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ZmGRAS46 Negatively Regulates Flowering Time in *Arabidopsis thaliana*

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Abstract: Flowering is an essential process in plant development, and there are six major flowering pathways: the photoperiodic pathway, gibberellin pathway, vernalization pathway, age pathway, autonomous pathway, and temperature pathway. In this study, we screened the transcriptome sequencing of early flowering mutants from the laboratory for the significantly differentially expressed *ZmGRAS46*, which belongs to the DELLA subfamily of the GRAS family. DELLA is involved in the gibberellin pathway to regulate plant flowering. However, it is not clear whether *ZmGRAS46* is involved in the gibberellin pathway which regulates plant flowering; therefore, in this experiment, we investigated the regulatory role of this gene in *Arabidopsis* flowering by overexpressing *ZmGRAS46*. It was found that overexpression of *ZmGRAS46* in *Arabidopsis* promotes the formation of rosette leaves and flower buds and delays flowering time in *Arabidopsis*, and experiments have shown that *ZmGRAS46* represses the expression of *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF CONSTANS1 (SOC1)*, *CONSTANS (CO)*, and *LEAFY (LFY)*. Our results indicated the possibility that *ZmGRAS46* represses flowering through the CO-FT-SOC1-mediated photoperiodic flowering pathway. The delayed flowering phenotype of overexpressing *ZmGRAS46 Arabidopsis* could be rescued by applying GA3. The experimental results indicate that *ZmGRAS46* depends on the GA3 pathway to regulate flowering in *Arabidopsis*.

Keywords: *Arabidopsis thaliana*; *ZmGRAS46*; GA3 pathway; flowering time

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1. Introduction

Flowering occurs in the apical meristematic tissue of the stem of plants. With the occurrence of a long evolution, plants have a mechanism for sensing and responding to changes in the external environment. Among the many mechanisms, the regulatory mechanism of flowering has a critical position [1]. *Arabidopsis thaliana* (*Arabidopsis thaliana* (L.) Heynh.), one of the model plants in plant molecular, cellular, and evolutionary biology, is favorable for cultivation, has a self-fertilizing reproductive system, has a relatively short generation time, and has easy access to germplasm material. Many recent research advances have been made to regulate flowering by mediating different signaling pathways. The autonomous flowering pathway enables *Arabidopsis* to promote flowering independently of day length by inhibiting the central flowering deterrent *FLOWERING LOCUS C (FLC)* [2]. Diurnal temperature changes regulate photoperiodic flowering by altering the pattern of *FT* accumulation [3]. ARGONAUTE5 physically and functionally interacts with miR156 to regulate flowering in *Arabidopsis* by mediating the aging pathway [4]. BraVRG is a new *cabbage* protein promoting *Arabidopsis* flowering through vernalization induction [5]. Gibberellin regulates flowering in *Arabidopsis* by mediating DELLA-BRAHMA-NF-YC [6].

Among them, the DELLA protein plays a significant role as a central regulator in gibberellin signaling [7].

DELLA proteins belong to a conserved subfamily of GRAS proteins, and the GRAS family of transcription factors has essential roles in plant growth and development, signal transduction, axillary bud and root development, mycorrhiza and rhizoma formation, and stress response. An analysis of the evolutionary tree of GRAS protein family members in *Arabidopsis thaliana* and the conserved structural domains of the proteins showed that there are 12 conserved sequences at the C-terminal end of GRAS proteins and several conserved structural domains at the N-terminal end, including the conserved DELLA structural domain [8,9]. DELLA proteins can regulate flowering by interacting with several transcription factors in the leaf and stem tip to regulate their activities [10]. Gomez shows that DELLA proteins are new players in the control of seed size and suggests that the modulation of DELLA-dependent pathways can improve crop yield [11]. Different expression patterns of the oilseed rape *BnaDELLA* gene were detected under biotic and abiotic stresses, suggesting that regulating *BnaDELLA* expression in oilseed rape is a promising approach to improving oilseed rape stress tolerance and harvest indexes [12]. RGL2 is one of the DELLA proteins, and studies have shown that RGL2 has a regulatory effect on flower development, mainly regulating the growth of stamens and the division of anthers, which significantly influences plant fertility [13]. There are few intron insertions in GRAS genes during the evolutionary process, among which the DELLA family has a more straightforward gene structure, which is more conserved, and the function of DELLA proteins has been widely studied in recent years, but *ZmGRAS46* in the DELLA subfamily has not been investigated, so this gene was selected in this study to investigate its regulatory role on flowering.

In recent years, global warming and an increase in temperature have affected the flowering period of plants; Krista et al. showed that the change in the flowering period had an impact on the nectar secretion of flowers, disrupting plant–pollinator interactions, thus affecting the entire ecosystem [14]. Late frost damage severely impacts the early flowering species Siberian plum, reducing yield [15]. Sorghum is a short-day plant with a solid photoperiodic response, so it becomes essential to develop the cultivation of photoperiod-insensitive cereals in temperate regions [16]. These studies have shown that changes in flowering time can significantly affect plant growth and development, so to solve the problem of early and late flowering for plant flowering, related research becomes essential.

In this study, we successfully cloned *ZmGRAS46* and obtained positive Arabidopsis plants by genetic transformation. Through expression pattern analysis and phenotypic observation, we demonstrated that *ZmGRAS46* delayed plant flowering by repressing *CO*, *FT*, *SOC1*, and *LFY*. This regulatory effect was dependent on the GA signaling pathway. In addition, this study provides new insights for the subsequent analysis of the molecular mechanism of the GRAS gene regulation of plant flowering.

2. Materials and Methods

2.1. Plant Materials and Treatments

All *Arabidopsis thaliana* seedlings used in this study were in Columbia-0 wild-type (Col-0 WT) background. The CDS region of *ZmGRAS46* was cloned, and the gene was ligated into the pCAMBIA3301 vector by seamless cloning. *Arabidopsis* was genetically transformed by the Agrobacterium-mediated method, and the overexpression-positive *Arabidopsis* plants, OE6 and OE8, which had the highest expression of *ZmGRAS46*, were selected for the present experiment.

