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## Abstract

**Growth-regulating factors (GRFs) belong to a small family of transcription factors that are highly conserved in plants. GRFs regulate many developmental processes and plant responses to biotic and abiotic stimuli. Despite the importance of GRFs, a detailed mechanistic understanding of their regulatory functions is still lacking. In this study, we used ChIP sequencing (ChIP-seq) to identify genome-wide binding sites of Arabidopsis GRF1 and GRF3, and correspondingly their direct downstream target genes. RNA-sequencing (RNA-seq) analysis revealed that GRF1 and GRF3 regulate the expression of a significant number of the identified direct targets. The target genes unveiled broad regulatory functions of GRF1 and GRF3 in plant growth and development, phytohormone biosynthesis and signaling, and the cell cycle. Our analyses also revealed that clock core genes and genes with stress- and defense-related functions are most predominant among the GRF1- and GRF3-bound targets, providing insights into a possible role for these transcription factors in mediating growth–defense antagonism and integrating environmental stimuli into developmental programs. Additionally, GRF1 and GRF3 target molecular nodes of growth–defense antagonism and modulate the levels of defense- and development-related hormones in opposite directions. Taken together, our results point to GRF1 and GRF3 as potential key determinants of plant fitness under stress conditions.**

**Keywords:** Arabidopsis, ChIP-seq, circadian rhythm, growth-regulating factors, growth–stress antagonism, phytohormones, RNA-seq.

## Introduction

Transcription factors are sequence-specific DNA-binding proteins that regulate the decoding of DNA sequences to mRNA. Some transcription factors have the ability to control, directly or indirectly, a significant number of genes involved in a multitude of cellular activities and functions, and thus are considered master regulators. The activity of these master regulators must be firmly controlled because their misregulation negatively impacts cellular reprogramming and fate. The plant-specific growth-regulating factors (GRFs) are a typical example of master regulators. GRFs belong to a small family of transcription factors that were first identified in rice two decades ago (van der Knaap *et al.*, 2000). These transcription factors contain highly conserved QLQ and WRC domains in the N-terminus and a highly variable C-terminal domain. The QLQ domain mediates the interaction of GRFs with GRF-interacting factors (GIFs), while WRC acts as a DNA-binding domain (Kim and Kende, 2004; Kim *et al.*, 2012). The C-terminal end of GRF proteins is essential for their transactivation activity (Kim and Kende, 2004) as GRFs with truncated C-terminal sequences displayed no transactivation function (Choi *et al.*, 2004; H. Liu *et al.*, 2014).

*GRF* genes are developmentally regulated and expressed in a cell- and tissue-specific manner as determined by their temporal and spatial expression patterns (Hewezi *et al.*, 2012; Kim *et al.*, 2012; Lee *et al.*, 2018). Functional studies revealed the involvement of *GRF* genes from different plant species in a multitude of developmental processes, including root and leaf development, stem elongation, floral organ and seed formation, meristem maintenance, cell expansion and proliferation, and plant longevity (Omidbakhshfard *et al.*, 2015). The involvement of *GRF* genes in regulating plant response to biotic and abiotic stimuli has also been reported (Hewezi *et al.*, 2012; Kim *et al.*, 2012; Casadevall *et al.*, 2013; Shang *et al.*, 2018), suggesting a role for these transcription factors in mediating the balance of plant growth and stress responses. Genetic analyses of *GRF* gene family members in Arabidopsis revealed that these transcription factors exert overlapping as well as unique functions. Because the *GRF* gene family is broadly present in monocots and eudicots, it has been suggested that the regulatory function of these family members may be conserved (Kim and Tsukaya, 2015). Recent functional analysis of GRFs in a number of plant species further supported this suggestion (Kuijt *et al.*, 2014; Wu *et al.*, 2014; Gao *et al.*, 2015). However, the genetic basis of the functional redundancy and specificity of GRFs remains poorly understood.

Several *GRF* genes in various plant species are post-transcriptionally regulated by miRNA396 (miR396) (Omidbakhshfard *et al.*, 2015). For example, in Arabidopsis, seven of the nine *GRF* genes contain the miR396-binding site and therefore their expression is post-transcriptionally regulated by miR396 (Jones-Rhoades and Bartel, 2004). Additionally, a reciprocal feedback circuit between GRFs and miR396 seems to be involved in stabilizing their transcript abundance (Hewezi and Baum, 2012). This tightly regulated homeostatic system has been shown to be fundamental to many of the aforementioned developmental processes (Liu *et al.*, 2009; Rodriguez *et al.*, 2010; Hewezi *et al.*, 2012; Baucher *et al.*, 2013; Casadevall *et al.*, 2013). Perturbation of any components of the GRFs–miR396 regulatory module frequently results in apparent growth and developmental defects (Rodriguez *et al.*, 2010; Hewezi *et al.*, 2012; H. Liu *et al.*, 2014; Lee *et al.*, 2018). Consistent with the key regulatory functions of GRFs, misregulation of Arabidopsis *GRF1* and *GRF3* was found to impact the expression of thousands of genes (Hewezi *et al.*, 2012), among which stress-, defense-, and growth-related categories were the most abundant (Hewezi *et al.*, 2012; J. Liu *et al.*, 2014). This finding provided further support for the notion that GRF1 and GRF3 are master regulators that integrate stress and defense signaling into developmental programs. Nevertheless, the molecular mechanisms underlying the multifaceted regulatory functions of GRF1 and GRF3 remain ill defined.

To gain a better understanding of the regulatory functions of GRF1 and GRF3 in mediating multiple growth and developmental processes and stress response, we used ChIP sequencing (ChIP-seq) to identify genome-wide binding sites of GRF1 and GRF3 and, accordingly, their direct downstream targets. RNA sequencing (RNA-seq) analysis revealed that GRF1 and GRF3 regulate the expression of a significant number of the identified direct targets. Our data emphasized the broad regulatory functions of GRF1 and GRF3 in various aspects of plant growth, development, and biotic and abiotic stress responses, and provided direct links to the regulated pathways. The results also provided unprecedented insights into possible roles of these transcription factors in mediating growth–defense antagonism and integrating environmental stimuli into developmental processes.

## Materials and methods

### *Plant materials and growth conditions*

35S:GRF1-green fluorescent protein (GFP) and 35S:GRF3-GFP transgenic lines were generated in the Arabidopsis triple mutant *grf1/grf2/grf3* background. *The grf1/grf2/grf3* mutant was constructed in the Wassilewskija (Ws) background (Kim *et al.*, 2003). Before planting, seeds were sterilized with commercial bleach (2.8% sodium hypochlorite) followed by four washes with sterilized double-distilled water. Plants were grown under long-day conditions (16 h light/8 h dark) at 24 °C.

### *Plasmid construction*

To construct 35S:GRF1-GFP, the GRF1 coding sequence was amplified using gene-specific primers containing an attB1.1 and attB2.1 overhang in the forward and reverse primers, respectively, and cloned into pDONR221 using the Gateway BP-reaction (Invitrogen). Using Gateway LR clonase (Invitrogen), the gene was then cloned into the binary vector pGWB551. The 35S:GRF3-GFP construct was generated by amplifying the coding sequence of GRF3 using gene-specific primers with *Eco*RI and *Hin*dIII restriction site overhangs in the forward and reverse primers, respectively. The amplified product was digested, and cloned into the corresponding restriction sites in the binary vector pEGAD. All the constructs were verified by sequencing. Primer sequences used for gene cloning are provided in Supplementary Table S1 at *JXB* online.

### *Generation of transgenic plants*

The binary vectors containing GFP-tagged GRF1 and GRF3, and the empty vector pGWB551 (35S:GFP) were transformed into *Agrobacterium tumefaciens* strain C58 by the freeze–thaw method. The *Agrobacterium* were then transformed into the *grf1/grf2/grf3* triple mutant plants by the floral dip method (Clough and Bent, 1998). Transgenic $T_1$ lines expressing the GRF1:GFP fusion or GFP alone were identified by screening $T_1$ seeds on hygromycin- (25 μg $ml^{-1}$) containing Murashige and Skoog (MS) medium. Similarly, transgenic $T_1$ lines overexpressing the GRF3:GFP fusion were identified by spraying 10-day-old $T_1$ plants with 120 μg $ml^{-1}$ BASTA (glufosinate ammonium, DuPont). Non-segregating $T_2$ lines were used for ChIP-seq and RNA-seq experiments.

### *ChIP DNA isolation, library preparation, and data analysis*

Seeds of 35S:GRF1-GFP, 35S:GRF3-GFP, and 35S:GFP transgenic plants were planted on MS medium at 24 °C under 16 h light/8 h dark conditions. Two-week-old whole plants were harvested in three independent replicates and immediately treated with 1% formaldehyde solution under vacuum for 25 min to covalently cross-link protein to DNA. Nuclei were isolated and lysed, and the chromatin was sheared using a focused ultrasonicator (Covaris M220) with the following settings: duty cycle 10%, intensity peak incident power 75 W, and cycles per burst 200 for 10 min. This resulted in the chromatin fragmentation of ~400 bp. Using anti-GFP antibody (5 mg $ml^{-1}$, Abcam), sonicated DNA was immunoprecipitated. Immune complexes were bound to protein A–agarose beads (GE Healthcare) and washed several times, and then eluted in elution buffer containing 1% SDS and 0.1 M $NaHCO_3$. Reverse cross-linking was carried out by adding 5 M NaCl to the eluted product and the samples were then incubated at 65 °C overnight. DNA was purified

following the phenol–chloroform extraction method and finally the DNA was re-suspended in nuclease-free water. The ChIP-seq libraries were prepared from the purified ChIP-DNA using the NEBNext Ultra II DNA Library Prep Kit (NEB E7645, Illumina) following the manufacturer's instructions. The libraries were barcoded, pooled together, and sequenced using the HiSeq 3000 system with 150 bp paired-end reads.

FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of the sequenced data, and the low-quality reads were trimmed using Trimmomatic (Bolger *et al.*, 2014). Since the *grf1/grf2/grf3* triple mutant was in the Ws background, we constructed the Ws genome by replacing Ws single nucleotide polymorphisms (SNPs) from the 1001 genome projects into the Columbia genome (TAIR10). Reads were then mapped to the Ws genome using Bowtie2 (Langmead and Salzberg, 2012). Binding peaks were called using MACS2 (Zhang *et al.*, 2008). Peaks were called separately for each of the three biological replications, and peaks identified in at least two replications were considered for the downstream analysis. The RSAT (Regulatory Sequence Analysis Tools) suite was used to identify the *cis*-binding motifs in the non-redundant binding sites of GRF1 and GRF3 (Thomas-Chollier *et al.*, 2012).

### *RNA-seq library preparation and data analysis*

Seeds of 35S:GRF1-GFP, 35S:GRF3-GFP, *grf1/grf2/grf3*, and Ws plants were planted on MS medium in a randomized complete-block design. Two-week-old plants were collected, frozen in liquid nitrogen, and ground into fine powder. mRNA was isolated using a magnetic mRNA isolation kit (NEB) following the manufacturer's protocol. RNA-seq libraries were prepared from 250 ng of mRNA using NEBnext mRNA library prep master mix (NEB) following the manufacturer's protocol. The 12 RNA-seq libraries were barcoded, pooled together, and sequenced using the HiSeq 3000 system with 150 bp paired-end reads.

FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to determine the quality of the sequencing reads. Low-quality reads were trimmed using Trimmomatic (Bolger *et al.*, 2014). After trimming, high quality reads were mapped to the Arabidopsis reference genome (TAIR10) using TopHat v2.0.14 (Trapnell *et al.*, 2009). The number of reads mapped to each annotated Arabidopsis gene was counted using HTSeq (Anders *et al.*, 2015). The count data were normalized and the differentially expressed genes (DEGs) were determined using the R package DESeq2 (Love *et al.*, 2014). A false discovery rate (FDR) of <0.05 was used to identify the DEGs. Gene Ontology (GO) term categorization and enrichment analysis of the DEGs were performed using the AgriGO database (Du *et al.*, 2010) with Fisher's statistical test and Bonferroni adjustment at a significance level of 0.05.

### *Bacterial infection assay*

*Pseudomonas syringae* pv tomato DC3000 (Pst DC3000) inoculation assay was performed as described in Hewezi *et al.* (2010). Growth of the bacteria was examined at 0, 3, and 5 days post-infection (dpi) by collecting 6 mm leaf discs. Three biological replicates of each line were used for counting the bacterial colonies. Data are presented as the $\log_{10}$ of the colony-forming units (CFU). Collected leaf tissues were also used for qRT-PCR.

### *RNA isolation and quantitative real-time reverse transcription–PCR (qRT–PCR) analysis*

Total RNA was isolated from 20 mg of tissue as described previously (Verwoerd *et al.*, 1989). Total RNA was treated with DNase I (Invitrogen) and ~25 ng RNA was used in each qRT–PCR. qRT–PCR was performed using Verso SYBR green One-Step qRT-PCR Rox mix (Thermo Scientific) according to the manufacturer's protocol. The dissociation curves were created using the following program: 95 °C for 15 s and 60 °C for 75 s, followed by a slow gradient from 60 °C to 95 °C. *Actin8* (AT1G49240) and *PP2AA3* (AT1G13320) were used as internal controls to normalize the mRNA level. The primers used for qRT–PCR assays are provided in Supplementary Table S1.

### *ChIP-qPCR*

ChIP-qPCR was carried out using a diluted ChIP-seq library (1/10) of 35S:GRF1-GFP, 35S:GRF3-GFP, and 35S:GFP. ChIP-qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) following the manufacturer's instructions. ChIP-qPCR was conducted with an initial hold at 50 °C for 2 min for UDG activation and 95 °C for 2 min for polymerase activation followed by 40 cycles of denaturation at 95 °C for 3 s and 60 °C for 30 s annealing and elongation. The dissociation curves were created using the following program: 95 °C for 15 s and 60 °C for 1 min, followed by a slow gradient from 60 °C to 95 °C. The enrichment analysis was performed by computing $2^{-\Delta Ct}$ where ΔCt is the difference in the Ct values between library DNA of the 35S:GRF1-GFP or 35S:GRF3-GFP plants and control plants (35S:GFP). Primers used for ChIP-qPCR assays are provided in Supplementary Table S7.

### *Hormone quantification*

Seeds of 35S:GRF1-GFP, 35S:GRF3-GFP, *grf1/grf2/grf3*, and Ws plants were planted on MS medium with four replications in a randomized complete-block design. A total of 100 mg of fresh tissues were harvested from 2-week-old plants. The tissues were flash-frozen in liquid nitrogen and sent to the Proteomics and Mass Spectrometry Facility (Danforth Plant Science Center, St. Louis, MO, USA) for hormone quantification. Eksigent ekspert™ microLC200 coupled to a Sciex 6500 QTrap® (Framingham, MA, USA) was used for hormone quantification.

## Results

### *Identification of GRF1- and GRF3-binding sites*

To identify genome-wide binding sites of GRF1 and GRF3, we performed ChIP-seq. For this, we generated transgenic plants overexpressing GRF1 or GRF3 tagged with GFP in the *grf1/grf2/grf3* triple mutant background under the control of the 35S promoter (35S:GRF1-GFP and 35S:GRF3-GFP). Transgenic lines with ~3- and 5-fold increases in the expression of *GRF1* or *GRF3* were selected. The selected lines showed no visible morphological irregularities and were indistinguishable from the Ws wild-type plants. Transgenic plants expressing GFP in the *grf1/grf2/grf3* mutant were used as negative controls. The 35S promoter was used to guide the ectopic expression of *GRF1* and *GRF3* because these transcription factors are expressed in numerous actively growing tissues at various developmental stages, making collection of the correct tissues where GRFs are expressed a real challenge if the native promoters are used. The *grf1/grf2/grf3* triple mutant was used to minimize potential binding site competition among these functionally redundant transcription factors. The ChIP-seq analysis was performed using 2-week-old plants with three biological replicates. Binding peaks of GRF1 and GRF3 were identified in each replicate separately and compared with those identified in control samples to eliminate non-specific binding peaks. Peaks were considered for downstream analysis only if they were identified in at least two biological replicates. Based on this criterion, 1108 and 2194 peaks were identified for GRF1 and GRF3, respectively. Obviously, the large majority of the identified peaks (81% for GRF1 and 73% for GRF3) were located in the intergenic regions that include gene promoters (Fig. 1A, B). Binding peaks were also identified much less frequently in gene body regions including exons, introns, and untranslated regions, suggesting that these regions may have

regulatory functions (Lin and Tam, 2001; Rose, 2008; Barrett *et al.*, 2012).

To identify direct targets of GRF1 and GRF3, we associated the binding peak regions with the closest protein-coding genes within 1 kb of the transcription start site (TSS) or the transcriptional termination site (TTS). Using this criterion, we identified 589 direct target genes of GRF1 and 1075 of GRF3 (Supplementary Tables S2, S3). Interestingly, 154 target genes were common to GRF1 and GRF3, a finding that is consistent with the redundant function of these transcription factors (Hewezi *et al.*, 2012; J. Liu *et al.*, 2014). Of these 154 common target genes, 139 have overlapping binding sites (Supplementary Table S4). The genome-wide distribution of the binding peaks relative to protein-coding genes revealed a

