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ZmD11 Gene Regulates Tobacco Plant Floral Development under Drought Stress

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Abstract: Maize is most sensitive to drought stress at the floral stage by reducing tassel and silk quality, and thus improving drought tolerance at this stage may help preserve yield. It has been reported that BRs (brassinosteroids) promote floral development under drought stress. However, the function of the brassinosteroid biosynthesis gene *ZmDWARF11* (*ZmD11*) on floral growth under drought stress has not been elucidated. This study found that under normal growth conditions, the heterologous over-expression of ZmD11 significantly enhanced both the vegetative growth and floral development of tobacco. Under drought stress, overexpressing ZmD11 reduced stress-induced tobacco flower size reduction, while it did not affect vegetative growth. After drought treatment, the activities of protective enzymes, including CAT (Catalase), SOD (Superoxide Dismutase), and POD (Peroxidase), were higher, while the content of MDA (Malondialdehyde) was lower in ZmD11 over-expression tobacco lines than that in the wild type control. The relative expression of dehydrin-related genes *NtLeat5* and *NtERD10* was increased in *ZmD11* over-expression tobacco lines compared to that in the control. In summary, we reported that ZmD11 plays a role in tobacco floral development under drought stress. Our data are valuable in understanding the functions of BRs in regulating plant floral development under drought stress.

Keywords: maize; ZmD11; brassinosteroids; floral development

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1. Introduction

Maize is vulnerable to drought stress throughout its entire growing period [1,2], but it is most sensitive to drought stress at the floral stage [3]. The cross-pollinated characteristics of maize mean that it is at great risk during tassel flowering and silk spinning time, which is critical to ensuring satisfactory pollination. If maize is under drought stress, the time of maize silk spinning is significantly delayed, affecting pollination and kernel yield [4]. The floral development of maize is now at increased risk due to global warming and drought, leading to the floral stage of maize becoming the bottleneck of maize yield improvement worldwide [5–8].

BRs have been reported to play prominent roles in the trade-off between plant growth and stress resistance [9]. There are two main pathways involved in the trade-off [10]. First, BRs and ABA (abscisic acid) act antagonistically through BIN2 (BRASSINOSTEROID INSENSITIVE 2) and BZR1/BES1 (BRASSINAZOLE RESISTANT 1/BRI1 EMS SUPPRESSOR 1) [11,12]. BIN2 activates the ABA response, phosphorylating SnRK2 (SNF1-related protein kinases 2), promoting the phosphorylation of ABI5 (ABSCISIC ACID-INSENSITIVE 5), while BZR1/BES1 inhibits the ABA response by blocking the synthesis of ABI5. BIN2 also phosphorylates BZR1/BES1 to promote the degradation of BZR1/BES1. Second, through RD26 (RESPONSIVE TO DESICCATION 26) and BES1, BRs and drought

stress play an antagonistic regulatory role. RD26 regulates the transcription of *BES1* downstream genes and inhibits the function of BRs, while BES1 inhibits RD26, reducing drought stress response [13]. In addition, SA and ABA are also important hormones in responding to drought stress [14].

There are several parameters to test plant drought stress response, such as CAT (Catalase) [15], SOD (Superoxide Dismutase) [16], POD (Peroxidase) [17], and MDA (Malondialdehyde) [18]. CAT, SOD, and POD are antioxidant enzymes; their activity increased due to drought stress. MDA is a lipid peroxidation marker; plants with lower amounts of MDA under drought conditions are generally considered to be more tolerant to drought. Dehydrin gens encode a family of proteins that are important in responding to drought stress [19]. Drought stress normally induces the accumulation of Late Embryogenesis Abundant (LEA) and Early Response to Dehydration (ERD) proteins in plant tissues. Thus, the transcription level of dehydrin genes is another valuable parameter of plant drought response [20].

Dwarf11 is a key enzyme for the biosynthesis of BRs and is functionally conserved in maize and rice [21,22]. Mutants of *dwarf11* show dwarf plant architecture and reduced kernel size, which is restored by exogenous BRs treatment [22]. The panicle-specific overexpression of OsD11 increased the number of spikelets whilst promoting grain volume and weight [21]. The heterologous expression of ZmD11 has been shown to restore the phenotype of the rice *osdwarf11* mutant and increase kernel length and 100-kernel weight [22]. Knocking down *OsD11* or applying BRs biosynthesis inhibitors to young panicles significantly reduce the content of BRs and the expression levels of the inflorescence meristem genes *OsAPO2* and *OsTAW1*, leading to obstruction of rice spikelet differentiation [23]. Under moderate drought stress, OsD11 increased the content of BRs and enhanced the growth of inflorescence meristem [23], indicating a potential role of OsD11 in regulating floral development under drought stress.