In this experiment, the WT, OE6, and OE8 seeds were vernalized in dark culture at 4 °C for 3 days. Then, the vernalized seeds were planted in nutrient soil containing vermiculite using a pipette gun and placed in an artificial climate chamber (22 °C, monochromatic white light, 400–700 nm continuous spectrum, 16 h light/8 h dark, 130 μmol/(m^2·s), 65%RH) to wait for their germination. *Arabidopsis* was sprayed with 200 μmol/L GA3 (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China) every three days starting. On the 15th day of cultivation, the growth of the treated *Arabidopsis* was observed and recorded.

2.2. Generation of Constructs and Transgenic Plants

For creating *ZmGRAS46* overexpression constructs, the protein-coding sequence of *ZmGRAS46* was PCR-amplified with primers and then cloned into the pCAMBIA3301 vector driven by a Cauliflower mosaic virus (CaMV) 35 S promote [17]. The recombinant plasmid was transformed into Agrobacterium tumefaciens GV3101, and *Arabidopsis thaliana* was dip-flower infested by the Agrobacterium-mediated method [18]. The T3 plants of positive lines were selected with Glyphosate (5 mg/L, Sigma, Kawasaki-shi, Japan), and the viable *Arabidopsis thaliana* plants were identified by PCR for the subsequent experiments. All primers used in this study are listed in Supplementary Table S1.

2.3. RNA Extraction, Reverse Transcription, and Gene Expression Quantification

The treated samples were prepared by freezing in liquid nitrogen and stored in a refrigerator at −80 °C for RNA extraction in subsequent experiments. RNA extraction by Trizol method, and cDNA was synthesized using the TransScript All-in-one First-Strand cDNA Synthesis SuperMix for qPCR (TRANS, Xiamen, China) following the manufacturer's instructions. Gene expression levels were quantified by quantitative real-time PCR (qRT-PCR) using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) with a Thermal Cycler Dice Real-Time System TP800 (TaKaRa, San Jose, CA, USA) following the manufacturer instructions. *AtActin2* was used as a reference. The expression value was calculated using the comparative CT method [19]. Three replications were performed for each sample and each experiment.

2.4. Measurement of Physiological and Biochemical Indicators

On the 15th of cultivation, 200 μmol/L GA3 was sprayed on WT, OE6, and OE8 Arabidopsis plants once every three days. SOD, POD, Pro, H_2O_2, O_2^-, and chlorophyll contents were measured in treated and untreated leaves when the plants entered flowering stage. The antioxidant enzyme activities of SOD and POD were measured according to Xiang's method [20]. The proline content was analyzed using the acid ninhydrin method [21]. The content of H_2O_2 and O_2^- was calculated by using the acetone method, and the specific method was referred to as Wang's method [22]. The absorbance of chlorophyll solution was measured at 649 nm and 665 nm, and the chlorophyll content was measured by Fitter's method [23].

2.5. Yeast Two-Hybrid (Y2H) Assay

Relatively high-scoring ZmMADS62 proteins were obtained by screening through STRING online software (Version: 11.0) analysis. In order to verify whether ZmGRAS46 and ZmMADS62 indeed interacted with each other, we constructed the pGBKT7-ZmGRAS46 decoy vector and the ZmMADS62-AD prey vector. After proving that the pGBKT7-ZmGRAS46 prey vector did not have toxicity and self-activating activity (Figures S1 and S2), ZmGRAS46-BK vector plasmid and ZmMADS62-AD vector plasmid were transformed into the same AH109 yeast sensory state at the same time. The transformed bacterial fluids were coated on SD/-Trp-Leu two-deficient solid medium. Single colonies were picked and put into the YPDA liquid medium at 29 degrees Celsius for two days. The cultured bacterial liquid, positive bacterial liquid, and negative bacterial solution were spotted on SD/-Trp-Leu two-deficient solid medium and SD/-Trp-Leu-Ade-His, SD/-Trp-Leu-Ade-His-X-α-Gal four-deficient solid yeast medium at 29 degrees Celsius for two days to observe the growth [24].

2.6. Statistical Analysis

All results in this study were performed in more than three replicates. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of experimental measurement data; Students' *t*-tests were used to confirm the variability of results between treatments, respectively. $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results

3.1. Overexpression of ZmGRAS46 Inhibition Flowering

Zhou et al. found that transcription factors can control plants' structure and flowering time [25]. In order to investigate the regulatory effects of *ZmGRAS46* on flowering, we transfected *Arabidopsis thaliana* with *ZmGRAS46* using an Agrobacterium-mediated method in this experiment. We obtained two overexpression plants (OE6 and OE8) with high expressions of *ZmGRAS46*, which were used in the subsequent experiments (Figure S3). In this study, we observed the phenotypes of overexpression plants OE6 and OE8. We found that the flowering time of the overexpression plants was significantly later than that of the wild-type plants (Figure 1A,B). The wild-type plants (WT) flowered about seven days earlier than the overexpression plants. The number of rosette leaves at flowering was about 10 in the wild-type *Arabidopsis thaliana*. However, the number of rosette leaves at flowering was around 26 in the positive *Arabidopsis thaliana* (Figure 1C,D). These results suggest that the overexpression of *ZmGRAS46*, in its early stages, focuses on promoting rosette leaf growth in *Arabidopsis* while delaying flowering in *Arabidopsis*.

