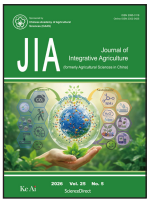
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RESEARCH ARTICLE

BrRRG regulates leaf size by controlling cell cycle gene expression in Chinese cabbage

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Highlights

- *RETARDED ROOT GROWTH* (*BrRRG*) is the gene responsible for defective leaf and root development in the *mini24* mutant.
- *BrRRG* was identified as a key regulator controlling leaf size *via E2Fa* and cell-cycle genes in Chinese cabbage.

Abstract

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) is an important leafy vegetable in the *Brassica* genus of the Brassicaceae family. The size of its edible leaves is an essential trait that determines its economic and nutritional values. However, the current understanding of leaf development in Chinese cabbage remains limited. Here, through forward genetic analysis of the mutant *mini24* with defective leaf and root development, we identified the *BrRRG* gene, which regulates cell division in Chinese cabbage by map-based cloning. We demonstrated that *BrRRG* impacts leaf size by regulating the expression of *E2Fa* transcription factors and cell cycle-related genes in Chinese cabbage. Furthermore, *BrRRG* was found to modulate Chinese cabbage's response to auxin (indole-3-acetic acid, IAA) and cytokinins hormones, revealing a distinct regulatory mechanism by which *BrRRG* coordinates the development of underground roots and aboveground leaves. Thus, these results indicate that mutation in *BrRRG* impairs the growth and development of Chinese cabbage.

Keywords: Chinese cabbage, *BrRRG*, cell division, cell cycle gene

1. Introduction

Chinese cabbage is a key leafy vegetable in the *Brassica* genus that is widely cultivated in East Asian countries and regions because of its nutritional value and economic importance. Originating in China with a long history of cultivation, Chinese cabbage remains one of the most consumed vegetables. The size and morphology of leaves directly determine the yield and commercial quality of Chinese cabbage. Previous research has demonstrated that organisms regulate organ size through intricate gene networks, with leaf development being governed primarily by two fundamental processes, namely cell proliferation and cell expansion (Johnson and Lenhard 2011; Bogre *et al.* 2008).

During early leaf development, cell division predominates. After reaching a certain number of cells, cell expansion follows (Gonzalez *et al.* 2012). Leaf blades develop from leaf primordia, which themselves arise from shoot apical meristem (SAM). Cell division rates vary in different cells across SAM, with cells in the peripheral zone (PZ) dividing more rapidly than those in the central zone (CZ), where cell division is more heterogeneous (Reddy *et al.* 2004). The cell cycle, controlled by cell cycle proteins, regulates this process; premature exit from cell cycle reduces the cell count, producing miniaturized organs. For example, *GRF-INTERACTING FACTOR1* (*GIF1*)/*ANGUSTIFOLIA3* (*AN3*) regulates cell division, and mutations in *gif1, gif2*, and *gif3* decrease cell numbers, leading to smaller leaves (Lee *et al.* 2009). After cell proliferation reaches a certain threshold,

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cell expansion begins, and coordinated regulation of these processes influences leaf size (Andriankaja *et al.* 2012). Cell expansion is driven by proteins, such as EXPANSINs (EXPs), ENDOTRANSGLYCOSYLASE/HYDROLASEs (XTHs), and PECTIN METHYLESTERASEs (PMEs), as well as reactive oxygen species (Xu *et al.* 2020; Zhang *et al.* 2025). Other proteins, such as GENERAL REGULATORY FACTOR 1/2 (GRF1/2), ENHANCER OF DA1-1/cytochrome P450 (EOD3/CYP78A6), ZINC-FINGER HOMEODOMAIN 5 (ZHD5), KUODA 1 (KUA1), and ARABIDOPSIS THALIANA HOMEOBOX 12 (ATHB12), also affect cell expansion (Hong *et al.* 2011; Fang *et al.* 2012; Lu *et al.* 2014; Hur *et al.* 2015, Omidbakhshfard *et al.* 2015; Tsukaya 2015). Overexpression of *GRF1* and *GRF2* increases cell size, resulting in larger leaves (Omidbakhshfard *et al.* 2018).

In recent years, research on Chinese cabbage leaf size has increased, and many genes have been identified. For example, *BrANT-1*, *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*BrARGOS*), and *BrGRF3* promote cell proliferation and organ enlargement (Elliott *et al.* 1996; Wang *et al.* 2010; Gu *et al.* 2016; Ding *et al.* 2018). *BrNGA1* influences both the rate and duration of cell proliferation in leaves, leading to reduced transcript levels of *CycB1-1* and *CycD3-1*, thereby controlling leaf size (Kwon *et al.* 2009). Brassinosteroids (BRs) also play a role in regulating cabbage leaf size. Genes such as *novel_15* and *novel_54* participate in BR biosynthesis, and they are upregulated in tetraploid cabbage, reducing BR levels by targeting *CYP90C1* and *CYP734A1* (Wang *et al.* 2018). Although leaf development in Chinese cabbage has been documented, the underlying regulatory mechanisms remain incompletely elucidated and warrant further investigation.

Driven by market demand, smaller plants have gained popularity among consumers. To provide new germplasm and genetic resources for the molecular breeding of such novel types of Chinese cabbage, we screened a small-leaf mutant, *mini24*, from an EMS mutant library (Sun *et al.* 2022). Through gene localization and expression analysis, *BrRRG* was identified as a key regulator of cell division in Chinese cabbage leaves. Further investigation revealed that *BrRRG* regulates *E2Fa* and cell cycle-related gene expression, leading to impaired leaf development in the *mini24* mutant.

2. Materials and methods

2.1. Plant materials

A mutant library of Chinese cabbage was generated through EMS mutagenesis of the wild-type (WT) A03 doubled haploid line (Sun *et al.* 2022). A mutant (*mini24*) exhibiting small leaves was isolated from the M6 generation and established through multiple generations of backcrossing. F_1 and F_2 populations were established from the cross between A03 and the *mini24* mutant for genetic analysis of leaf size. In August 2021, 100 plants each of A03 and *mini24* from the M6 generation were cultivated in the same plastic tunnel at Hebei Agricultural University, China, (22±3)°C, 16-h/8-h day/night photoperiod. After 10 days of leaf development, whole leaves were harvested for RNA extraction and sequencing.