Our group previously generated a maize large grain mutant *tc19* and reported that the content of BRs and the expression of the BRs biosynthesis gene *ZmD11* both increased in *tc19* [24], indicating that there might be a role of *ZmD11* in regulating maize floral development. However, the function of *ZmD11* in maize floral development under drought conditions is still unclear. Here, we found that the heterologous expression of *ZmD11* promotes the size of floral organs in tobacco under drought stress.

2. Materials and Methods

2.1. Plant Materials and Growth

Maize inbred line Chang7-2 and tobacco (*Nicotiana benthamiana*) seeds were collected from the Laboratory of Qingdao Agricultural University Maize Molecular Breeding. Maize and tobacco seedlings were both grown in the Qingdao Agricultural University greenhouse (temperature at about 23 °C to 26 °C, humidity at about 50% to 80%, and light intensity at about 18–25 cd). Maize was grown in 6.5 cm × 6.5 cm growing pots; maize seedlings at the V3 stage were used for BR treatment. All the tobacco seedlings were grown on plates with MS medium for 7 days. Three tobacco seedlings were transferred to new plates with MS medium facing root length analysis. The other tobacco seedlings were transferred to 6.5 cm × 6.5 cm growing pots growing for another 30 days facing root fresh weight and shoot fresh weight analysis. Tobacco drought stress was carried out at both the one-month-old seedling stage and floral stage. Tobacco plants in the control were irrigated every 3 days with 50 mL of water. Tobacco drought stress was imposed by stopping irrigating for 7 days and 15 days, respectively.

2.2. Gene Clone and Transformation

ZmD11 gene clone was performed by using Takara PrimerSTAR Max DNA Polymerase (R045Q). The primers for gene clone are listed in Supplementary Table S1.

After PCR and electrophoresis, DNA gels were purified by using the Vazyme FastPure Gel DNA Extraction Mini Kit (DC301-01). After DNA electrophoresis, the gel containing

the DNA fragment of interest was cut under UV light and put in a tube. An equal volume of Buffer GDP was added to the tube. This was incubated at 50~55 °C for 7–10 min. Brief centrifugation occurred. We transferred no more than 700 µL of sol solution to the adsorption column in a 2 mL collection tube and centrifuged at 12,000 rpm for 30 to 60 s. We discarded the filtrate and placed the adsorption column in the collection tube. We added 300 µL of Buffer GDP to the adsorption column and incubated for 1 min. Centrifuge occurred at 12,000 rpm for 60 s. We discarded the filtrate and placed the adsorption column in the collection tube. We added 700 µL of Buffer GW to the adsorption column. Centrifuge occurred at 12,000 rpm for 60 s. We discarded the filtrate and placed the adsorption column back into the collection tube. Centrifuge occurred at 12,000 rpm for 2 min. We placed the adsorption column in a 1.5 mL sterilized centrifuge tube, added 20–30 µL of Elution Buffer to the center of the adsorption column, and incubated for 2 min. Centrifuge occurred at 12,000 rpm for 1 min. We discarded the adsorption column and stored the DNA at −20 °C.

The purified ZmD11 DNA fragments were cloned into pMD19T using the Takara pMD™19-T Vector Cloning Kit (6013). We mixed 1 µL pMD19-T vector, 1 µL control insert, and 1 µL sterilized water. We added 5 µL of Solution I. Reaction occurred at 16 °C for 30 min. We added the whole amount (10 µL) to 100 µL of JM109 competent cells and left them on ice for 30 min. We heated at 42 °C for 45 s and placed in ice for an additional 1 min. We added 890 µL of SOC medium and incubated for 60 min at 37 °C with shaking. Incubation occurred on L-agar plate medium containing X-Gal, IPTG, and Amp to form a single colony. We counted the white and blue colonies. The pMD19T plasmid was transformed into DH5α chemically competent cells, which were sprayed on LB medium with ampicillin and incubated overnight at 37 °C. The positive clones were screened by colony PCR and Sanger sequencing, propagated by using LB medium with ampicillin, and purified by using the Vazyme FastPure Plasmid Mini Kit (DC201-01).