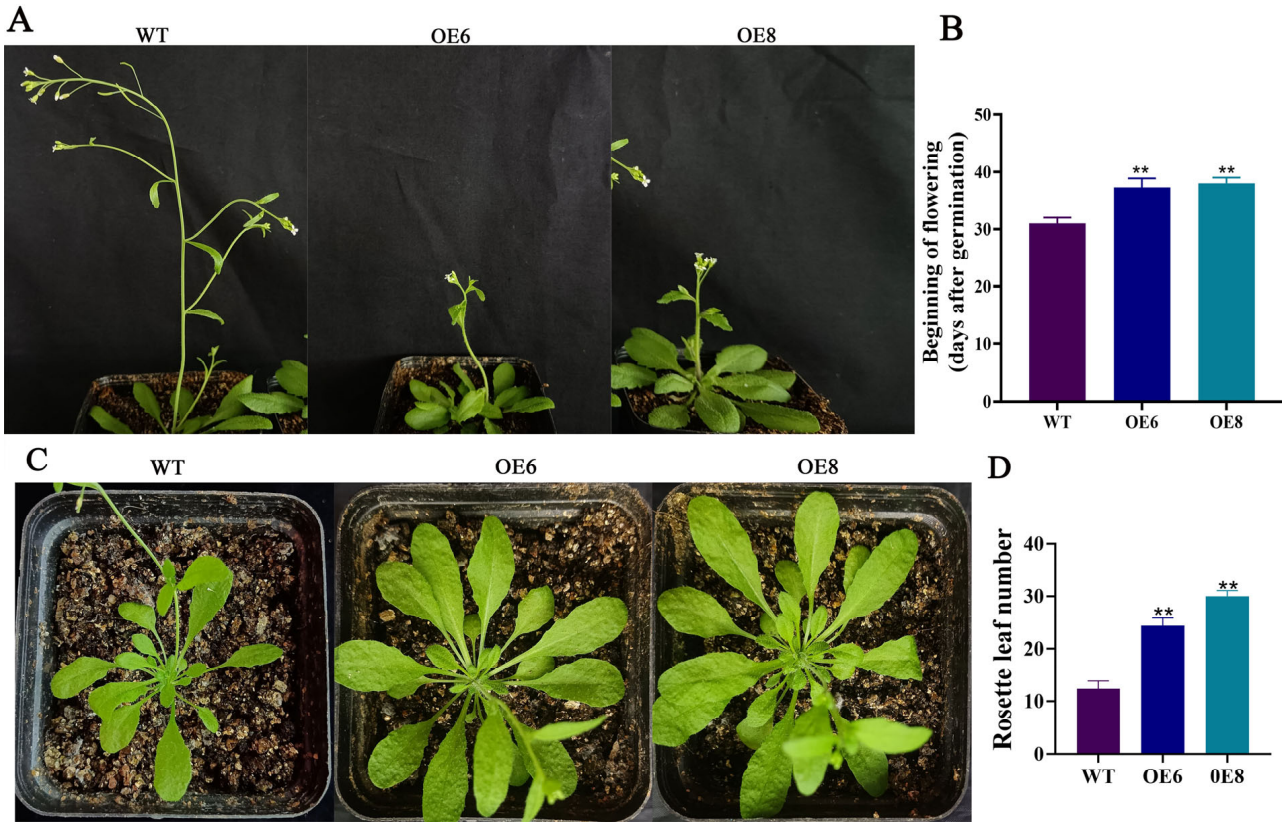

Figure 1. Flowering phenotypes of *Arabidopsis* seedlings with different *ZmGRAS46* backgrounds. (**A**,**B**) The flowering phenotypes of Col-0 wild-type (WT) and *ZmGRAS46*-overexpressing (OE6, OE8) lines under long-day (16 h/8 h light/dark) conditions. (**C**,**D**) Phenotypes of rosette leaves of Col-0 wild-type (WT) and *ZmGRAS46*-overexpressing (OE6, OE8) lines under long-day (16 h/8 h light/dark) conditions. Using Student's *t*-test, asterisks indicate statistically significant differences (** $p < 0.01$). Data are shown as mean ± SD from three independent experiments.

3.2. ZmGRAS46 Promotes Flower Bud Formation and Inhibits Floral Branch Elongation

During *Arabidopsis thaliana* flowering, plants OE6 and OE8 had approximately 12 flower buds, while wild-type *Arabidopsis* had around 7 buds. The number of buds of the positive plants was significantly more than that of the wild-type *Arabidopsis* plants (Figure 2A,B). The length of the main branches of wild-type *Arabidopsis* plants was significantly longer than that of OE6 and OE8 by about eight centimeters (Figure 2C,D). The results showed that

the overexpression of *ZmGRAS46* promoted the formation of flower buds and inhibited the elongation of main branches.