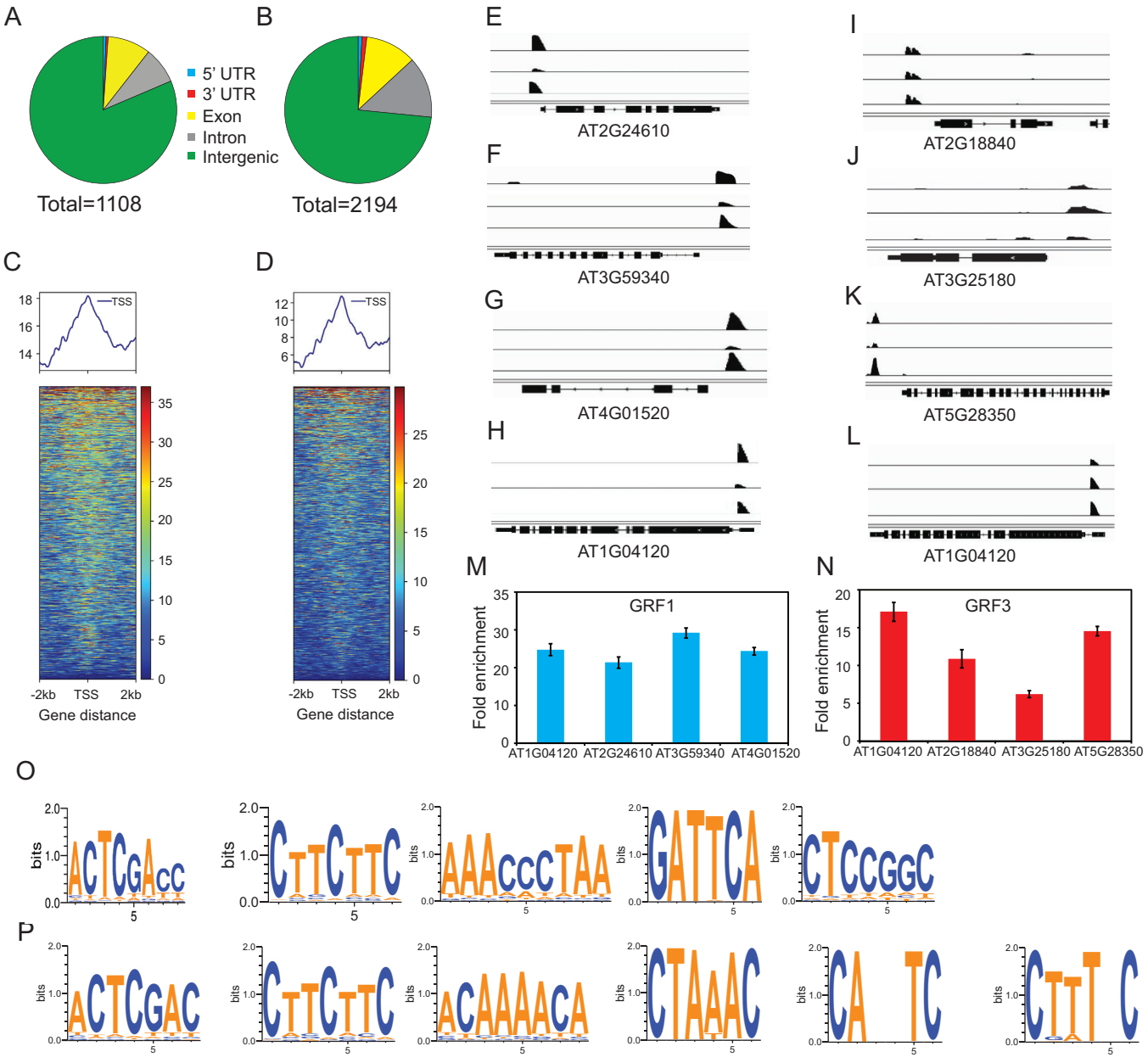


**Fig. 1.** Genome-wide analysis of GRF1- and GRF3-binding sites identified by ChIP-seq. (A, B) Distribution of binding sites of GRF1 (A) and GRF3 (B) in various genic and intergenic features of the Arabidopsis genome. The majority of the binding sites are located in the intergenic region that also includes the promoter, determined as 1000 bp upstream of the transcription start site (TSS). (C, D) Heatmaps of ChIP-seq signal densities of GRF1 (C) and GRF3 (D) across 2 kb windows showing enrichment near the TSS. The numbers in the bars on the right indicate signal intensity, which reflects the intensity of the binding sites in the overexpression lines compared with GFP control plants. Target sequences are ordered based on signal strength. (E–L) Representative examples of ChIP-seq binding profiles of selected genes targeted by GRF1 (E–H) or GRF3 (I–L). (M, N) ChIP-qPCR showing fold enrichment of binding of GRF1 or GRF3 to the target genes indicated in (E–L). Fold values reflect enrichment in the GRF1–GFP and GRF3–GFP overexpression plants relative to GFP control plants. Data are presented as the mean ±SE ($n$=3). (O, P) Consensus DNA-binding motifs identified in the binding regions of GRF1 (O) or GRF3 (P). Note that ACTCGAC and CTTCTTC were identified as common binding motifs for GRF1 and GRF3. (This figure is available in color at *JXB* online.)

high frequency near the TSS (Fig. 1C, D). Representative examples of ChIP-seq binding profiles of GRF1 and GRF3 in the promoters of seven putative target genes are shown in Fig. 1E–H and 1I–L, respectively. The ChIP-seq binding profiles of these seven genes were further verified using ChIP-qPCR. There was at least a 6-fold enrichment of the binding regions in the 35S:GRF1-GFP and 35S:GRF3-GFP plants compared with the control (Fig. 1M, N).

To identify DNA-binding motifs of GRF1 and GRF3, the DNA sequences of the binding peak regions were retrieved and analyzed using RSAT peak motifs. The analysis resulted in the identification of five over-represented DNA-binding motifs for GRF1 (Fig. 1O). Approximately 51% of the peak regions contained at least one of these motifs and 18% contained two or more motifs. For GRF3, six DNA-binding motifs were identified (Fig. 1P), and 70.5% of the peak regions contained at least one of these motifs and ~33% contained two or more motifs. These binding motifs were highly enriched at the center of the binding summits (Supplementary Fig. S1). Interestingly, two of the identified DNA-binding motifs (ACTCGAC and CTTCTTC) were common to both GRF1 and GRF3, suggesting that these transcription factors may redundantly regulate the same genes by binding to these common motifs (Fig. 1O, P). Interestingly, the common DNA-binding motif ACTCGAC is quite similar to that recently identified for GRF7 and GRF9 (CTGACA) (Kim *et al.*, 2012; Omidbakhshfard *et al.*, 2018), providing additional evidence for the redundant functions of GRFs.

### *Identification of differentially expressed genes in GRF1 and GRF3 overexpression lines*

Binding of GRF1 and GRF3 to the promoters of their target genes is expected to exert a transcriptional regulatory function leading to gene expression changes. To examine the extent to which GRF1 and GRF3 regulate the expression of the identified direct targets, we generated and analyzed RNA-seq libraries of 2-week-old plants that included 35S:GRF1-GFP, 35S:GRF3-GFP, the *grf1/grf2/grf3* triple mutant, and the wild-type Ws. Principal component analysis (PCA) of the normalized transcripts of RNA-seq data revealed clear separation of the overexpression lines from the *grf1/grf2/grf3* triple mutant and wild-type Ws, and high similarity between the three biological replicates (Supplementary Fig. S2). At an FDR of 0.05, 2596 and 2212 DEGs were identified in the 35S:GRF1 and 35S:GRF3 plants, respectively, when compared with the *grf1/grf2/grf3* mutant (Supplementary Tables S5, S6). A set of 1470 genes was shared between the two gene lists from which 1468 genes were similarly regulated in the overexpression lines, a finding that further confirms the redundant function of these two transcription factors. Similarly, a set of 826 genes was identified as differentially expressed in the *grf1/grf2/grf3* mutant when compared with the wild-type Ws plants (Supplementary Table S7). In addition, we made use of the DEGs previously identified using microarray analysis of root tissues of 35S:GRF1 (2293 genes), 35S:GRF3 (2410 genes), and the *grf1/grf2/grf3* mutant (3944 genes) (Hewezi *et al.*, 2012). Taking into consideration genes regulated in the triple mutant, a total number of 3018 and 5102 DEGs were considered as GRF1-regulated genes based on RNA-seq and microarray analyses, respectively (Fig. 2A). Similarly, a total number of 2532 and 5558 DEGs were considered as GRF3-regulated genes based on RNA-seq and microarray analyses, respectively (Fig. 2A). Of the 589 GRF1 target genes, 156 (26.5%, 45 from RNA-seq, 88 from microarray, and 23 from both RNA-seq and microarray data sets) overlapped with the DEGs, and thus we refer to these genes as GRF1-regulated targets (Fig. 2A; Supplementary Table S8). Similarly, of the 1075 GRF3 target genes, 279 genes (26%; 83 from RNA-seq, 146 from microarray, and 50 from both RNA-seq and microarray data sets) overlapped with the DEGs, and were considered as GRF3-regulated targets (Fig. 2B; Supplementary Table S9).

We performed GO term classification and enrichment analyses of the DEGs identified from our RNA-seq analysis. We observed that the down-regulated genes in GRF1 and GRF3 overexpression lines were enriched in molecular functions relevant to numerous aspects of plant development, including anatomical structure development, meristem development, leaf development, plant organ development, post-embryonic development, reproductive structure development, shoot development, as well as auxin-mediated signaling, wounding, and stress responses (Fig. 2C). On the other hand, up-regulated genes were enriched in molecular functions related to plant responses to biotic and abiotic stimuli (Fig. 2C). Of note is that some GO terms related to stress responses and phytohormone signaling were enriched in both up-regulated and down-regulated genes (Fig. 2C). These results provided a suggestion for the involvement of these two transcription factors in mediating the trade-off between plant development and stress responses.

We also performed GO term classification and enrichment analyses of the direct targets of GRF1 and GRF3. The direct targets of GRF1 and GRF3 were enriched for genes involved in various aspects of plant growth and development that include, among others, floral organ and post-embryonic development, leaf and root development, epidermal cell differentiation and development, meristem maintenance, and cell wall origin (Fig. 2D). Genes involved in the regulation of hormone levels (metabolism, biosynthesis, and transport) and responses to hormone stimuli as well as biotic and abiotic stresses were also enriched among the direct targets of GRF1 and/or GRF3 (Fig. 2D). The analysis is consistent with the known role of several GRFs in the regulation of plant development and stress responses, and suggests that the identified direct targets are of biological significance.