The ZmD11 DNA sequences were then cloned into pCAMBIA1300 by using Takara *Xba* I (1093S) and Takara *Bam*H I (1010S) restriction enzymes. Clonal screening, propagation, and purification were performed by using the same methods as that of pMD19T. The positive clones were transformed into LBA4404 *Agrobacterium* chemically competent cells and then transformed into tobacco using the leaf disk method. The transgenic tobacco plants were analyzed by PCR. The *ZmD11* over-expressed tobacco transgenic lines were indicated as OX lines. The primers for the PCR analysis are listed in Supplementary Table S1.

2.3. Relative Water Content

Tobacco leaves with 0.5 g sample fresh weight (Wf) were soaked in 0.5 g distilled water for 8 h to obtain fully turgid weight (Wt). Then, the leaves were dried in an oven at 75 °C for about 24 h to determine dry weight (Wd). The relative water content (RWC) was calculated as follows: RWC (%) = (Wf − Wd)/(Wt − Wd) × 100% [25].

2.4. Enzyme Activity

The activity of Micro Superoxide Dismutase (SOD), Peroxidase (POD), Malondialdehyde (MDA), Catalase (CAT), and proline (Pro) was tested using the kits bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

The kits were the SOD Assay Kit (BC0175). We weighed about 0.1 g of tissue, added 1 mL of extract, and homogenized in an ice bath. Centrifuge occurred at 8000× *g* at 4 °C for 10 min, when we then took the supernatant and put it on ice. The spectrophotometer was warmed up for more than 30 min; the wavelength was adjusted to 560 nm. Reagents 1, 2, and 4 were bathed at 25 °C for more than 5 min. We added the samples and reagent 3. After mixing well and standing at room temperature for 30 min, the absorbance value A of each tube was measured at 560 nm. We calculated ΔA. We calculated the percentage of inhibition and SOD enzyme activity. SOD activity (U/g fresh weight) = 11.4 percent inhibition/(1 − percent inhibition) × sample dilution factor.

We used the POD detection kit (BC0095). We weighed approximately 0.1 g of tissue, added 1 mL of extract, and homogenized in an ice bath. Centrifuge occurred at 1000× *g*

at 4 °C for 10 min, when we then took the supernatant and put it on ice for testing. We preheated the spectrophotometer or microplate reader for more than 30 min and adjusted the wavelength to 470 nm. Reagents 1, 2, and 3 were left at 25 °C for more than 10 min. We added the above reagents to the tube, mixed immediately, transferred 200 μL to a microglass cuvette, and recorded the absorbance value A_1 at 470 nm for 30 s and the absorbance value A_2 after 90 s. $\Delta A = A_2 - A_1$. POD (U/g fresh weight) = 4900ΔA/Sample mass (g/mL).

We used the MDA content detection kit (BC0025). We weighed about 0.1 g of tissue, added 1 mL of extract, and homogenized in an ice bath. Centrifuge occurred at 8000× *g* at 4 °C for 10 min, when we then took the supernatant and put it on ice for testing. We pipetted 0.3 mL of reagent into a 1.5 mL centrifuge tube, added 0.1 mL of sample, and mixed well. This was kept warm in a water bath at 95 °C for 30 min, and then it was placed in an ice bath and cooled, and centrifuge occurred for 10 min at room temperature. We pipetted 200 μL of the supernatant into a microquartz cuvette and measured the absorbance at 532 nm and 600 nm, denoted as A_{532} and A_{600}, $\Delta A = A_{532} - A_{600}$. This was calculated according to the fresh weight of the sample. MDA content (nmol/g fresh weight) = 25.8ΔA/W.