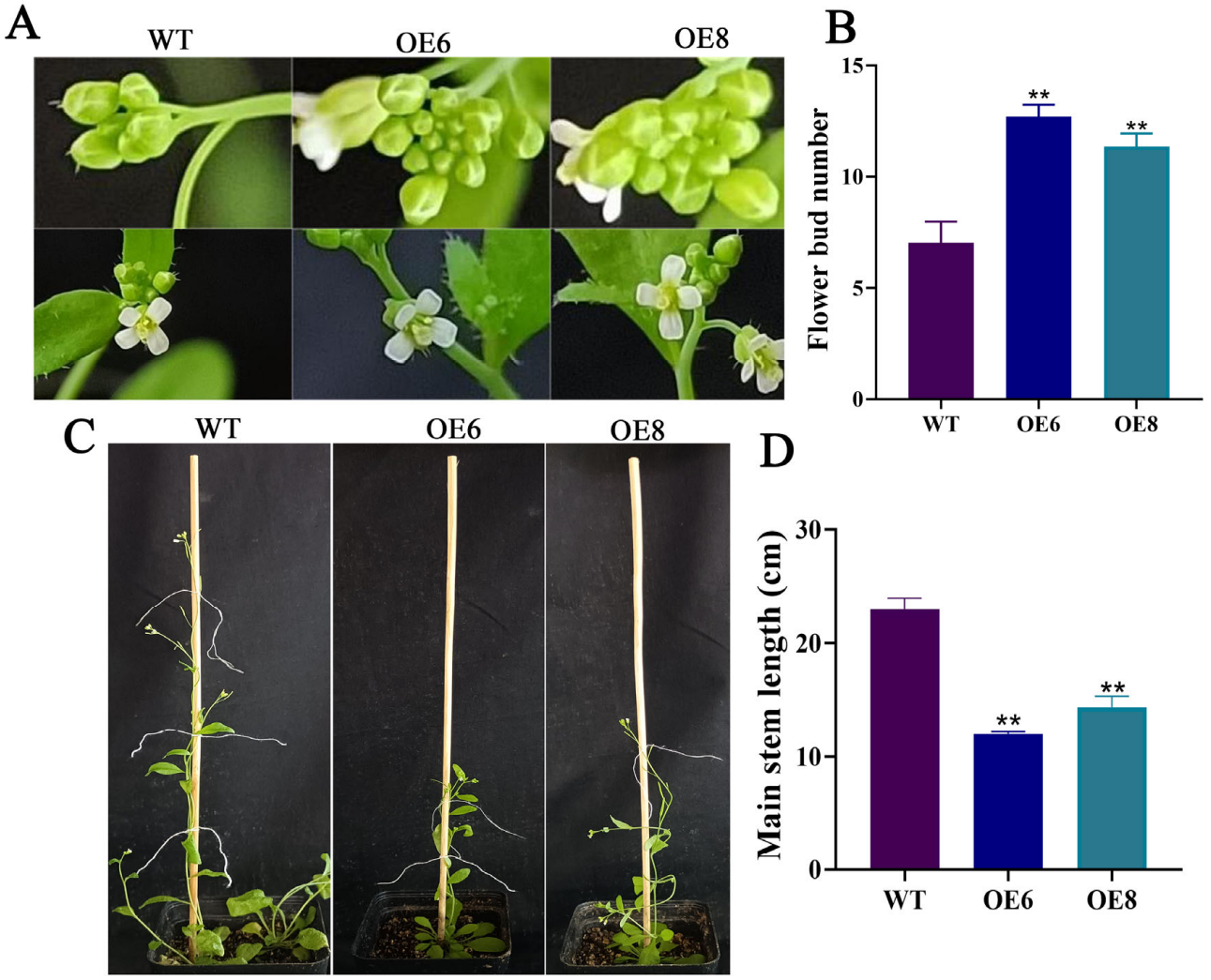

Figure 2. Phenotypic observation of *Arabidopsis* with different *ZmGRAS46* backgrounds. (**A**,**B**) Observation on bud of Col-0 wild-type (WT) and *ZmGRAS46*-overexpressing (OE6, OE8) lines under long-day (16 h/8 h light/dark) conditions. (**C**,**D**) Length of main branches of Col-0 wild-type (WT) and *ZmGRAS46*-overexpressing (OE6, OE8) lines under long-day (16 h/8 h light/dark) conditions. Using Student's *t*-test, asterisks indicate statistically significant differences (** $p < 0.01$). Data are shown as mean ± SD from three independent experiments.

3.3. Determination of Physiological and Biochemical Indices in T3 Generation of Arabidopsis thaliana Plants Transgenic for ZmGRAS46 after GA3 Treatment

The *GA20ox* and *GA3ox* genes catalyze the formation of active GA, and the *GA2ox* gene can render gibberellins irreversibly inactive [26–28]. The experiment involved spraying gibberellin onto wild-type and overexpressed *Arabidopsis thaliana* OE6 and OE8 until they entered the flowering stage. Then, *Arabidopsis thaliana* flower buds and blossoms were sampled. A fluorescence qPCR revealed that the expressions of *GA2ox2*, *GA3ox2*, *GA20ox1*, and *GA20ox2* were decreased in positive *Arabidopsis thaliana* OE6 and OE8 and that the expression of *GA3ox1* was elevated in flower buds and decreased in blossoms (Figure 3A,B). It suggests that exogenous gibberellin spraying inhibited endogenous gibberellin synthesis, altering the changes of gibberellin in plants.