### *GRF1 and GRF3 target auxin biosynthesis, transport, and signaling genes*

Our analyses point to complex regulatory functions for GRF1 and GRF3 that determine auxin level and response. Among the direct targets of GRF1 and GRF3 are several genes involved in auxin biosynthesis, inactivation, transport, and signaling (Fig. 3A). For example, genes encoding enzymes such as CYP79B2, YUCCA4, ALDEHYDE OXIDASE 1 (AAO1), and NITRILASE 1 (NIT1), which play key roles

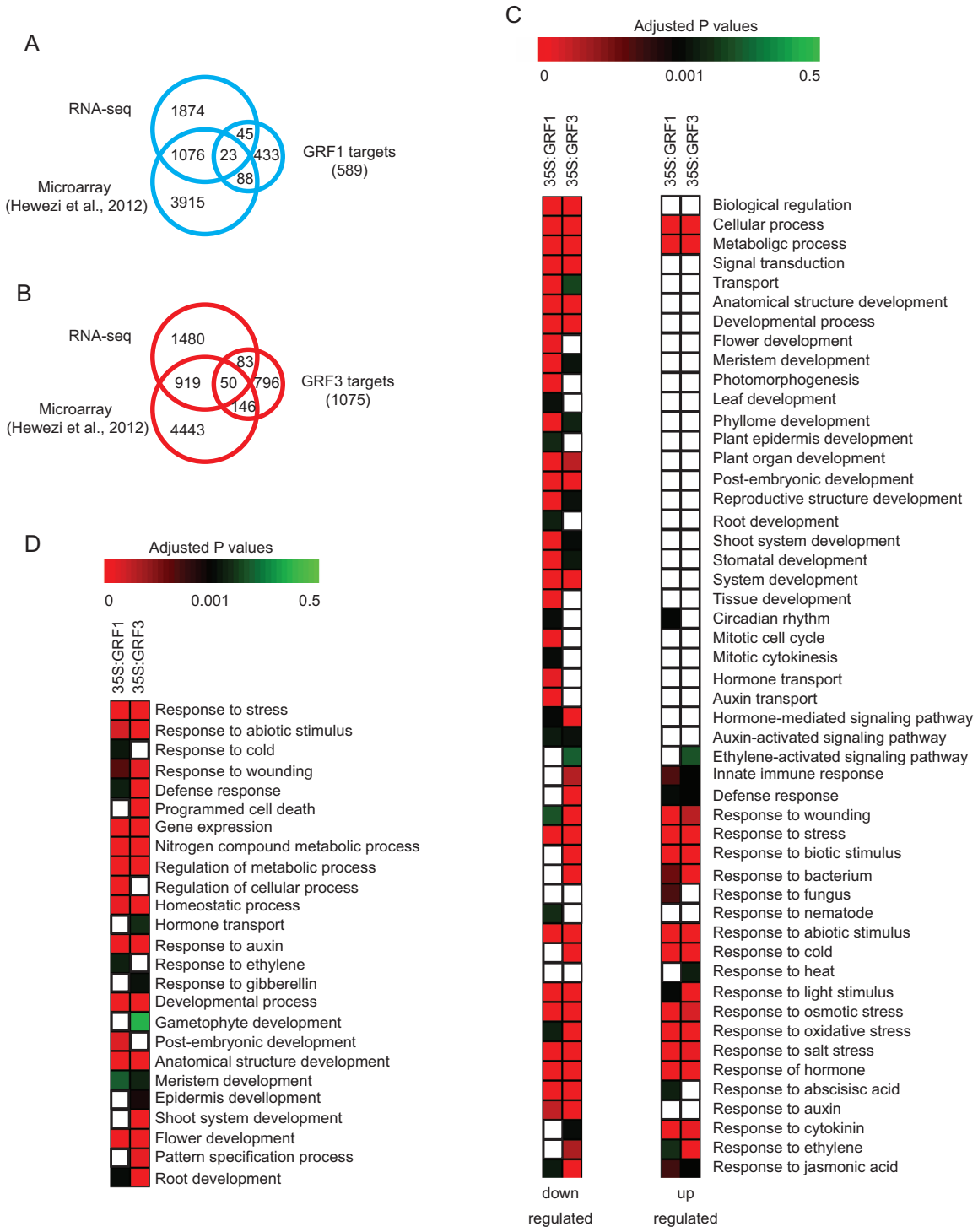


**Fig. 2.** Functional classification and Gene Ontology analysis of the differentially expressed genes and the direct targets of GRF1 and GRF3. (A) Venn diagram showing the overlap between GRF1-regulated genes and its direct target genes. (B) Venn diagram showing the overlap between GRF3-regulated genes and its direct target genes. (C) Gene Ontology analysis of GRF1 and GRF3 differentially expressed genes. The analysis was performed separately for up-regulated and down-regulated genes. (D) Gene Ontology analysis of GRF1 and GRF3 direct targets. GO term enrichment analysis was performed using the AgriGO database with Fisher's statistical test and Bonferroni adjustment at a significance level of 0.05. GO terms represented in white are not statistically significant. (This figure is available in color at *JXB* online.)

in auxin biosynthesis via the tryptophan pathway (Mano and Nemoto, 2012), were among the direct targets of GRF3 (Fig. 3A). GRF1 also seems to contribute to the regulation of auxin level through direct control of *GH3.5* and *GH3.6*, which have been shown to function in maintaining the auxin level at physiological concentrations required for various aspects of plant growth and development (Staswick *et al.*, 2005; Zhang *et al.*, 2007). In addition, several genes encoding functions related to auxin transport that include *PIN2 PROMOTER BINDING PROTEIN 1* (*PPP1*), *XAANTAL2* (*XAL2*),

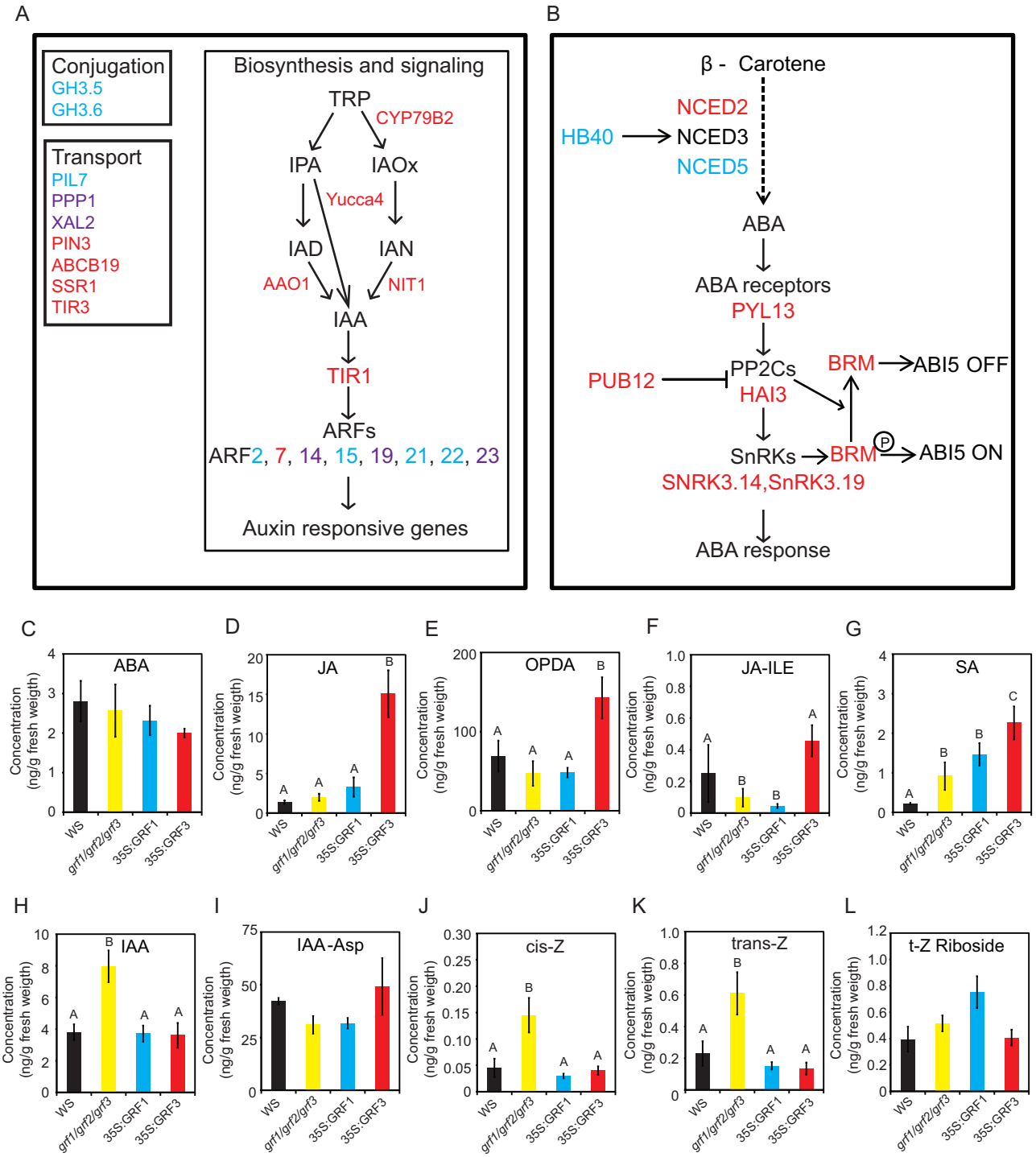


**Fig. 3.** GRF1 and GRF3 regulate the level of defense- and development-related hormones in opposite directions. (A, B) Direct target genes of GRF1 and GRF3 that are involved in auxin biosynthesis, transport, and signaling (A) and ABA biosynthesis and signaling (B). (C–L) Concentration of ABA (C), JA (D), OPDA (E), JA-Ile (F), SA (G), IAA (H), IAA-Asp (I), *cis*-zeatin (J), *trans*-zeatin (K), and *trans*-zeatin riboside (L) in 2-week-old Ws, *grf1/grf2/grf3* mutant, 35S:GRF1-GFP, and 35S:GRF3-GFP plants. Data were obtained from four biological samples and are represented as the mean ±SE. Bars with different letters are statistically significantly different at $P<0.05$. (This figure is available in color at *JXB* online.)