We used the CAT Assay Kit (BC0200). We weighed about 0.1 g of tissue, added 1 mL of extract, and homogenized in an ice bath. Centrifuge occurred at 8000× *g* at 4 °C for 10 min, where we then took the supernatant and placed it on ice for testing. The spectrophotometer was warmed up for more than 30 min, the wavelength was adjusted at 240 nm, and the distilled water was zeroed. The configuration of the CAT detection working solution occurred as follows: 20 mL of reagent 1 is added to each bottle of reagent 2 (100 μL) and mixed well as a working solution; reagents that are not used up are stored at 4 °C for one week. The CAT assay solution was tested in a water bath at 37 °C (mammals) or 25 °C (other species) for 10 min prior to the assay. A total of 1 mL CAT detection solution was placed in a 1 mL quartz cuvette, where 35 μL of sample was then added and mixed for 5 s. The initial absorbance value A1 at 240 nm and the absorbance value A2 after 1 min are determined immediately at room temperature. Calculate $\Delta A = A1 - A2$. CAT (U/g fresh weight) = 678ΔA/W.

The proline content determination kit was used (BC0295). We weighed about 0.1 g of tissue, added 1 mL of extract solution for ice bath homogenization, and then put boiling water in the bath; we shook the extract for 10 min, centrifuged at room temperature for 10 min, took the supernatant, and then cooled it down. The spectrophotometer was warmed up for more than 30 min; the wavelength was adjusted to 520 nm. We took 0.5 mL of supernatant, 0.5 mL of reagent 1, and 0.5 mL of reagent 2 in a covered test tube, incubated in a boiling water bath for 30 min, and shook once every 10 min. After cooling, we added 1 mL of reagent 3 to the test tube, shook for 30 s, and let stand for a while to transfer the pigment to reagent 3. We pipetted 1 mL of the upper solution into a 1 mL glass cuvette, allowed colorimetry to occur at a wavelength of 520 nm, and recorded the absorbance value. Based on the absorbance value and concentration of the standard, we established a standard curve. The proline content of the sample was calculated by the standard curve (y is the proline content, μg/mL; x is the OD value).

2.5. BR, SA, and ABA Content Determination

The BR, SA, and ABA content determination was performed by Sangon Biotech Shanghai Co., Ltd. (Shanghai, China) using kits. The kits were BR ELISA kit (KT4963-48T), SA ELISA kit (KT7911-48T), and ABA ELISA kit (KT4924-48T), respectively. In general, blank wells, standard wells, and sample wells were set up, respectively. We dispensed 50 μL of the standard on the microchip-coated plate, 40 μL of sample diluent, followed by 10 μL of the sample to be tested (final sample dilution is 5-fold). We sealed the plate with a sealing film and incubated at 37 °C for 30 min. We diluted the concentrated washing solution 30× with distilled water and set it aside. We carefully removed the sealing film, discarded the liquid, spun dry, filled each well with washing solution, let it stand for 30 s and then discarded it, repeated this 5 times, and then pat it dry. We added 50 μL of enzyme labeling reagent per well, except for blank wells. We incubated and washed it again. Then, we added 50 μL

of chromogenic agent A and then added 50 µL of chromogenic agent B, gently shook and mixed, and incubated in the dark at 37 °C for 10 min. We added 50 µL of stop solution per well. The OD value of each well was measured on a wavelength of 450 nm.

2.6. BR Treatment

24-Epibrassinolide (IE0110) was bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). In total, 10 µM 24-Epibrassinolide was used for the exogenous treatment [22,26]. A total of 1 mg 24-Epibrassinolide was dissolved in 2.08 mL sterilized water to prepare the stock solutions; the stock solutions were diluted to 10 µM to make working solutions. Maize plant at the V3 stage was used for the 24-Epibrassinolide treatments. To make sure that the plants were evenly treated with BR, the treatments were repeated three times; 1 mL working solution was used each time. Maize plant leaves were collected at 0, 1, 3, 6, 9, and 12 h after the 24-Epibrassinolide treatment for RNA isolation.

2.7. RNA Extraction, Reverse Transcription and qRT-PCR

The maize and tobacco leaves were collected for RNA isolation. RNA was extracted by using the standard Trizol method. cDNA was generated by using Promega Eastep® RT Master Mix (LS2052). In general, we mixed the gDNA Remover with 10× DNase I buffer formulated into a 5× *g* DNA Remover Master Mix. After gentle mixing of the reaction solution, a 2 min incubation at 37 °C adequately removed gDNA residue from the RNA sample. A total of 1 µg RNA was used for reverse transcription. The mixture was incubated at 37 °C for 15 min and then incubated at 85 °C for 5 s. TB Green® Fast qPCR Mix (RR430S) was used for the qRT-PCR. We used the Applied Biosystems 7500 Fast Real-Time PCR System. We used the following factors: 95 °C for 30 s, 1 cycle; 95 °C for 3 s, 60 °C for 12 s, 40 cycles. Primers are listed in Supplementary Table S1.