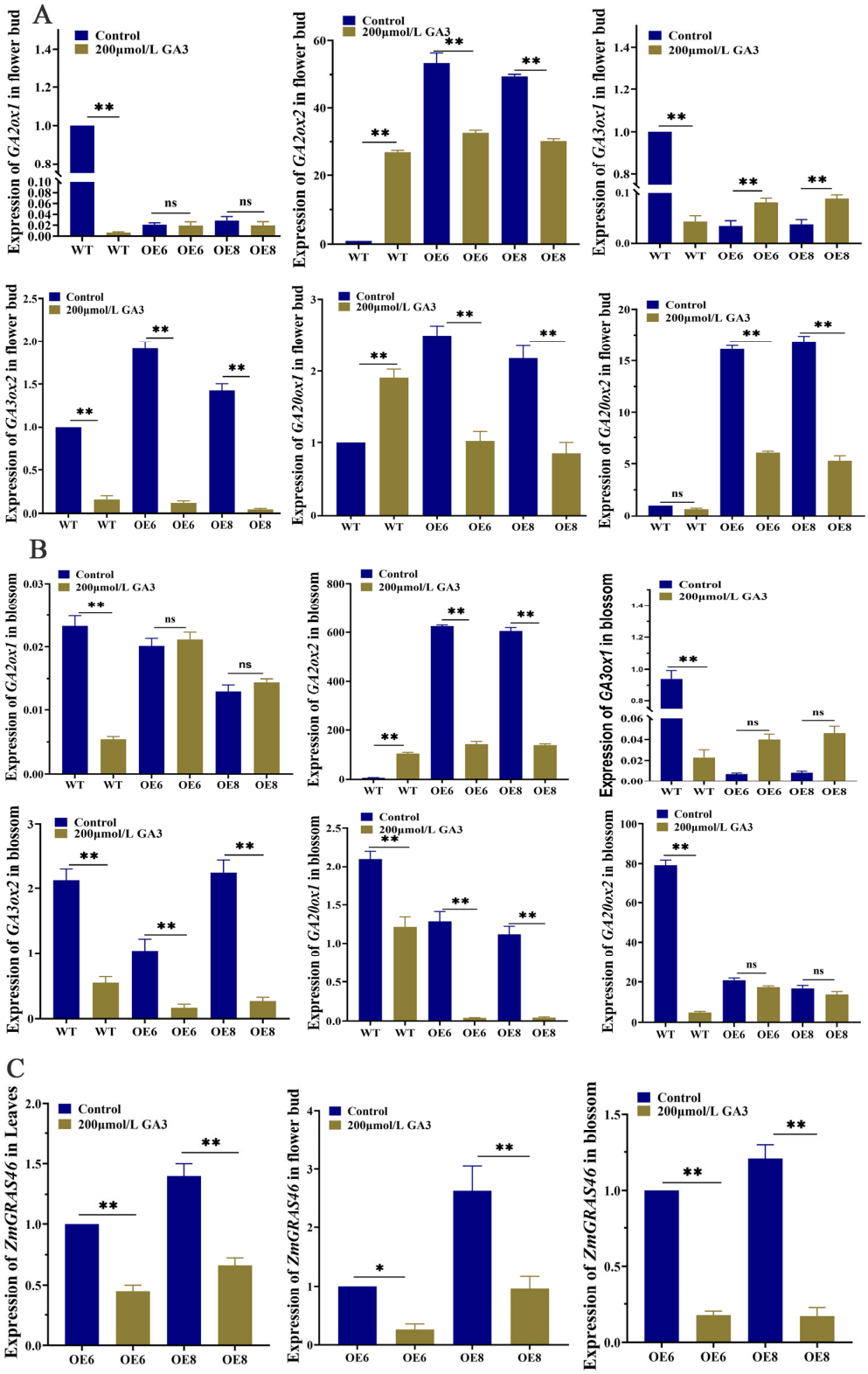

Figure 3. Differential expression of genes related to GA3 synthesis and metabolism in *Arabidopsis* seedlings with different *ZmGRAS46* backgrounds. (**A**) Differential expression of GA3 anabolic genes in *Arabidopsis* buds. (**B**) Differential expression of GA3 anabolic genes in *Arabidopsis* blossom. (**C**) Expression analysis of *ZmGRAS46* in different conditions. Using Student's *t*-test, asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; ns: not significant). Data are shown as mean ± SD from three independent experiments.

Bao S et al. showed that ubiquitination degraded DELLA when bound to GA [7]. In order to verify whether the expression of *ZmGRAS46* was changed, this experiment sampled treated *Arabidopsis* leaves, flowers, and buds and found that the expression of *ZmGRAS46* was reduced (Figure 3C), indicating that the application of GA3 would reduce the expression of *ZmGRAS46*, and in order to explore what would happen to *Arabidopsis* positive for the reduction of the *ZmGRAS46* content, a later stage of the *Arabidopsis* was treated and the phenotypes were observed and recorded.

The application of gibberellins affects plants' physiological and biochemical indices, and excessive concentrations affect the peroxide scavenging capacity of plants [29]. In order to verify whether the application of 200 μmol/L GA3 gibberellin has any adverse effect on the growth condition of plants, we measured the physiological and biochemical indexes of *Arabidopsis thaliana*. In this experiment, *Arabidopsis* leaves just entering the flowering stage were sampled. *Arabidopsis* leaves without GA3 treatment were the control group. In contrast, GA3-treated *Arabidopsis* leaves were the experimental group. The results of the measurements showed that spraying with GA3 could increase POD, SOD, Pro, and total chlorophyll contents in wild-type and overexpressed *Arabidopsis thaliana* OE6 and OE8. They would reduce H_20_2 and O_2^- in wild-type *Arabidopsis thaliana* and OE6 and OE8 plants (Figure 4). The results showed that spraying 200 μmol/L GA3 was beneficial to plant growth and that it could be possible to spray 200 μmol/L GA3 for the subsequent experiments.

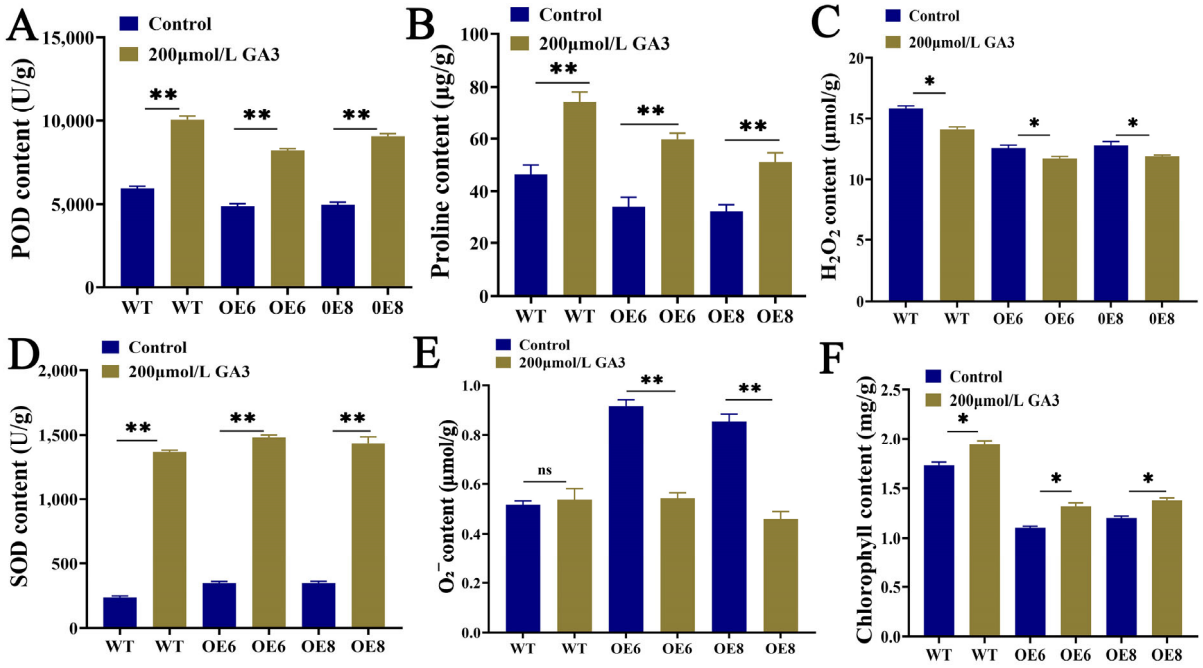

Figure 4. Determination of physiological and biochemical indexes of *Arabidopsis* with different *ZmGRAS46* backgrounds. (**A**) Analysis of POD activity. (**B**) Analysis of Pro activity. (**C**) Analysis of H_2O_2 activity. (**D**) Analysis of SOD activity. (**E**) Analysis of O_2^- activity. (**F**) Analysis of total chlorophyll content. Using Student's *t*-test, asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; ns: not significant). Data are shown as mean ± SD from three independent experiments.

3.4. ZmGRAS46 Is Dependent on the GA Pathway to Regulate Flowering

Wild-type plants and OE6 and OE8 plants were planted in nutrient soil containing vermiculite, and 200 μmol/L GA3 was sprayed on *Arabidopsis thaliana* starting at 15 days of plant growth and every three days, and the growth of *Arabidopsis thaliana* after spraying was observed. Observations on the 30th of cultivation showed that untreated wild-type *Arabidopsis* had formed flower buds, but OE6 and OE8 had not yet formed flower buds, whereas GA3-treated WT, OE6, and OE8 plants had elongated main branches (Figure 5A).