2.2. Candidate mutant gene mapping *via* MutMap and KASP

Using fresh leaves from 30 F_2 lines with smaller leaves and 30 WT individuals derived from the cross between the WT and the *mini24* mutant, genomic DNA was extracted using the CTAB method (Aboul-Maaty and Oraby 2019). Equal amounts of DNA from each plant were pooled for both the mutant and WT samples. The two DNA sample pools were sent to NOVOgene Biotechnology for resequencing *via* the Illumina HiSeq™ PE150 instrument. The effective sequencing data were aligned to the reference genome *via* BWA software. The *Brassica rapa* Chiifu V3.0 genome served as the reference genomic sequence, and SNP analysis was performed on the sequencing results, applying a SNP index threshold of ≥0.6 to filter candidate SNP loci (Novogene Co., Ltd., Beijing, China).

Kompetitive Allele Specific PCR (KASP) was employed to verify the mutation sites in the WT and *mini24* plants. SNP markers identified in the mutant gene from MutMap were selected for KASP analysis across three WT plants, three *mini24* lines, two F_1 lines, and an F_2 population consisting of 180 individuals. The KASP results were subsequently used to analyze the ratios of SNPs associated with different leaf types (Neelam *et al.* 2013).

2.3. Differential interference contrast microscopes

For leaf cell size observation, the maximum outer leaves of 45-day-old plants were harvested and cleared in clearing solution (80 g chloral hydrate, 30 mL water, and 10 mL glycerol) for 5–7 days. The samples were examined *via* a DIC microscope (Leica DM2500, Germany) and photographed with a cooled CCD digital imaging system (Olympus BH2, Japan).

2.4. RNA-Seq analysis

Total RNA from leaf sections was extracted *via* the Eastep™ Total RNA Extraction Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The RNA purity and concentration were assessed *via* a NanoPhotometer® spectrophotometer (IMPLEN, Munich, Germany) and a Qubit® RNA Assay Kit with a Qubit 2.0 fluorometer (Life Technologies, ThermoFisher, Waltham, MA, USA). The cDNA library was prepared and sequenced as previously described, with RNA-Seq data analyzed according to established methods.

2.5. Virus-induced gene silencing (VIGS) in Chinese cabbage

A 40-bp sequence of *BrRRG* was designed and reverse-complemented to obtain an 80-bp palindrome sequence (5′→3′ TTAACTTCCGACGCTTCTCAG CGTTCCCTTCTCCCATTTC and 3′→5′ GAAATGGGAGA AGGGAACGCTGAGAAGC GTCGGAAGTTAA). The *BrRRG*-silencing vector (pTY-*BrRRG*) was synthesized and constructed by GeneScript according to a previous study. The empty pTY vector served as the negative control. Two-week-old Chinese cabbage seedlings were used for the VIGS assay. The pTY-*BrRRG* and pTY-S empty vectors (5 μg)

were coated with gold powder and subsequently bombarded into Chinese cabbage leaves *via* a PDS1000/He gene gun (Bio-Rad, Hercules, CA, USA), as previously described. One month later, qRT-PCR was performed to confirm the silencing effect of *BrRRG* in Chinese cabbage.

2.6. Quantitative real-time PCR

A 1-μg aliquot of total RNA from each sample was used for reverse transcription and first-strand cDNA synthesis *via* the PrimeScript™ RT Reagent Kit with gDNA Eraser (RR092A, TAKARA, Tokyo, Japan). SYBR Green Master Mix (Vazyme, Nanjing, China) was used for qRT-PCR analyses. qRT-PCR was performed in three biological replicates with three technical replicates on a LightCycler® 96 (Roche, Switzerland) under the following conditions: 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 10 s at 57°C, and 10 s at 72°C. Following PCR, a melting curve was generated for 10 s at 95°C, 60 s at 60°C, and 1 s at 97°C. The $2^{-\triangle\triangle Ct}$ method was used to calculate relative gene expression levels between WT and *mini24* plants, with the *Actin* gene from Chinese cabbage serving as an internal reference. The gene-specific primers used for qRT-PCR are presented in Appendix A.

3. Results

3.1. Identification of the *mini24* mutant

A mutant with smaller leaves, named *mini24*, was identified from an EMS-induced Chinese cabbage mutant population. The root length of *mini24* was markedly shorter than that of the WT roots at the seedling stage (Fig. 1-A–D). Compared with the WT plant leaves, *mini24* exhibited significantly shorter maximum leaf length, maximum leaf width, overall smaller plant spread, and fewer leaves at the rosette stage (Fig. 1-E–H). Furthermore, *mini24* had a significantly smaller leaf area than the WT (Fig. 2-A, B and E). However, observations of leaf palisade cells revealed that the cell area in *mini24* was significantly larger than that of WT (Fig. 2-C, D and F), with a concurrent reduction in cell number per unit leaf area (Appendix B). Thus, these findings suggest that the reduction in cell number is responsible for the smaller leaves in *mini24*. The increased cell area may represent a compensatory response to the reduced cell number.

3.2. Mapping candidate genes responsible for the small leaf phenotype in *mini24*

In the cross between *mini24* and the WT, all F_1 plants developed WT phenotypes. The F_2 population (477 individual plants) derived from self-pollinated F_1 generation segregated in the ratio of approximately 3:1, with about three-quarters (346) of the F_2 plants displaying the WT phenotype and one-quarter or so (131) showing the *mini24* phenotype (Table 1). This Mendelian segregation pattern indicates that the impaired leaf development phenotype in *mini24* is controlled by a single recessive gene.