*SHORT AND SWOLLEN ROOT 1* (*SSR1*), *TRANSPORT INHIBITOR RESPONSE 3* (*TIR3*), *PIN-FORMED 3* (*PIN3*), and *ARABIDOPSIS THALIANA ATP-BINDING CASSETTE B19* (*ABCB19*) were also direct targets of GRF1 or GRF3 (Fig. 3A). Furthermore, GRF1 or GRF3 also target eight of the 23 Arabidopsis auxin response factor genes (ARFs), which confer specificity to transcriptional regulation of auxin-responsive genes (Chapman and Estelle, 2009). The enrichment of genes with functions related to auxin transport, signaling, and response among the DEGs (Fig. 3A) further supports the multiple regulatory functions of GRF1 and GRF3 for auxin biosynthesis, conjugation, transport, and signaling.

### GRF1 and GRF3 target abscisic acid biosynthesis and signaling

Similar to auxin regulation, direct targets of GRF1 and GRF3 included genes that are involved in abscisic acid (ABA) biosynthesis, perception, and signaling (Fig. 3B). For example, GRF3 targets *PALE CRESS* (*PAC*), which is involved in the biosynthesis of carotenoids, the precursors of ABA synthesis (Holding *et al.*, 2000). Conversion of neoxanthin to xanthoxin is a key limiting step in ABA biosynthesis (Iuchi *et al.*, 2001; Seiler *et al.*, 2011). Genes encoding enzymes that are involved in this reaction, including *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 2* (*NCED2*) and *NCED5*, are among the direct targets of GRF3 and GRF1, respectively. While *NCED3*, the major contributor to this conversion, was not directly targeted by GRF1 or GRF3, its regulator *HB40* (*HOMEOBOX PROTEIN 40*) was identified as a direct target of GRF1. In addition, the *LIPID TRANSFER PROTEIN 3* (*LTP3*), which regulates the activity of *ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*) (Gao *et al.*, 2016), is targeted by GRF1. The function of GRF1/3 in regulating ABA signaling is supported by the identification of the ABA receptor *PYR1-LIKE 13* (*PYL13*) and ABA co-receptor *HIGHLY ABA-INDUCED PP2C GENE 3* (*HAI3*) among the direct targets. In addition, a number of key regulators of ABA signaling were identified as direct targets. This included SWI/SNF chromatin-remodeling ATPase *BRAHMA* (*BRM*), *LIPID PHOSPHATE PHOSPHATASE 2* (*LPP2*), *PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 4* (*PERK4*), *PLANT U-BOX 19* (*PUB19*), and *PUB12*, which were previously reported to mediate ABA responses to various abiotic stresses (Liu *et al.*, 2011; Kong *et al.*, 2015).

### GRF1 and GRF3 modulate the levels of defense- and development-related hormones in opposite directions

Our finding that GRF1 and 3 target various phytohormone-related genes prompted us to quantify the level of various phytohormones in 2-week-old *GRF1* and *GRF3* overexpression lines as well as in the *grf1/grf2/grf3* triple mutant (Fig. 3C–L). The GRF3 overexpression plants had significantly higher concentrations of defense-related hormones such as salicylic acid (SA), and jasmonic acid (JA), its precursor 12-oxo-phytodienoic acid (OPDA), and the active JA-conjugate JA-isoleucine (JA-Ile), compared with the *grf1/grf2/grf3* triple mutant and/or Ws plants (Fig. 3D–F). These results are in line with our ChIP-seq results showing that GRF3 targets the JA biosynthesis gene *LIPOXYGENASE 2* (*LOX2*), and *EDS5*, an essential factor for SA biosynthesis (Bell *et al.*, 1995; Serrano *et al.*, 2013). In addition, several genes involved in JA biosynthesis such as *ALLENE OXIDE CYCLASE1* (*AOC1*), *AOC2*, *JASMONATE RESISTANT1* (*JAR1*), *lipoxygenase2* (*LOX2*), and *acyl-CoA oxidase2* (*ACX2*) are among the DEGs identified in the overexpression lines. We also detected a non-significant increase of the level of JA and SA in the GRF1 overexpression plants as compared with the *grf1/grf2/grf3* triple mutant (Fig. 3D–G). In contrast, both *GRF1* and *GRF3* overexpression lines exhibited significantly lower levels of the growth-related hormones such as auxin (indole-3-acetic acid, IAA), and *cis*- and *trans*-zeatin-type cytokinins compared with the *grf1/grf2/grf3* triple mutant (Fig. 3H–K). These results are in agreement with the finding that GRF3 targets several auxin biosynthesis genes such as *CYP79B2*, *YUCCA4*, *ALDEHYDE OXIDASE 1* (*AAO1*), and *NITRILASE 1* (*NIT1*), and the cytokinin biosynthesis gene *ISOPENTENYLTRANSFERASE 4* (*IPT4*) (Kakimoto, 2001; Mano and Nemoto, 2012). Together, these results provide evidence that these transcription factors, particularly GRF3, modulate the levels of defense- and development-related hormones in opposite directions, possibly to govern defense/development trade-offs.

### GRF1 and GRF3 regulate circadian rhythm core genes

Plant growth and development are rhythmically regulated processes through a coordinated interaction between growth and circadian clock pathways (Covington *et al.*, 2008), and GRF1 and GFR3 seem to play a key role in this coordination. Among the GRF1- or GRF3-bound genes, we identified several core components of the circadian oscillator, including *LATE ELONGATED HYPOCOTYL* (*LHY*) and *TIMING OF CAB EXPRESSION 1* (*TOC1*) (Oakenfull and Davis, 2017) (Fig. 4A). The identified targets also included five genes that contribute to the regulation of the circadian core components, namely *PSEUDORESPONSE REGULATOR 2* (*PRR2*), *PRR3*, *EARLY FLOWERING 3* (*ELF3*), *TIME FOR COFFEE* (*TIC*), and *LWD2* (Oakenfull and Davis, 2017) (Fig. 4A). Among the GRF1- or GRF3-bound targets, we additionally identified the *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*), *PIF3-like1* (*PIL1*), *PIL5*, *PHOTOPERIODIC CONTROL OF HYPOCOTYL 1* (*PCH1*), and *PHYTOCHROME AND FLOWERING TIME 1* (*PFT1*), which have been recently shown to connect light signaling and the circadian clock to regulate photoperiod-responsive growth (Huang *et al.*, 2016; Zhu *et al.*, 2016; Oakenfull and Davis, 2017). Interestingly, *LHY*, *PRR3*, and *TIC* were among the DEGs identified in *GRF3* overexpression plants. Furthermore, the expression level of 10 targets of GRF1 and GRF3 associated with the regulation of clock function was quantified every 4 h in the wild-type Ws, *grf1/grf2/grf3* triple mutant, and GRF1 and GRF3 overexpression plants growing under a 16 h light/8 h dark regime. With the exception of *TOC1* and *PIF4*, the expression of eight targets, namely *LHY*, *TIC*, *PRR2*, *PRR3*, *ELF3*, *LWD2*, *PIL1*, and *PIL5*, exhibited clear rhythmic expression at at least one time point in Ws plants. However, these rhythmic patterns were disrupted in the *grf1/grf2/grf3* mutant and GRF1 and GRF3 overexpression plants (Fig. 4B–K). These results indicate that GRF1 and GRF3 contribute to the regulation of core clock genes.

### GRF1 and GRF3 target genes associated with the cell cycle

The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit ubiquitin ligase, which plays key roles in the