We found that there was no significant difference in flowering time between GA3-treated wild-type plants, and both OE6 and OE8 plants flowered at about 26 days of growth (Figure 5B). This suggests that spraying GA3 can change the flowering time of *ZmGRAS46* overexpression and advance OE6 and OE8, which originally delayed flowering, to a similar flowering time as the wild type. When the main branches were elongated, we recorded and observed the length of the main branches and found that the length of the main branches of the overexpression plants was significantly elongated after spraying GA3 with no significant difference compared with the wild type, indicating that the spraying of GA3 could change the length of the main branches with the overexpression of *ZmGRAS46* so that the length of the main branches, which was shorter than that of the wild type, was similar to the length of the main branches of the wild-type plants (Figure 5C,D). It was shown that *ZmGRAS46* was dependent on the GA pathway to regulate flowering, and spraying gibberellin reduced the expression of *ZmGRAS46*, restoring the phenotype of the overexpression of *ZmGRAS46* that delayed flowering as well as inhibited floral branch elongation.

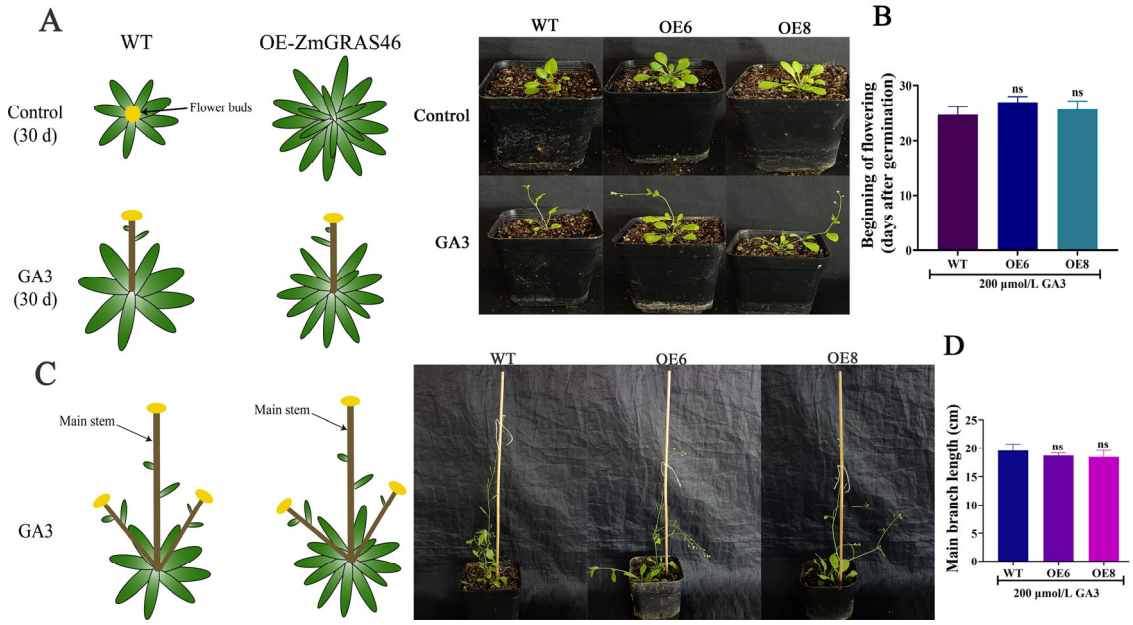

Figure 5. Flowering phenotypes of *Arabidopsis* seedlings with different *ZmGRAS46* backgrounds after GA3 treatment. (**A**,**B**) Flowering phenotypes of Col-0 wild-type (WT) and *ZmGRAS46* overexpressing (OE6, OE8) strains after GA3 treatment under long-daylight (16 h/8 h light/dark) conditions. (**C**,**D**) Primary branch length of Col-0 wild-type (WT) and *ZmGRAS46* gene overexpressing (OE6, OE8) strains under long daylight (16 h/8 h light/dark) conditions after GA3 treatment condition. Using Student's *t*-test, asterisks indicate statistically significant differences (ns: not significant). Data are shown as mean ± SD from three independent experiments.

3.5. ZmGRAS46 Regulates Diverse Genes That Are Involved in Flowering Regulation

To investigate the role of *ZmGRAS46* in regulating the flowering pathway, this experiment sampled flower buds and flowers of GA3-treated and untreated *Arabidopsis thaliana*. The results by fluorescence quantification showed that the expression of *CO*, *FT*, *SOC1*, and *LFY* was less than that of WT plants in OE6 and OE8, indicating that the overexpression of *ZmGRAS46* inhibited the formation of *CO*, *FT*, *SOC1*, and *LFY* to delay flowering. Our results indicated the possibility that *ZmGRAS46* represses flowering through the CO-FT-SOC1-mediated photoperiodic flowering pathway. *CO*, *FT*, *SOC1*, and *LFY* expressions were elevated in overexpressed OE6 and OE8 plants after GA3 treatment. This indicates that spraying GA3 can reduce the expression of *ZmGRAS46* and promote the elevated expression of flowering-related genes *CO*, *FT*, *SOC1*, and *LFY*, which contributed to the

early flowering of *Arabidopsis thaliana*, demonstrating that *ZmGRAS46* is dependent on the GA pathway for the regulation of flowering (Figure 6).