To identify candidate genes responsible for the *mini24* phenotype, MutMap analysis was performed using the F_2 population from *mini24*×WT crosses. Two DNA sample pools from 30 F_2 (WT×*mini24*) progenies with a smaller leaf phenotype and 30 WT individuals were re-sequenced. The

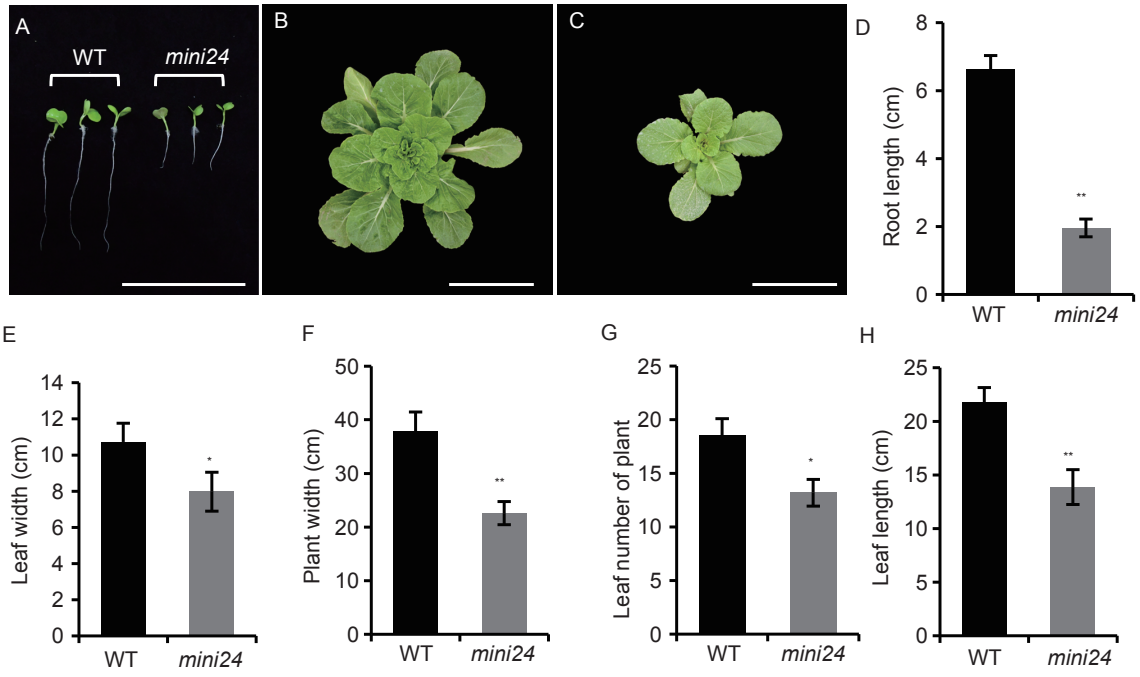


Fig. 1 Phenotypes of WT and *mini24* plants. A, root length of WT (left) and *mini24* (right) plants. Primary roots of WT and mini24 seedlings grown on Murashige and Skoog medium are shown at 10 days after germination. Bar=5 cm. B and C, WT (B) and *mini24* (C) plants at the rosette stage (35 days after colonization). Bar=10 cm. D, primary root length of WT and *mini24* seedlings at 10-day stage of Chinese cabbage. Data are shown as means±SD (n=10). **, P<0.01 indicates statistical significance by Student's t-test. E–H, leaf width (E), plant width (F), leaf number of plant (G), and leaf length (H) measured at the rosette stage. Data are shown as means±SD (n=20). **, P<0.01 and *, P<0.05 indicate statistical significance by Student's t-tests.

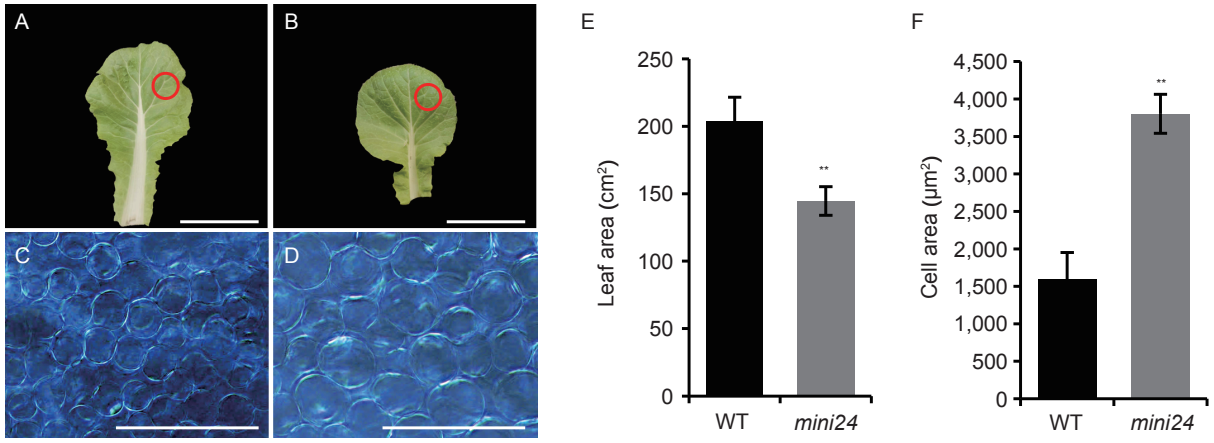


Fig. 2 Cell size of *mini24* plants. A and B, phenotypes of the largest outer leaves in WT (A) and *mini24* (B) plants. Leaves were sampled from plants grown in the greenhouse at 45 days after germination. Red circles indicate the regions used for quantifying palisade mesophyll cell area. Bar=10 cm. C and D, microscopic observations of leaf lamina cells in the largest outer leaves of WT (C) and *mini24* (D). Bar=200 μm. E, area of the largest outer leaves in WT and *mini24* plants. F, cell area of the largest outer leaves in WT and *mini24*. Data are shown as means±SD (n=10). **, P<0.01 indicates statistical significance by Student's t-test.