2.8. Statistical Analysis

Data were analyzed using Microsoft Office 2007 and DPS 17.10. Bar charts were drawn by using GraphPad Prism 8.

3. Results

3.1. The Expression of ZmD11

It has been reported that highly concentrated exogenous BR represses plant growth through the negative feedback on BRs biosynthesis [26,27]. To find whether highly concentrated exogenous BRs represses the activity of brassinosteroid biosynthesis gene *ZmD11*, maize seedlings were treated with highly concentrated exogenous BRs, and the transcriptional level of *ZmD11* was tested. The results demonstrated that the expression of *ZmD11* reduced after one hour of BRs treatment and stayed low over several hours (Figure 1A), indicating that the expression of *ZmD11* is negatively regulated by highly concentrated BRs.

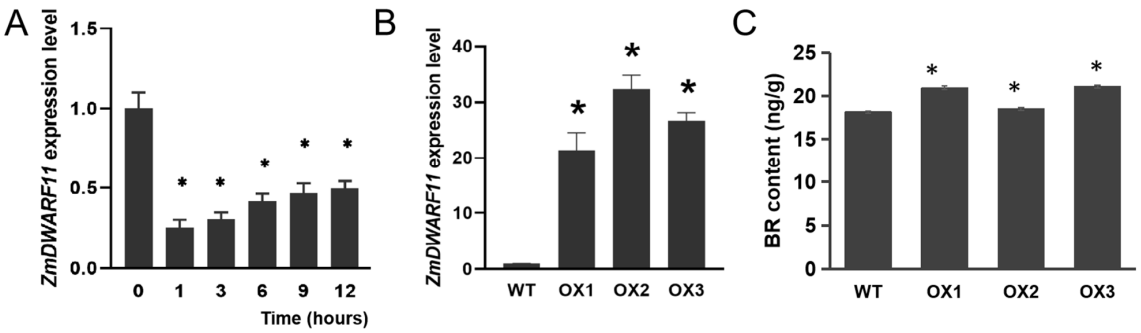

Figure 1. The expression of *ZmD11* and endogenous BR content. (**A**) The expression of *ZmD11* is inhibited by high concentrations of exogenous BRs. (**B**) The expression level *ZmD11* in over-expression lines. (**C**) The endogenous BR content in transgenic lines. WT indicates wild type; OX indicates *ZmD11* over-expression of transgenic lines. At least three biological replicates were used. * $p < 0.05$.

To study the biological function of ZmD11 in regulating plant growth, an experiment was performed to construct the *ZmD11* gene over-expression vector and transform it into tobacco. It was found that the expression of *ZmD11* is more than 20 times higher in transgenic lines than in the wild type control (Figure 1B).

Then, to test whether the endogenous BRs content has been affected in transgenic lines, we measured the endogenous BRs content. It was found that the endogenous BRs content in the wild type control was about 18 ng/g, while in transgenic lines, it was an average of 20 ng/g (Figure 1C). The endogenous BRs content has been increased by about 11% in transgenic lines. The increased endogenous BRs content is consistent with the over-expression of *ZmD11*.

3.2. Over-expression of ZmD11 Increased Vegetative and Floral Organ Size in Tobacco

To find out how ZmD11 affects the growth of tobacco, a morphological analysis, including root length, root fresh weight, and shoot fresh weight, was conducted using the transgenic tobacco lines. We found that under normal growth conditions, root length in transgenic lines was longer than that of the wild type (Figure 2A,B). In addition, the root fresh weight and shoot fresh weight of transgenic plants were bigger than those of the wild type (Figure 2C–E). This indicates that the over-expression of *ZmD11* increases plant tissues size in tobacco.