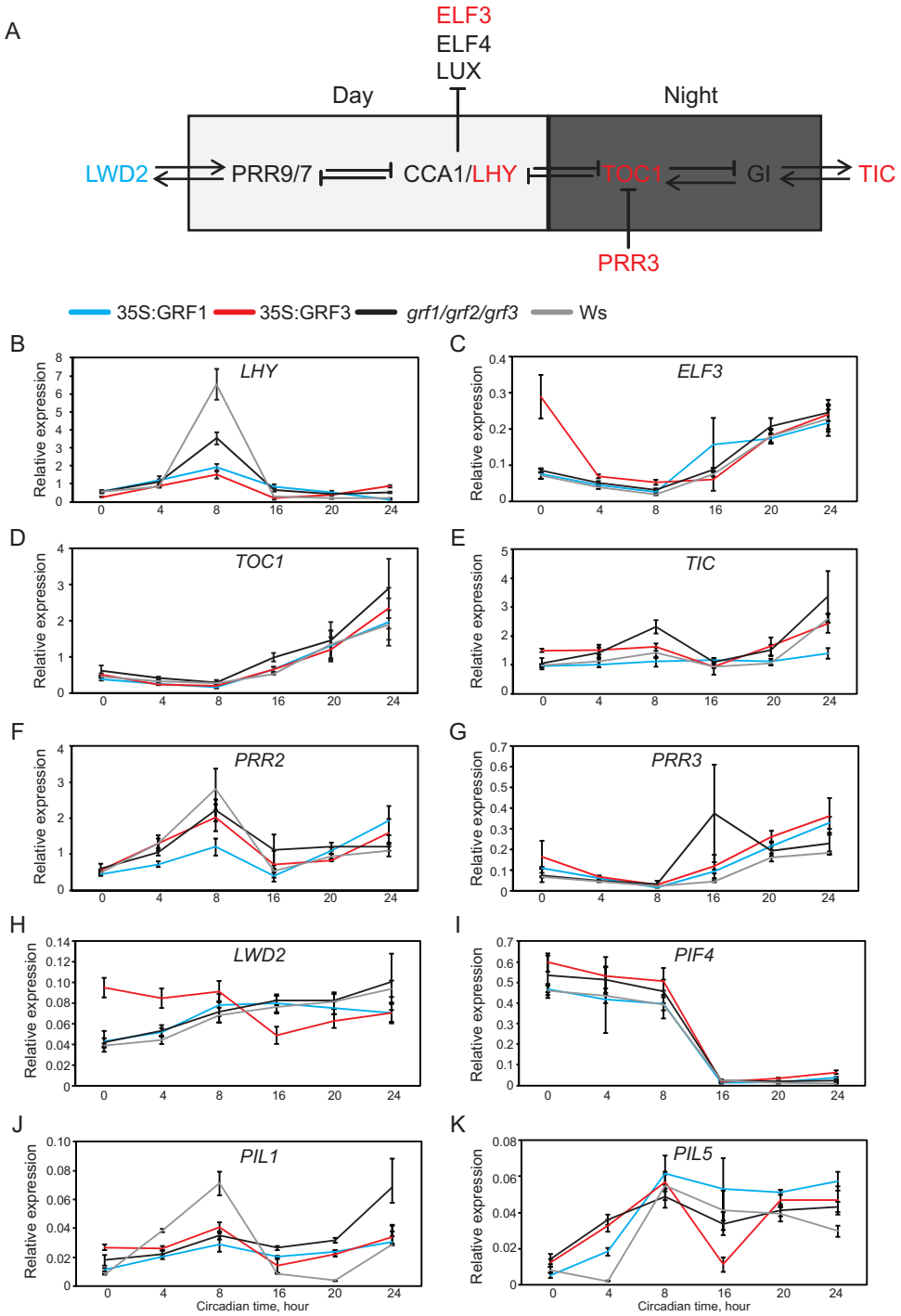


**Fig. 4.** GRF1 and GRF3 target core genes of the circadian rhythm pathway. (A) Schematic representation of the core circadian clock pathway in which direct targets of GRF1 or GRF3 are included. (B–K) qRT–PCR quantification of the indicated clock genes in wild-type Ws, *grf1/grf2/grf3* mutant, and GRF1 or GRF3 overexpression plants growing under a 16 h light/8 h dark regime. The expression levels were measured every 4 h in three biological samples and normalized to internal controls. The data represent mean values ±SE of three biological replicates. (This figure is available in color at *JXB* online.)

cell cycle and endoreduplication (Peters, 2006; Marrocco *et al.*, 2009). We found that GRF1 targets *APC2*, which codes for a subunit of the APC/C complex. In plants, the substrate specificity of APC/C is determined by CELL CYCLE SWITCH PROTEIN 52 (CCS52) and CELL DIVISION CYCLE 20 (CDC20) proteins. Our result revealed that both GRF1 and GRF3 target *CCS52A2*; one of three CCS52-encoding genes in Arabidopsis. In addition, GRF1 was found to bind to *ARABIDOPSIS RESPONSE REGULATOR 2* (*ARR2*), which directly regulates the expression of *CCS52A1* (Takahashi *et al.*, 2013). CCS52A1 and CCS52A2 have been shown to regulate the transition from the mitotic to the endoreduplication cycle during plant development (Lammens *et al.*, 2008; Larson-Rabin *et al.*, 2009; Takahashi *et al.*, 2013). Of the five *CDC20* genes identified in Arabidopsis, only *CDC20.1* and *CDC20.2* are functional and participate in

cell cycle-related cellular processes (Kevei *et al.*, 2011). Our analysis revealed that GRF3 targets *CDC20.2* in addition to *KIP-RELATED PROTEIN 5* (*KRP5*), which acts as a positive regulator of endoreduplication and regulates the expression of *CDC20.1* (Jegu *et al.*, 2013).

Other GRF3- or GRF1-bound targets that play roles in endoreduplication included *MITOTICARREST DEFICIENT 1* (*MAD1*), *NUCLEAR POREANCHOR* (*NUA*), and *CDT1*. MAD1 inhibits premature exit from cell division through the formation of a complex with MAD2 and NUA (Ding *et al.*, 2012; Bao *et al.*, 2014). CDT1 has also been shown to be involved in the events that regulate the transition from mitotic cell cycles to the endoreduplication cell cycle during plant development (Castellano Mdel *et al.*, 2004). Additional targets with key functions in the cell cycle included *CYCLIN B1;4*, *CYCLIN B2;2*, *SKP2A*, *CYCLIN DEPENDENT KINASE GROUP C2* (*CDKC2*), *ELONGATA3*, *NEURAL PRECURSOR CELL EXPRESSED*, *DEVELOPMENTALLY DOWN-REGULATED GENE 1* (*NEDD1*), and *TCP FAMILY TRANSCRIPTION FACTOR 4* (*TCP4*) (Jurado *et al.*, 2008; Zeng *et al.*, 2009; Aggarwal *et al.*, 2011; Skylar *et al.*, 2013; Zhao *et al.*, 2017).

### *GRF1 and GRF3 target genes associated with biotic and abiotic stresses*

Plants respond to biotic and abiotic stimuli by adjusting their developmental programs. As such, developmental programs and stress signaling are tightly linked despite the fact that few regulatory factors controlling this interaction have been discovered (Fan *et al.*, 2014; Campos *et al.*, 2016; Major *et al.*, 2017). Interestingly, we revealed that GRF1 and GRF3 target numerous genes associated with a number of abiotic stress responses, particularly drought and salt stress (Supplementary Tables S2, S3). For instance, several positive regulators of drought tolerance including *CYTOCHROME BC1 SYNTHESIS* (*BCS1*), *RBOHI*, *GENERAL CONTROL NON-REPRESSIBLE 4* (*ATGCN4*), *ARGININE DECARBOXYLASE 2* (*ADC2*), *ERF DOMAIN 53* (*ERF53*), *HARDY* (*HRD*), *WRKY57*, and *NAC DOMAIN CONTAINING PROTEIN 67* (*NAC067*) were among the target genes of GRF1 or GRF3 (Alcazar *et al.*, 2010; Abogadallah *et al.*, 2011; Cheng *et al.*, 2012; Jiang *et al.*, 2012; Zhang *et al.*, 2014; Rahman *et al.*, 2016; He *et al.*, 2017; Kaundal *et al.*, 2017). Similarly, we also observed that several genes that encode functions related to salinity tolerance were targeted by GRF1 and GRF3 (Supplementary Tables S2, S3). Targeting regulators of drought and salt stress by GRF1 and GRF3 came as no surprise considering that both stresses impact plant growth and development, and components of their signal transduction pathways often cross-talk with each other (Bechtold and Field, 2018).

In addition to targeting abiotic stress-related genes, GRF1 and GRF3 were found to target a plethora of genes with functions related to biotic stress (Supplementary Tables S2, S3). These genes can be grouped into two main categories that include programmed cell death and basal defense responses. Among the identified targets with function associated with programmed cell death were *CYTOCHROME BC1 SYNTHASE 1* (*BCS1*), which accelerates cell death rates by amplifying SA signaling (Zhang *et al.*, 2014); *CYSTATIN-1* (*CYS1*), which suppresses nitric oxide-mediated cell death (Belenghi *et al.*, 2003); *LAZARUS 5* (*LAZ5*), encoding a TIR-class NB-LRR R protein that regulates autoimmune cell death (Palma *et al.*, 2010); *ACCELERATED CELL DEATH 2* (*ACD2*), encoding a chlorophyll breakdown enzyme that controls cell death during bacterial infection (Yao and Greenberg, 2006; Pattanayak *et al.*, 2012); and *IAP-LIKE PROTEIN* (*ILP*), encoding a RING finger protein that attenuates cell death triggered by the bacterial effector AvrRpt2 (Kim *et al.*, 2011).

Among the GRF1- and GRF3-bound targets that are involved in basal defense responses, we identified several defensin protein genes (*PDF1.3*, *PDF2.3*, and *PDF2.5*), and pathogenesis-related protein genes (*PR6*, *PR9*, *PR14*, and *thaumatin-like proteins*). Further targets included *CONSTITUTIVE DISEASE RESISTANCE 1* (*CDR1*), encoding an aspartic protease that activates SA-dependent defense responses after pathogen infection (Xia *et al.*, 2004); *AVRPPHB SUSCEPTIBLE 1* (*PBS1*), encoding a receptor-like cytoplasmic kinases that incorporates defense signaling from various immune receptors (Zhang *et al.*, 2010); *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*), encoding a salicylic acid transporter required for elevated expression of basal genes (Nawrath *et al.*, 2002; Serrano *et al.*, 2013); and *WRKY28*, which is required for activation of the SA biosynthesis gene *ISOCHORISMATE SYNTHASE 1* (*ICS1*) (van Verk *et al.*, 2011). Genes encoding functions involved in systemic acquired resistance (*EARLY ARABIDOPSIS ALUMINIUM INDUCED 1*) (Cecchini *et al.*, 2015) and induced systemic resistance (*BETA GLUCOSIDASE 42*) (Nawrath *et al.*, 2002; Xia *et al.*, 2004; van Verk *et al.*, 2011; Qi *et al.*, 2014; Zamioudis *et al.*, 2014) were also identified among the GRF1- and GRF3-bound targets.