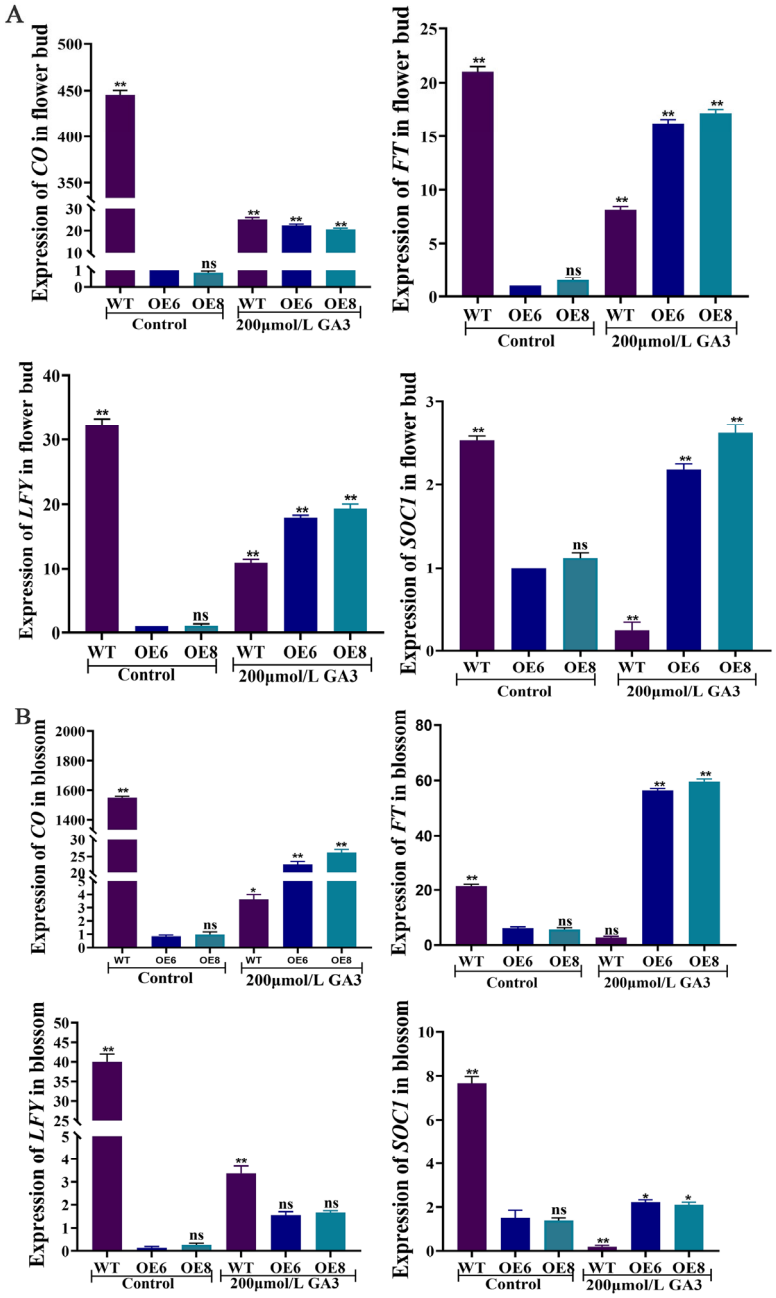

Figure 6. Differential expression of flowering-related genes in *Arabidopsis* seedlings with different *ZmGRAS46* backgrounds. (**A**) Differential expression of flowering-related genes CO, FT, LFY, and SOC1 in *Arabidopsis* flower buds. (**B**) Differential expression of flowering-related genes CO, FT, LFY, and SOC1 in *Arabidopsis* blossom condition. Using Student's *t*-test, asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; ns: not significant). Data are shown as mean ± SD from three independent experiments.

3.6. Interaction of ZmGRAS46 and ZmMADS62 Proteins

Delayed flowering affects fruit yield. Li et al. showed that MADS-box regulates fruit ripening time and quality [30,31]. In order to investigate the effect of *ZmGRAS46* on fruit quality, this experiment was conducted by screening and validation to find *ZmMADS62* in the MADS-box family. ZmGRAS46-BK vector plasmid and ZmMADS62-AD vector plasmid were simultaneously transformed into the same AH109 yeast sensory state. After

the transformation, the cultured, positive, and negative bacterial spots were incubated in an SD/-Trp-Leu two-deficient solid medium and SD/-Trp-Leu-Ade-His and SD/-Trp-Leu-Ade-His-X-α-Gal four-deficient solid yeast medium for two days at 29 °C, respectively. The results showed that the pGADT7-T + pGBKT7-53 positive control and ZmGRAS46-BK + ZmMADS62-AD experimental group grew colonies on both two- and four-deficient media, and the colonies turned blue on four-deficient media coated with X-α-gal. In contrast, the pGADT7-T + pGBKT7-lam negative control had colonies on two-deficient media growth growing colonies on all four-deficient media (Figure 7), suggesting that the ZmGRAS46-ZmMADS62 module interaction may play a role in regulating plant yield.

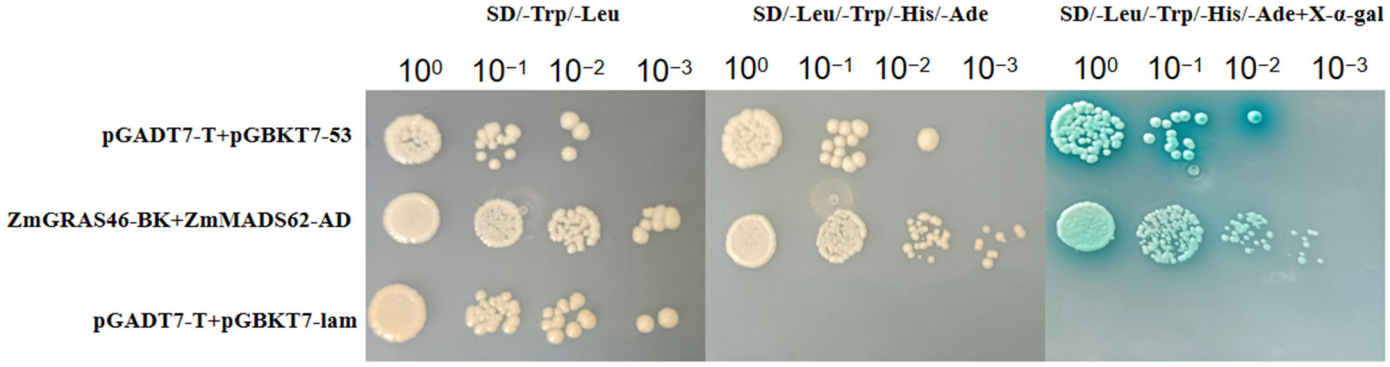

Figure 7. Interaction validation of ZmGRAS46-ZmMADS62. Positive control: pGADT7-T + pGBKT7-53; experimental group: ZmGRAS46-BK + ZmMADS62-AD; negative control: pGADT7-T + pGBKT7-Lam.

4. Discussion

A complex regulatory network synergistically regulates plant flowering. In this study, we showed that *ZmGRAS46* in the GRAS family can affect the flowering phenotype of Arabidopsis thaliana and repress the expression of *CO*, *FT*, *SOC1*, and *LFY*. The treatment by GA3 demonstrated that this gene mediates the GA pathway to regulate plant flowering.