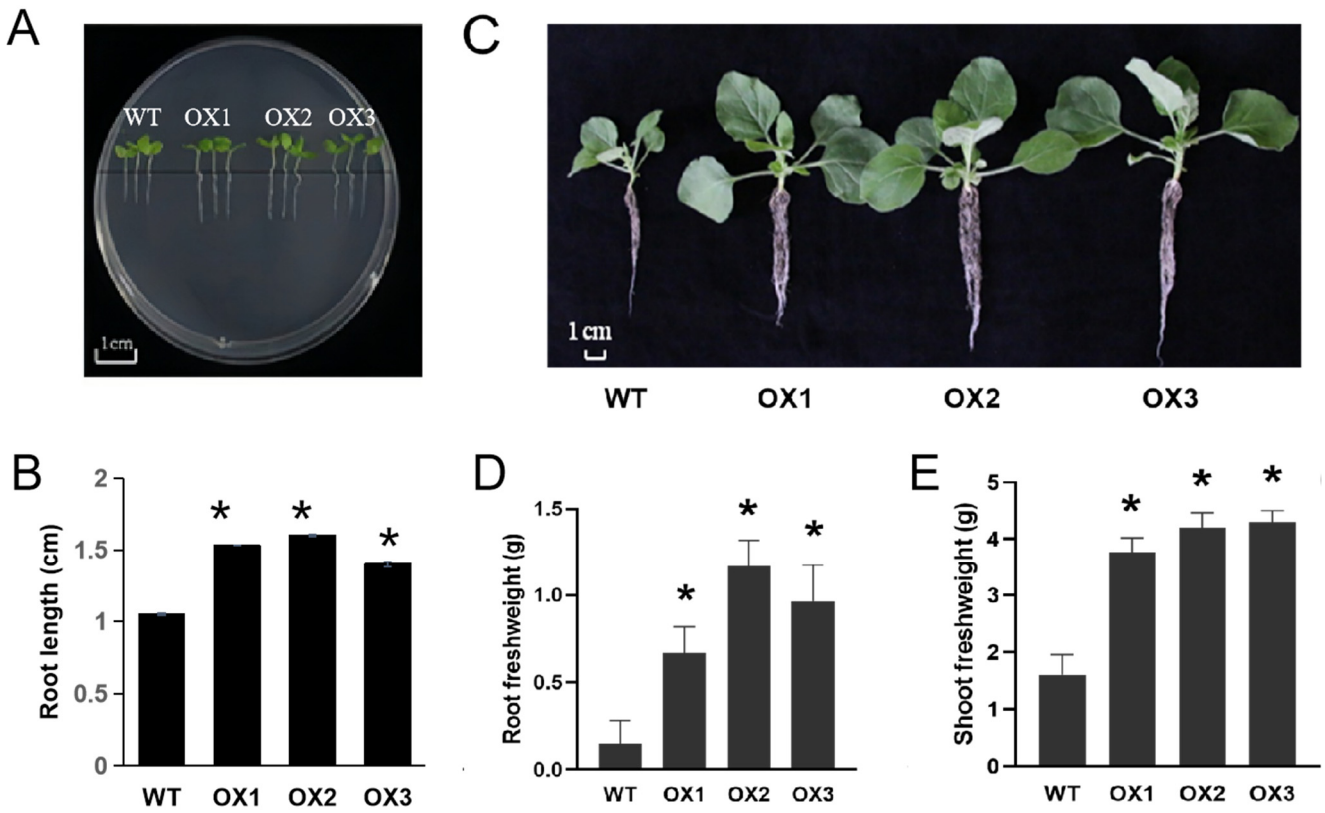

Figure 2. Over-expression of ZmD11 promotes seedling growth. (**A**) Photos of tobacco root after 7 days of growth. (**B**) Root length after 37 days of growth. (**C**) Photos of tobacco root and shoot after days of growth. (**D**) Root fresh weight after days of growth. (**E**) Shoot fresh weight after days of growth. WT indicates wild type; OX indicates *ZmD11* over-expression of transgenic lines. At least three biological replicates were used. * $p < 0.05$.

To discover whether the over-expression of ZmD11 affected the growth of floral organs, we measured the weight of the floral organ. We found that the floral organ weight in the wild type was about 0.13 g, while it was about 0.15 g in transgenic lines (Figure 3A,B). This indicates that the over-expression of ZmD11 enhanced the growth of floral organs.