### *GRF1 and GRF3 enhance plant resistance to Pst DC3000*

To provide additional evidence for the involvement of GRF1 and GRF3 in plant defense, we analyzed the phenotype of GRF1 and GRF3 overexpression lines, wild-type Ws, and the *grf1/grf2/grf3* triple mutant after infection with Pst DC3000. Both GRF1 and GRF3 overexpression plants showed enhanced resistance compared with the *grf1/grf2/grf3* triple mutant at 3 and 5 dpi (Fig. 5A), consistent with a recent report showing that an miR396-resistant variant of *GRF3* increased plant resistance to Pst DC3000 (Beltramino *et al.*, 2018). These results raised the possibility that the enhanced resistance phenotype could be due to activation of positive regulators of plant defense response that are targeted by GRF1 and GRF3. To test this possibility, we quantified the expression of *WRKY28*, *PBS1* (targets of GRF1), *EARLI1*, and *EDS5* (targets of GRF3) in the Pst DC3000-infected leaf tissues of the overexpression lines at 3 and 5 dpi. WRKY28, PBS1 EARLI1, and EDS5 have been previously identified as positive regulators of plant defense response (Swiderski and Innes, 2001; van Verk *et al.*, 2011; Serrano *et al.*, 2013; Cecchini *et al.*, 2015). The transcript levels of *WRKY28* and *PBS1* were significantly

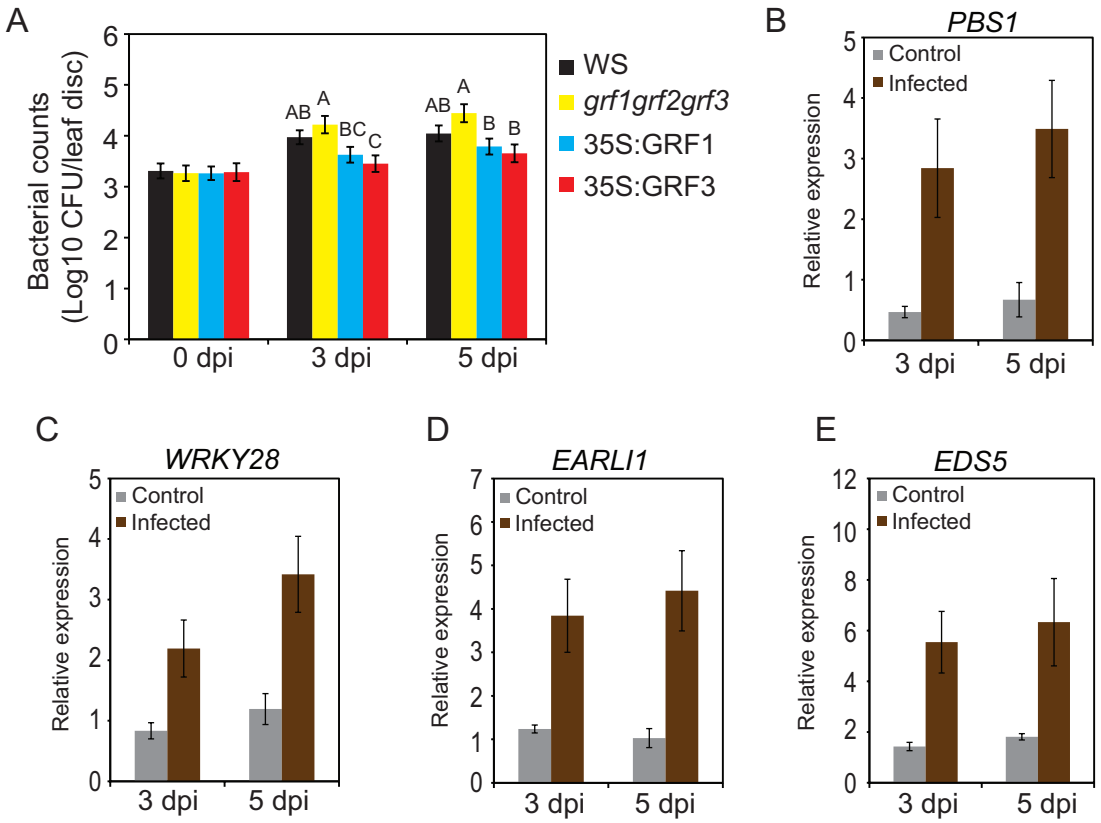


**Fig. 5.** Transgenic Arabidopsis plant overexpressing *GRF1* or *GRF3* exhibited enhanced resistance to *Pseudomonas syringae* pv tomato (Pst DC3000). (A) *GRF1* and *GRF3* overexpression plants showed enhanced resistance to Pst DC3000 in comparison with the *grf1/grf2/grf3* triple mutant at 3 and 5 dpi. *GRF1* and *GRF3* overexpression lines, Ws, and *grf1/grf2/grf3* mutant plants were inoculated with Pst DC3000. At 0, 3, and 5 dpi, 6 mm leaf discs were collected, ground, and plated on King's B media. Colonies were counted at 3 d and 5 d after plating. Data are the mean ±SE of the three biological replicates. (B–E) qRT–PCR quantification of *PBS1* (B) and *WRKY28* (C) in GRF1 overexpression plants, and of *EARLI1* (D) and *EDS5* (E) in *GRF3* overexpression plants under non-infected and Pst DC3000-infected conditions relative to *grf1/grf2/grf3* triple mutant plants. Leaf tissues were collected at 3 and 5 dpi from both infected and control plants. The experiment was conducted with three biological replicates, and the data represent the mean ±SE. (This figure is available in color at *JXB* online.)

increased in GRF1 overexpression plants in response to Pst DC3000 infection at 3 and 5 dpi compared with the non-infected leaf tissues (Fig. 5B, C). Similarly, the transcript levels of *EARLI1* and *EDS5* were significantly increased in GRF3 overexpression plants at both time points (Fig. 5D, E). Together, these data suggest that GRF1 and GRF3 regulate the defense response in part through direct activation of positive regulators of plant defense.

## Discussion

In this study, we identified genome-wide binding sites and genes regulated by GRF1 and GRF3, providing insight into the regulatory function of these transcription factors in various developmental processes and stress responses. Consistent with the redundant functions of GRFs (Kim *et al.*, 2003; Rodriguez *et al.*, 2010), we observed a significant overlap between the identified direct targets of GRF1 and GRF3. Identifying the binding motifs of GRF1 and GRF3 provides insights into the genetic basis of the functional redundancy of these transcription factors. We determined that GRF1 and GRF3 share a common *cis*-binding motif through which a common set of target genes can be equally regulated by GRF1 or GRF3. We also discovered unique DNA-binding motifs for each of these two transcription factors that would allow GRF1 and GRF3 to regulate their target genes in a specific manner. The identification of several DNA-binding motifs for GRF1 and GRF3 may indicate their association with a variety of cofactors that determine the binding specificity. Since transcriptional regulation frequently involves the formation of multiprotein complexes (Singh, 1998), it is plausible that GRF1 and GRF3 recruit these cofactors through their QLQ protein–protein interaction domain (Kim and Tsukaya, 2015).

Our RNA-seq analysis revealed that ~20% of the identified targets were differentially expressed in the *grf1/grf2/grf3* triple mutant, and GRF1 or GRF3 overexpression plants. The low percentage of regulated targets can be explained by the fact that many of these targets are expressed in specific tissues and organs that were not included in our tissue samples; the floral organs, for instance. In addition, several target genes of GRF1 and GRF3 are known to be regulated by biotic and abiotic stresses. Thus, developmentally and stress-regulated genes cannot be identified in the 2-week-old plants grown under standard conditions. Recent studies also reported on small overlaps between the direct targets of various transcription factors and DEGs (Sun *et al.*, 2015; Birkenbihl *et al.*, 2017).

Phytohormones are fundamental to almost all aspects of plant growth and development (Kamiya, 2010). GRF1 and GRF3 seem to exhibit strong regulatory capacity on the biosynthesis and signal transduction of phytohormones by targeting multiple core genes involved in these processes. Auxin is a key developmental growth regulator (Zhao, 2010), and regulating auxin levels and outputs may provide GRF1 and GRF3 with the ability to define various growth and developmental programs. Despite the fact that levels of ABA were not significantly altered in the overexpression lines growing under normal growth conditions, ABA levels and responses may be modulated in these lines under stress conditions taking into consideration that several genes associated with ABA biosynthesis and signaling are among the identified direct targets. We also found that GRF1 binds to the promoter of GRF7. GRF7 has been identified as a negative regulator of ABA signaling associated with drought and salinity stress (Kim *et al.*, 2012). This finding illustrates an additional level of regulation by GRF1 of ABA-mediated abiotic stress responses. Thus, regulating ABA levels and responses under stress conditions may enable GRF1 and GRF3 to integrate stress signaling into developmental programs to modulate growth traits under unfavorable conditions. For example, the GRF3-targeted *BRM*, which regulates vegetative phase change (Xu *et al.*, 2016) and flower patterning (Wu *et al.*, 2012), is regulated by ABA (Peirats-Llobet *et al.*, 2016), thus allowing GFR3 to quickly adjust growth traits to environmental cues.

One of our most striking findings is that GRF1 and GRF3 modulate the levels of defense- and development-related hormones in opposite directions, possibly to govern defense–development trade-offs. This function can be mediated through mutual regulation of core genes involved in hormone synthesis and outputs. This coordination could also be established by targeting integrative nodes that synchronize antagonistic interactions between various phytohormones. For example, the interaction between auxin, ABA, and JA signaling can be mediated by ARF2 and MYC2, which are targets of GRF1 and GRF3, respectively. ARF2 was identified as a connective hub between auxin and ABA signaling pathways in controlling plant growth and development (Wang *et al.*, 2011). MYC2, a central node of the JA signaling pathway, was found to positively regulate ABA signaling but to inhibit auxin biosynthesis (Abe *et al.*, 2003; Dombrecht *et al.*, 2007; Kazan and Manners, 2013; Huang *et al.*, 2017).