Table 1 Segregation ratios of F_1 and F_2 between WT and *mini24* plants

	Total plants	WT plants (normal plant)	Mutant plants (mini plant)	Segregation ratio	χ^2 value	P
F_1	5	5	0	5:0		
F_2	477	346	131	2.46:1	1.544	0.214

Chinese cabbage reference genome (*Brara_Chiifu_V3.0*; http://brassicadb.cn) was used for comparison. After filtering for single nucleotide polymorphisms (SNPs) with an SNP index≥0.6, a candidate genomic region between positions 24.38 and 24.42 Mb on chromosome A07 was identified in the F_2 population. Within this region, two candidate SNP loci were detected (Table 2). Genotyping of these SNPs *via* KASP markers across the entire F_2 population revealed that the *BraA07g034270.3C* locus (A07:24384352) exhibited 100% cosegregation with the *mini24* phenotype (Fig. 3-A; Table 2).

The *BraA07g034270.3C* gene contains seven exons and six introns, and it encodes a 373-amino-acid protein and includes a single conserved structural domain (DUF155). This gene is an ortholog of *RETARDED ROOT GROWTH* (*RRG*, *AT1G69380*) in *Arabidopsis thaliana*, which encodes a mitochondrion-localized protein required for cell division in the root meristem (Zhou *et al.* 2011). Loss of RRG function in *Arabidopsis* results in shorter root length. The homologous gene in Chinese cabbage, located on chromosome A07, is named *BrRRG*. In the *mini24* mutant, a single nucleotide substitution (G to A) occurs at position 454 in the third exon of *BrRRG*, leading to premature termination of the protein at position 152 (Fig. 3-B and C).

Sequence analysis of the BrRRG protein across four *Brassica* species (*B. oleracea*, *B. juncea*, *B. nigra*, and *B. napus*) and *Arabidopsis* revealed that the DUF155 domain is highly conserved. Unlike *Arabidopsis*, which has only one copy of the *RRG* gene, *Brassica* species typically have one or two copies. In Chinese cabbage, two copies of *BrRRG* were identified, with the second copy, *BrRRG1*, located on chromosome A02 (Fig. 3-D).

3.3. Silencing of the *BrRRG* gene reduces leaf area in Chinese cabbage

To further confirm that *BrRRG* is responsible for the impaired leaf development in the *mini24* mutant, VIGS of *BrRRG* was performed in WT plants. The leaf area of the *BrRRG*-silenced plants (pTY-*BrRRG*) was significantly smaller than that of the control plants (pTY-S) (Fig. 4-A and B). Consistent with predictions, pTY-*BrRRG* exhibited cellular enlargement relative to pTY-S (Fig. 4-A–C). Additionally, qRT-PCR analysis confirmed that the expression of *BrRRG* was significantly lower in pTY-*BrRRG* plants than controls (Fig. 4-D). These findings provide strong evidence that impaired leaf development in *mini24* is caused by a point mutation in the *BrRRG* gene.

3.4. *BrRRG* regulates DNA replication pathways and hormone signaling pathways

To identify the genes regulated by *BrRRG*, the expression patterns of *BrRRG* and *BrRRG1* were analyzed *via* data from the *Brassica* database. We found that their expression levels were similar across roots, stems, leaves, flowers, and meristematic tissues in Chinese cabbage. During leaf development, *BrRRG* expression was consistent throughout all leaf parts, with slightly higher levels observed in leaves than in petioles, suggesting that *BrRRG* is involved in all developmental stages of Chinese cabbage (Guo *et al.* 2021) (Appendix C). Consequently, total RNA was extracted from the leaves of both *mini24* and WT plants, and altered gene transcript levels were assessed through RNA sequencing (RNA-seq). The analysis revealed that 657 genes were downregulated and that 1,353 genes were upregulated ($|\log_2\text{FoldChange}|\geq 1$) in the *mini24* mutants compared with the WT (Fig. 5-A).

KEGG pathway enrichment analysis of the differentially expressed genes revealed the following three significantly

Table 2 KASP analysis of candidate mutation site

Gene ID	Variant	Chromosome	SNP site	Wild type site	Mutant site	KASP consistent rate (%)
BraA07g034270.3C	Stopgain	A07	24384352	G	A	100
BraA07g034330.3C	Nonsynonymous	A07	24405317	G	A	95.7

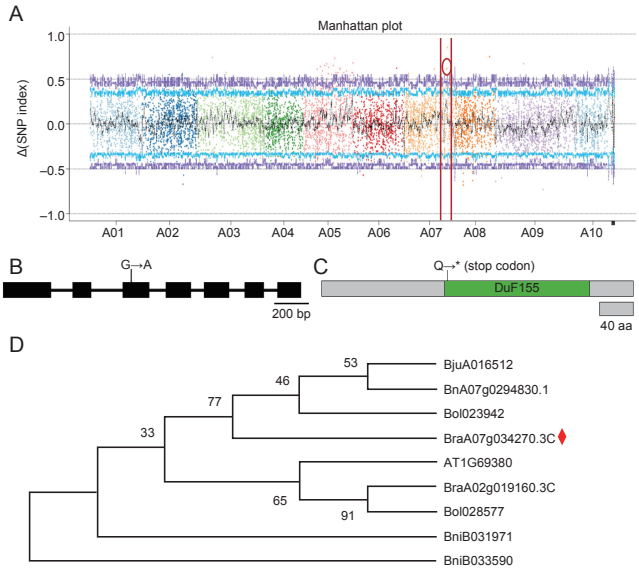


Fig. 3 Identification of candidate loci and genes responsible for leaf size in *mini24*. A, identification of genomic regions harboring causal mutations for *mini24* using MutMap. SNP index plots for *mini24*. Each symbol corresponds to an SNP, and the *x*-axis corresponds to the chromosomal position across 10 chromosomes. The black regression line is the average value of the SNP index based on a sliding window analysis. The SNPs on chromosome WT marked by a red circle between two red lines are the predicted causal mutations for *mini24*. Chromosomal positions of two mutant genes identified on chromosome 07 *via* MutMap. The purple line indicates the threshold for genetic screening. B and C, schematic diagram of the WT (*BrRRG*) and mutant (m*BrRRG*) *RRG* genes (B) and their encoded proteins (BrRRG and mBrRRG, respectively) (C). Asterisk (*) indicates the pre-mature stop codon. D, phylogenetic analysis of BrRRG proteins (marked with a red diamond) among Chinese cabbage, four other *Brassica* species and *Arabidopsis*. A phylogenetic tree was built using 7 RRG proteins *via* the neighbor-joining method. ClustalW in MEGA was used for multiple alignment *via* the default setting and phylogenetic construction.

affected pathways (padj<0.05): the eukaryotic ribosomal biogenesis pathway, the mustard oleosin biosynthesis pathway, and the DNA replication pathway. The top 20 biological pathways enriched in the KEGG analysis also included phytohormone signaling, phenylpropanoid biosynthesis, zeatin biosynthesis, and mismatch repair pathways (Fig. 5-B).