BrLAS is a GRAS transcription factor in kale-type oilseed rape that alters the phenotype of *Arabidopsis*, delays the flowering time of the plant, and can respond to it under drought stress [32]. The *GAI1-like* gene in the GRAS family delays plant flowering and reduces plant height in Rhus chinensis [33]. *SlGRAS26*, a GRAS transcription factor in *Solanum lycopersicum*, responds to abiotic stress and alters plant height, affecting the initiation of inflorescence meristematic organization [25]. The above studies showed that the GRAS family can affect the flowering time of plants, which is consistent with the results of the present study that show *ZmGRAS46* delayed plant flowering and altered plant phenotypes (Figures 1 and 2), which is inconsistent with the above studies, although the present study did not investigate as to whether *ZmGRAS46* responded to abiotic stresses. In the study on upland cotton, *GhGRAS55* of the GRAS family was found to affect plant germination and promote early plant maturation [34]. For the GRAS family, the effect of early and late plant maturity in this study is consistent with the function of this study. However, inconsistently, *ZmGRAS46* did not affect plant germination.

Many members of the GRAS family regulate flowering through the GA pathway, and in studies on kale, it was found that *BraRGL1* repressed the transcriptional activation of the *BraLFY* gene with *BraSOC1*. However, the presence of GA3 enhanced the activation of *BraSOC1*, suggesting that the BraRGL1-BraSOC1 module regulates mossing and flowering in cabbage through GA signaling [35]. Plants overexpressing *SlGARS7* (SlGRAS7-OE) in tomato exhibited various phenotypes associated with many behaviors, including plant height, root and shoot length, and flowering time. It was observed that many genes related to growth hormone and gibberellin (GA) were down-regulated, and sensitivity to GA3/IAA was altered in SlGRAS7-OE seedlings [36]. The overexpression of a tomato miR171 target gene SlGRAS24 impacts multiple agronomical traits via regulating gibberellin and auxin homeostasis [37]. Consistent with these, this study found that *ZmGRAS46* depends on

the GA signaling pathway to regulate flowering (Figure 5). This study did not investigate other hormonal pathways, such as growth hormone, which provides a new direction for subsequent research.

CONSTANS (CO), a zinc fiber protein, is a crucial regulator for plant flowering, and light signaling has a stabilizing effect on CO proteins, which play a central role in regulating photoperiodic flowering [38,39]. In this study, the expression of flowering-related genes was determined, and the overexpression of *ZmGRAS46* suppressed *CO* expression and reduced *CO* expression in *Arabidopsis* (Figure 6). This suggests *ZmGRAS46* may mediate the photoperiodic pathway to regulate flowering in *Arabidopsis*, but the role of *ZmGRAS46* in the photoperiodic pathway regulation of flowering was not investigated in this study. In follow-up experiments, we will change the photoperiod and perform different light treatments to investigate whether ZmGRAS46 mediates the photoperiodic pathway to regulate plant flowering. Li et al. showed that *SOC1*- and *FT*-overexpressed repressors integrate the gibberellin (GA) signaling pathway and the *FLOWERING LOCUS C (FLC)*-mediated vernalization pathway in regulating flowering time. In this study, the overexpression of *ZmGRAS46* altered the expression of *SOC1* and *FT* but not *FLC*, and follow-up experiments are needed to investigate whether *ZmGRAS46* mediates the vernalization pathway [40].

Crops need to have high yields. Gautam et al. showed that early flowering mutants were obtained by the mutagenesis of rice with gamma rays. EMS can affect rice yield, suggesting that crops' early maturity is of some research significance [41]. Wheat yield is affected by environmental, management, and genotypic factors. To cope with different climatic changes, farmers must have different varieties, e.g., early, mid–late, and late flowering varieties, and sow according to seasonal conditions [42,43]. The publication of related studies indicates the importance of research on the effect of flowering time on yield. Li et al. showed that MADS-box transcription factors regulate fruit ripening, that *SOC1* in the MADS-box mediates plant flowering, and that this family also has a role in influencing yield [25,44]. The K-domain of a blueberry's *SUPPRESSOR of CONSTITUTIVE EXPRESSION OF CONSTANS 1 (VcSOC1K)* has similarities to five MADS-box genes in maize. The constitutive expression of *VcSOC1K* has been demonstrated to be very effective in improving maize grain yield [45]. The expression of the MADS-box transcription factor gene, *Zmm28*, resulted in increased vegetative growth, photosynthetic capacity, and nitrogen utilization in maize plants, and these positive changes led to a significant increase in grain yields [46]. Since the MADS-box family impacts plant yield, ZmMADS62, a family member, was chosen as the prey vector in this study. The experimental results showed that ZmGRAS46 could interact with ZmMADS62 (Figure 7). However, it was not investigated whether the ZmGRAS46-ZmMADS62 module regulated yield, and the effect of ZmGRAS46 on yield will be investigated in subsequent experiments.

5. Conclusions

In this study, we showed that *ZmGRAS46* negatively regulated flowering in Arabidopsis thaliana by inhibiting the expression of *CO*, *FT*, *SOC1*, and *LFY*. The overexpression of *ZmGRAS46* affected the plant's phenotype, which promoted rosette leaf growth, facilitated bud formation, and inhibited the elongation of the main stem. The application of GA3 rescued the delayed flowering phenotype of *Arabidopsis*. It promoted the elongation of flowering shoots, demonstrating that *ZmGRAS46* regulates flowering in *Arabidopsis* by mediating the GA pathway. In recent years, global warming and the increase in temperature have changed the flowering period of plants, affecting their growth and development, and it has become necessary to study their flowering period. This study elucidated the mechanism by which *ZmGRAS46* is mediated in the GA pathway to regulate plant flowering, and it provided a theoretical basis for solving the problem of early and late flowering.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010155/s1, Figure S1: Toxicity validation of pGBKT7-ZmGRAS46 prey vector; Figure S2: Validation of pGBKT7-ZmGRAS46 prey vector self-activation;

Figure S3: Analysis of relative expression of *ZmGRAS46* in eight T3 generation positive overexpressing *Arabidopsis thaliana* plants; Table S1: All primer sequences used in this study.

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