The cell cycle is fundamental for organ growth and plant development, and hence cellular activities that control the cell cycle and plant growth are expected to be highly interconnected. While it is currently unknown how this interconnectivity is achieved, our data suggest that inputs from GRF1 and GRF3 may be involved. The finding that both transcription factors target key regulators of mitotic cycles and endocycles is consistent with the notion that key factors controlling organ growth frequently exhibit the capacity to govern the transition between various cellular growth stages (Sablowski and Carnier Dornelas, 2014). Recently, it has been shown that GRFs control cell proliferation in developing leaves and the shoot apical meristem (SAM), a function that was associated with mitotic cyclin expression and TCP4 activity (Rodriguez *et al.*, 2010; Schommer *et al.*, 2014; Omidbakhshfard *et al.*, 2018). TCP4, which promotes cell maturation through the control of mitotic cell cycle progression (Palatnik *et al.*, 2003; Efroni *et al.*, 2008), is among the identified targets of GRF3.

Prior studies have implicated GRFs in meristem organization and maintenance (reviewed in Omidbakhshfard *et al.*, 2015), although the underlying mechanisms remain elusive. Among the direct targets of GRF1/3, we uncovered a number of key regulators involved in SAM organization, including, for example, components of the CLE signaling pathway, ENHANCER OF SHOOT REGENERATION 1 (ESR1), WUSCHEL RELATED HOMEOBOX 9A (WOX9A), the chromatin-remodeling protein SPLAYED, and BELL-type ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1) (Kwon *et al.*, 2005; Wu *et al.*, 2007; Gómez-Mena and Sablowski, 2008; Dolzblasz *et al.*, 2016; Iwase *et al.*, 2017). Because meristem cells divide at different rates, coordination between core meristem organizers and the cell cycle machinery would be necessary to maintain a functional SAM (Andersen *et al.*, 2008). Consistent with such a hypothesis, the cell cycle switch protein CCS52A2, which is targeted by both GRF1 and GRF3, has been shown to regulate the expression of *WUS* and *CLV3* (Liu *et al.*, 2012). Our finding that GRF1 and GRF3 target core components of the cell cycle machinery and meristem organization provides a suggestion for a possible role for these transcription factors in mediating such synchronization between cell proliferation, differentiation, and morphogenesis during plant growth and development. Since phytohormone signaling is known to impact the cell cycle, SAM organization, and developmental programs (Gaillochet and Lohmann, 2015), this synchronizing function can also be indirectly executed by GRF1/GRF3 through modulation of hormone signals, particularly auxin and cytokinin, both of which were modulated in the overexpression lines.

Our analysis revealed that genes involved in various aspects of plant development as well as those with stress- and defense-related functions are most predominant among the GRF1- and GRF3-bound targets. This finding sheds light on a possible role for these transcription factors in coordinating the trade-offs between plant growth and stress responses, as has been previously suggested (J. Liu *et al.*, 2014). In agreement with this suggestion, GO terms associated with defense and stress categories were enriched among the up-regulated genes identified in the GRF1 and GRF3 overexpression plants, whereas growth- and development-related categories were enriched among the down-regulated genes. Additionally, as mentioned above, levels of defense- and development-related hormones were modulated in opposite directions in the overexpression lines. Furthermore, these transcription factors have been shown to be involved in modulating plant response to cyst nematode infection in both Arabidopsis and soybean (Hewezi *et al.*, 2010; Noon *et al.*, 2019). Thus, it is reasonable to postulate that under normal developmental conditions, GRF1/GRF3 inhibit their direct targets that are involved in stress and disease resistance, allowing investment of energy for plant growth. In contrast, under biotic and abiotic stress conditions, GRF1/GRF3 may inhibit growth traits to direct metabolic resources towards cellular processes associated with stress tolerance. Since plants are generally subjected to a variety of biotic and abiotic stress factors simultaneously, the direct control by GRF1 and GRF3 of genes with functions related to these stresses may enable

effective control of growth and developmental traits under combined stress conditions.

Recent studies have elucidated a number of molecular nodes that integrate biotic cues and hormonal signals with developmental programs to maximize plant fitness and survival. For example, MYC2, EDS5, and HOMOLOG OF BEE2 INTERACTING WITH IBH 1 (HBI1) have been shown to coordinate the trade-off between growth and immunity (Kazan and Manners, 2013; Chandran *et al.*, 2014; Fan *et al.*, 2014; Malinovsky *et al.*, 2014; Smakowska *et al.*, 2016). The identification of these molecular nodes among the GRF1- and GRF3-bound targets suggests a regulatory function for GRF1 and GRF3 in balancing the growth–defense antagonism (Fig. 6). Our analysis revealed a possible role for GRF1 and GRF3 in integrating environmental stimuli into developmental processes through targeting core components of clock and photosensory pathways. Considering that various *PRR* and *PIF* genes (*PRR2*, *PRR3*, *PIF4*, *PIL1*, and *PIL5*) were found to be directly bound by GRF1 or GRF3 and their rhythmic expression patterns observed in wild-type plants were disrupted in the GRF1 and GRF3 overexpression lines, it is reasonable to speculate that GRF1 and GRF3 regulate the growth-promoting activity of PIFs and PRRs in a photoperiod-responsive manner to presumably prevent hypertrophy as recently described (Martin *et al.*, 2018).

Taking into consideration the many cellular activities and molecular functions that GRF1 and GRF3 mediate, one would expect that their expression should be tightly controlled. Interestingly, we found that GRF1 and GRF3 bind to their own promoters, presumably to control their own transcript levels. This suggestion is in agreement with previous studies showing that several GRFs including GRF1 and GRF3 negatively regulate their own expression through a feedback regulatory loop (Hewezi and Baum, 2012; Omidbakhshfard *et al.*, 2018). This fine-tuning mechanism may operate when the expression levels of GRF1 or GRF3 attain critical thresholds of unexpected changes during certain developmental circumstances or during plant exposure to biotic or abiotic stress stimuli for instance. In conclusion, our analyses revealed the ability of GRF1 and GRF3 to coordinate such growth–stress/defense interactions by directly targeting various components of growth and stress pathways, and molecular nodes of growth–defense antagonism as well as through the regulation of biosynthesis and signaling of growth- and stress-promoting hormones.

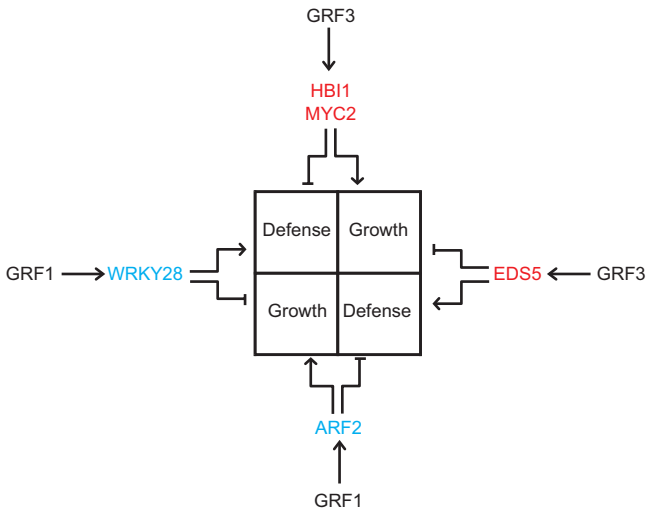


**Fig. 6.** Model for GRF1 and GRF3 inputs into the regulation of growth–defense trade-offs. In addition to targeting key genes involved, many aspects of plant growth, development, and stress responses, GRF1 and GRF3 target molecular nodes that regulate growth–defense antagonism, including HBI1, MYC2, ARF2, EDS5, and WRKY28 for instance. HBI1, MYC2, and ARF2 have been shown to activate growth signaling but suppress plant defense. In contrast, EDS5 and WRKY28 activate plant immunity but inhibit plant growth traits. Thus, targeting molecular nodes that function in opposite directions of growth–defense antagonism provides GRF1 and GRF3 additional layer of tight control over plant growth and immunity to optimize plant fitness and survival. (This figure is available in color at *JXB* online.)

## Accession numbers

ChIP-seq and RNA-seq data reported in this manuscript have been submitted to the NCBI GEO database under the accession number GSE128171.

## Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Distribution of identified DNA-binding motifs of GRF1 and GRF3 in the binding regions. The identified motifs of GRF1 and GRF3 are highly enriched at the center of the binding summits.

Fig. S2. Principal component analysis of the normalized RNA-seq data of wild-type Ws, *grf1/grf2/grf3* mutant, 35S:GRF1-GFP, and 35S:GRF3-GFP plants.

Table S1. Primer sequences used in the current study.

Table S2. List of GRF1 direct target genes.

Table S3. List of GRF3 direct target genes.

Table S4. List of 154 direct target genes common to GRF1 and GRF3.

Table S5. List of differentially expressed genes identified in 2-week-old 35S:GRF1-GFP plants.

Table S6. List of differentially expressed genes identified in 2-week-old 35S:GRF3-GFP plants.

Table S7. List of differentially expressed genes identified in 2-week-old *grf1/grf2/grf3* mutant plants.

Table S8. List of 156 GRF1-regulated targets.

Table S9. List of 279 GRF3-regulated targets.

## Acknowledgements

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## Author contributions

TH and TJB conceived and designed the original research plans; SP performed the experiments; JL provided assistance to SP; TB-S contributed new reagents and analytic tools; SP and TH analyzed the data and wrote the manuscript; and TH supervised the experimental work. The authors have no conflicts of interest to declare.

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