Notably, 11 genes within the DNA replication pathway were significantly upregulated, including the *MINICHROMOSOME MAINTENANCE* (*MCM*) gene, DNA polymerase *POL2A*, DNA polymerase *δ*, *REPLICATION FACTOR C 2* (*RFC2*), *REPLICATION FACTOR C 4* (*RFC4*), DNA primase *POLA3*, and *PROLIFERATING CELL NUCLEAR ANTIGEN 1* (*PCNA-1*) (Fig. 5-C).

In the hormone signaling pathway, the differentially expressed genes were enriched primarily in the auxin and cytokinin pathways, both of which are known to influence cell division. In the auxin response pathway, 16 genes were significantly upregulated, including 4 *AUX1* genes, 3 *AUX/IAA* genes, 1 Auxin *RESPONSE FACTOR* (*ARF*) gene, 2 *GRETCHEN HAGEN3* (*GH3*) genes, and 7 *Small Auxin-Up RNA* (*SAUR*) genes (Fig. 5-D). Each of these components plays distinct roles in the auxin response pathway. The AUX1 gene encodes an auxin endocytosis transporter responsible for the transport of indole-3-acetic acid (IAA). The GH3 gene encodes an auxin-binding enzyme that responds to auxin signals. The SAUR gene family consists of auxin-responsive genes, with 60 to 140 members typically present in most higher plant species (Stortenbeker and Bemer 2019). Additionally, four type-A *Arabidopsis Response Regulator* (*ARR*) genes were significantly upregulated in the cytokinin pathway (Fig. 5-E). Our endogenous hormone assays revealed no significant changes in IAA and CK levels (Appendix C). We examined cytokinin sensitivity in *mini24 via* 6-BA treatment, demonstrating that root elongation inhibition by 0.1 μmol L^{-1} 6-BA was significantly weaker in *mini24* than in WT (Fig. 5-F), thereby confirming reduced cytokinin sensitivity in the *mini24* plant.

3.5. *BrRRG* regulates the expression of cell cycle genes

In eukaryotic organisms, DNA replication in plants occurs during the S phase of mitosis, and both DNA replication and proper cell cycle progression are critical for cell proliferation and differentiation (Limas and Cook 2019; Ratnayeke *et al.* 2023). Type-A *ARR* genes, which are part of the cytokinin signaling pathway, also promote S-phase progression, and several key cell cycle genes, including *CDC2, CycD3,* and various B-type cyclins, are regulated by cytokinins (Jeleńska *et al.* 2000; Dewitte *et al.* 2007). On the basis of these findings, we hypothesized that cell division in the *mini24* mutant may be altered, which is closely associated with cell cycle regulation. The present study focused on the differential expression of genes within the cyclin family of cell cycle regulators between *mini24* and the WT. Nine cyclin genes, including *CYCD3-1*, *CYCD3-2*, *CYCB1-2-1*, *CYCB1-2-2*, *CYCA1-1*, *CYCB1-3*, *CYCB2-4-1*, *CYCB2-4-2*, and *CYCB2-2*, were significantly upregulated in *mini24* (Fig. 6-A and B). Furthermore, qRT-PCR analysis of *BrRRG*-silenced plants (pTY-*BrRRG*) demonstrated that *BrRRG* silencing resulted in the upregulation of cyclin genes (Fig. 6-C).

Previous studies on *Arabidopsis rrg* mutants have shown that mutations in RRG lead to prolonged cell cycle duration in root meristematic tissues. The expression levels of several cell cycle-related genes, including *CYCD4-1*, *Histone H4*, *CYCB1-1*, *CDKB1-1*, *E2Fa*, *DPa*, *DEL1*, and *WEE1*, were analyzed. qRT-PCR analyses indicated that while *CYCD4-1*, *E2Fa*,

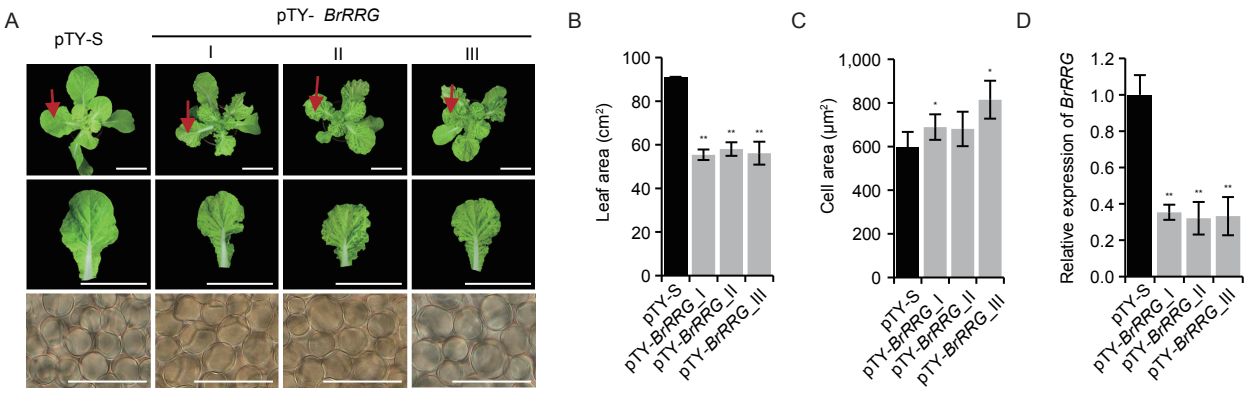


Fig. 4 Silencing of *BrRRG* reduces leaf size in Chinese cabbage. A, phenotypes of pTY-S and *BrRRG*-silenced (pTY-*BrRRG*) plants, showing the sixth leaf and its corresponding palisade mesophyll cells. Plant and leaf bars=10 cm. Cell bar=100 μm. Images were digitally extracted for comparison. VIGS of *BrRRG* was performed on Chinese cabbage plantlets at 5 days after seed germination in the greenhouse. Plants were photographed at 30 days after VIGS. B, area of the sixth leaf after *BrRRG* was silenced at 30 days post VIGS. The black columns represent the negative control pTY-S, and the gray columns represent the *BrRRG*-silenced plants. C, cell area of the sixth leaf after *BrRRG* was silenced at 30 days post VIGS. The black columns represent the negative control pTY-S, and the gray columns represent the *BrRRG*-silenced plants. D, silencing efficiency assessment of *BrRRG*. Data are shown as means±SD (n=3). *, P<0.05; **, P<0.01 indicates statistical significance by Student's t-tests.

DPa, and *DEL1* expression remained largely unchanged in the *rrg* mutant, the expression of *Histone H4*, *CYCB1-1*, *CDKB1-1*, and *WEE1* was significantly downregulated (Zhou *et al.* 2011). Similarly, in the Chinese cabbage *mini24* mutant, the *E2Fa* presented varying degrees of upregulation, whereas the expression of other genes, such as *CYCB1-1*, remained largely unchanged (Fig. 6-A). These findings were corroborated by qRT-PCR in the *BrRRG*-silenced plant (pTY-*BrRRG*), which confirmed the significance of these findings.

4. Discussion

4.1. *BrRRG* regulates cell division in Chinese cabbage leaves

For leafy vegetables, such as Chinese cabbage, leaf size directly determines their economic value. In recent years, several genes involved in regulating cell division have been identified in Chinese cabbage. For example, the overexpression of *BrGRFs* has been shown to increase cell proliferation, resulting in the production of larger flowers, cotyledons, and other organs (Hong *et al.* 2017). Similarly, the overexpression of the Chinese cabbage *BrANT-1* gene in *Arabidopsis* results in increased cell proliferation and larger leaves (Ding *et al.* 2018). In *Arabidopsis*, *RRG* regulates cell division in root tip meristematic tissues. Disruption of RRG function leads to a reduction in the number of dividing cells, decreased cell production rates, and impaired endoreplication, all of which affect meristem size and root growth (Zhou *et al.* 2011). However, the effect of RRG on leaf cell division has not been reported.

Here, we identified a small-leaf mutant *mini24* that carries a loss-of-function mutation of *B. rapa* RRG. Mutation in *BrRRG* resulted in smaller leaves, and larger cells may indicate a compensatory mechanism in response to suppressed *BrRRG*-mediated cell division. An additional line of evidence indicates that transcript levels of auxin-responsive genes *SAUR50* and *IAA14* modulate cellular expansion (Wang *et al.* 2025). In Chinese cabbage, *BrRRG* is a multicopy gene expressed across various tissues, suggesting that it underwent duplication during the evolution of the Chinese cabbage genome. In the present study, silencing *BrRRG* in WT also led to reduced leaf size with increased cell area, further supporting the role of *BrRRG* in regulating leaf cell division in Chinese cabbage.

4.2. *BrRRG* influences *E2Fa* and cell cycle gene expression

In *Arabidopsis*, mutations in *RRG* have been shown to significantly downregulate the expression levels of *Histone H4*, *CYCB1-1*, *CDKB1-1*, and *WEE1* (Zhou *et al.* 2011). However, the expression of *E2Fa* remains largely unchanged. The observed increase in *E2Fa* expression in the *mini24* Chinese cabbage mutant suggests that the regulatory mechanisms of *BrRRG* may differ between the aboveground and belowground tissues compared to *Arabidopsis*.

Multiple *E2F* transcription factors are present in *Arabidopsis*, where they bind to the promoters of target genes to promote transcription (Ramirez-Parra *et al.* 2003; Vlieghe *et al.* 2003; Naouar *et al.* 2009). However, a single mutation in *E2Fa* or *E2Fb* does not induce growth inhibition in *Arabidopsis* (Nisa *et al.* 2023), and the overexpression of *E2Fa* has been linked to delayed growth and flowering (Kosugi and Ohashi 2003), and contained fewer but larger cells, suggesting that rosette leaf cell division was negatively regulated by the ectopically expressed *AtE2Fa* (He *et al.* 2004). The target genes of *E2Fa* encompass a broad range of genes related to DNA replication and mismatch repair. *E2F* transgenic plants have demonstrated that *E2F* genes regulate the expression of various genes, including those encoding cell cycle proteins and MCM (Springer *et al.* 2000; Ni *et al.* 2009; Herridge *et al.* 2014). Numerous studies have established that MCM genes are essential for DNA replication and that knockdown of the MCM2-7 complex leads to the

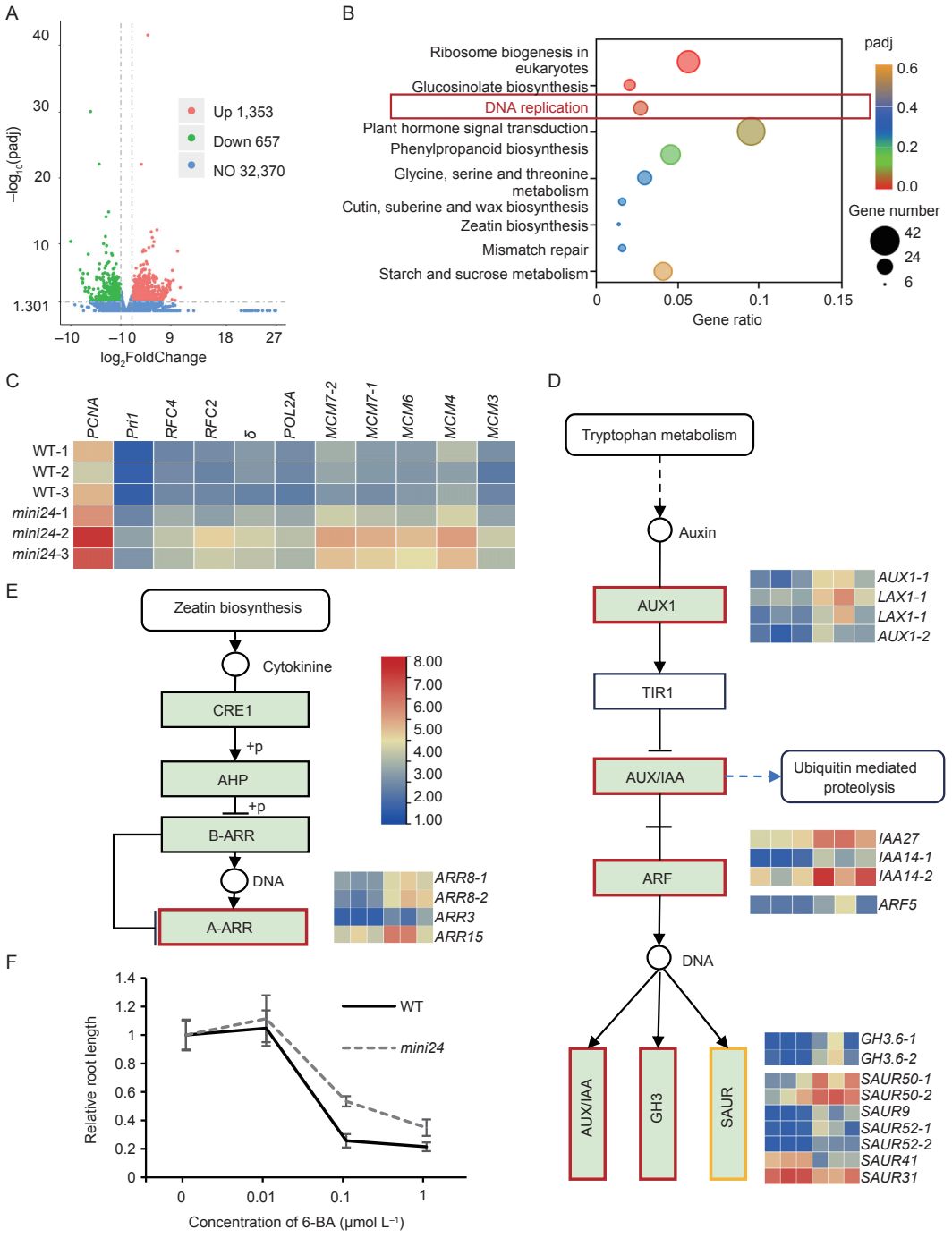


Fig. 5 Transcriptome analysis of WT and *mini24* at the seedling stage. A, volcano plot showing the number of significantly differentially expressed genes. Statistical significance was analyzed *via* two-tailed Student's *t*-tests (**, P<0.01, $|\log_2\text{FoldChange}|\geq 1$). B, significant KEGG enrichment analysis of the growth pathway in WT and *mini24* plants. The *x*-axis shows the ratio of the number of DEGs in a KEGG pathway to the total number of DEGs, and the KEGG pathways are shown on the *y*-axis. The size of the dots represents the number of DEGs in the pathway, and the color of the dots represents the adjusted *P*-value. C, DEG analysis of DNA replication pathways in *mini24* plants. Upregulated genes are highlighted red. D and E, DEG analysis of the IAA (D) and CK (E) response pathways in *mini24* plants. The red border represents upregulated genes, the blue border represents downregulated genes, and the green border represents both up- and downregulated genes. Arrows are for legend only. In the heatmaps, boxes on the left 1, 2, and 3 refer to the WT, and those on the right 1, 2, and 3 refer to *mini24*. The color bar indicates the expression level. F, the primary root length of WT and *mini24* seedlings germinated and grown on medium containing various concentrations of BA as indicated. The root length in the absence of BA was set as 100% for each genotype (n=15).

upregulation of components within the DNA repair pathway, which is crucial for leaf development in plants (Springer *et al.* 2000; Ni *et al.* 2009; Herridge *et al.* 2014).

In *A. thaliana*, 49 cyclins were identified and classified into A, B, D, H, L, T, U, and SDS types (Wang *et al.* 2004). The cyclins A1 and B1 have been suggested to be involved in the

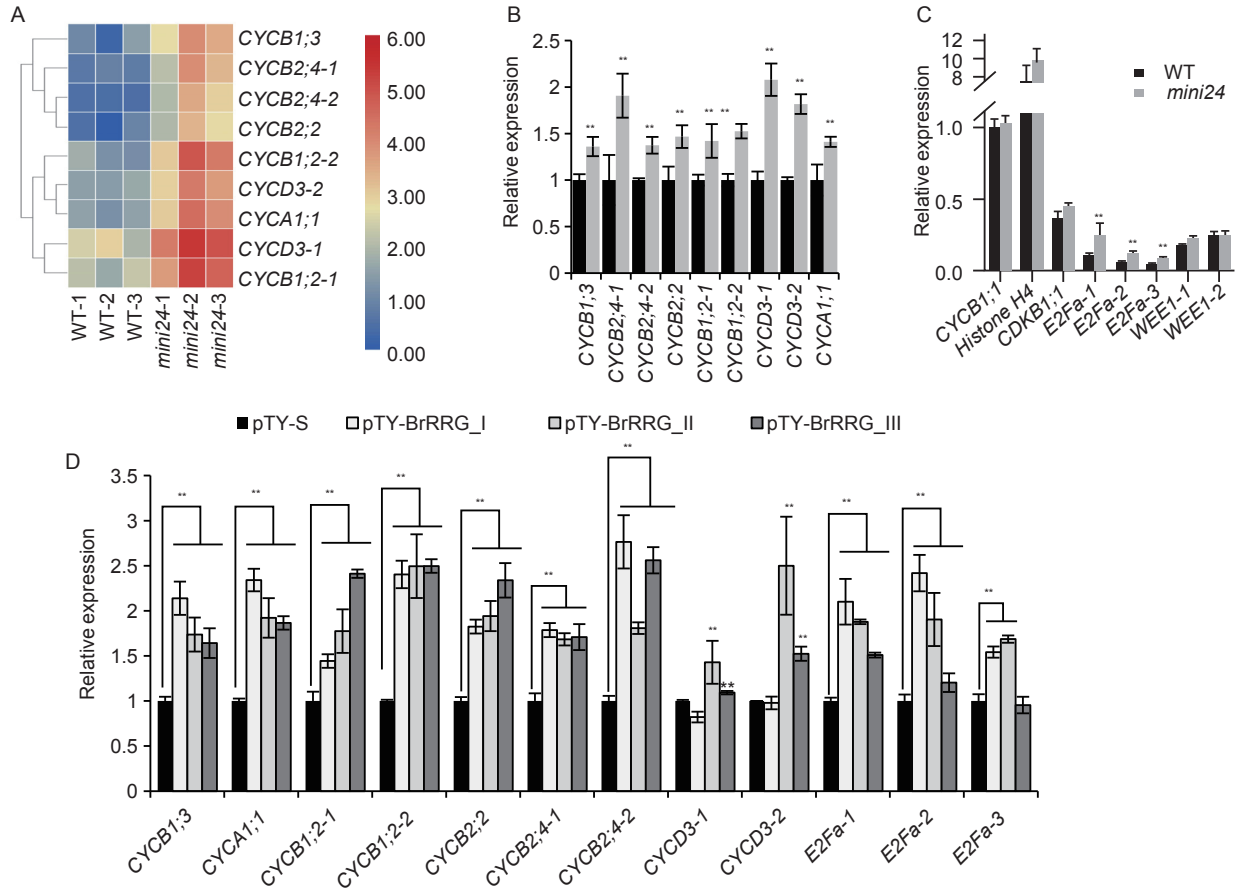


Fig. 6 Analyses of the expression of cell cycle-related genes in *mini24* mutants and *BrRRG*-silenced plants. A, heatmap showing the relative levels of the 9 different genes in the CYC gene families of WT and *mini24* at the seedling stage. B, relative expression levels of cycle family genes in the WT and *mini24* plants. The error bars represent ±SDs from three biological replicates (n=3 plants for each replicate). *, P<0.05 and **, P<0.01, Student's t test compared with the WT. C, expression levels of cell cycle-related genes in the WT and *mini24* plants. qRT-PCR data are shown as averages from triplicate repeats and three biological replicates. The expression level of the first gene in WT was 1 for analysis. **, P<0.01 and *, P<0.05, Student's t-test compared with the WT plant. D, relative expression levels of *E2Fa* genes and *Cyclin* genes (*CYCD3-1, CYCD3-2, CYC1B-1, CYC1B-2, CYCA1-1, CYCB1-3, CYCB2;4-1, CYCB2;4-2,* and *CYCB2-2*) in the leaves of *BrRRG*-knockdown plants. Data are shown as mean±SD (n=3). **, P<0.01 indicates statistical significance by Student's t-test.

progression from G2 to M phase in plants (Mironov *et al.* 1999; John *et al.* 2001), D-type cyclins are conserved in animals and plants and are proposed to be sensors of growth conditions and to trigger the G1/S transition by activating the RBR/E2F-DP pathway (Gutierrez *et al.* 2002; Trimarchi and Lees 2002). The upregulation of CYCs regulates the length of the cell cycle, the number of cell cycles and the premature cessation of cell cycles, thereby affecting leaf development and size (Breuer *et al.* 2010; De Veylder *et al.* 2011; Shi *et al.* 2021). Overexpression of *MYB48* or upregulation of *miR139a* reduces organ and plant size and modifies the morphology of the plant (Wang *et al.* 2015; Shi *et al.* 2021). In the present study, transcriptome sequencing revealed elevated expression of genes associated with the DNA replication pathway, and similarly revealed increased the expression of cell cycle-related genes that belong to the CYCA, CYCB and CYCD gene families. Verification through VIGS indicated that these genes presented increased transcript levels following *BrRRG* silencing. These findings implied that *BrRRG* represses the expression of *E2Fa* and certain cell cycle proteins in Chinese cabbage.

Notably, the *E2F* transcription factor has been identified as a key target of the auxin hormone, playing a crucial role in determining cell proliferation, as well as the transition from the mitotic cycle to cell expansion and DNA replication in the nucleus (Vandepoele *et al.* 2005; Vlieghe *et al.* 2005). In line with these findings, the present transcriptomic analysis revealed the upregulation of genes associated with the auxin signaling pathway, suggesting a potential regulatory mechanism through which *E2Fa* transcription factors influence plant growth (Fig. 6-E). Furthermore, the observed reduced cytokinin sensitivity in *mini24* suggests that *BrRRG* may additionally regulate cytokinin signal transduction in plants. These findings indicated that in plant leaves, *BrRRG* may also indirectly promote the expression of auxin- or cytokinin-related genes to regulate leaf size, warranting further experimental investigation into the underlying mechanisms involved.

Overall, the present study demonstrated that *BrRRG* regulates the expression of *E2Fa* and cell cycle genes (*CYCD3*, *CYCB1-2*, *CYCA1-1*, *CYCB1-3*, *CYCB2-4*, and *CYCB2-2*), thereby influencing the size of Chinese cabbage leaves. These findings provide a foundation for future

investigations into the mechanisms of cell division during the growth and development of Chinese cabbage and offer valuable genetic resources for breeding efforts.

5. Conclusion

In the EMS-induced *mini24* mutant of Chinese cabbage, a premature stop codon was identified in the *BrRRG* gene, leading to disrupted cell division and reduced plant size. This discovery highlights the regulatory role of *BrRRG* in leaf cell division in Chinese cabbage. Comparative transcriptomic analysis between *mini24* and the WT plants suggested that *BrRRG* might be involved in auxin and cytokinin signaling pathways. Moreover, silencing *BrRRG* in wild-type Chinese cabbage demonstrated that *BrRRG* influences the expression of *E2Fa* transcription factors and cell cycle-related genes during leaf development.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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