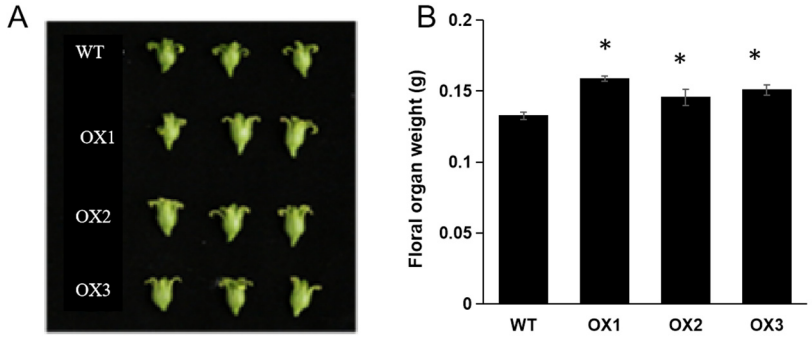

Figure 3. Over-expression of ZmD11 promotes tobacco floral development. (**A**) Photos of flower after 52 days of growth. (**B**) Floral organ weight. WT indicates wild type; OX indicates ZmD11 over-expression of transgenic lines. Three replicates were used. * $p < 0.05$.

3.3. Over-Expression of ZmD11 Increases Floral Size under Drought Stress

A series of experiments were conducted to determine how the BRs content is linked to drought stress responses. Transgenic tobacco lines and wild type lines were tested under the stress influence at both seedling and floral stages to determine if ZmD11 can regulate plant growth under drought stress.

It was observed that during the seedling stage, after drought stress treatment, the leaves became curly and yellow in both transgenic lines and wild type (Figure 4A). It was therefore concluded that there is no difference between the transgenic lines and wild type. At the floral stage, the inflorescence development was inhibited in both the wild type and transgenic lines. The petals were shorter and the stamens were invisible in wild type, while the inflorescence size in transgenic plants was relatively bigger (Figure 4A). It is therefore proposed that the response to drought stress might be different at floral stages between transgenic lines and the wild type control.

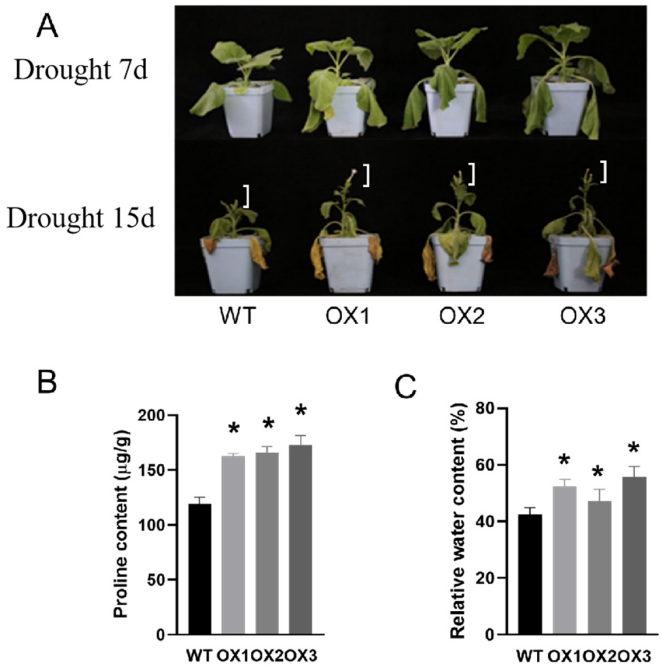

Figure 4. The activity of protective enzymes and the transcription level of dehydrin genes. (**A**) Photos of floral organs during drought stress. (**B**) Proline content in tobacco leaves. (**C**) Relative water content in tobacco leaves. Drought 7 d is the 7th day of drought stress treatment, and Drought 15 d is the 15th day of drought stress treatment. WT indicates wild type; OX indicates ZmD11 over-expression of transgenic lines. Three replicates were used. * $p < 0.05$.

We further tested the proline content and relative water content in leaves. We found that the proline content was about 120 μg/g in the leaves of the wild type, while it was more than 160 μg/g in transgenic lines (Figure 4B). The relative water content was about 40% in the leaves of the wild type, while it was about more than 50% in the leaves of transgenic lines (Figure 4C). This indicates that the over-expression of ZmD11 increases plant drought stress resistance at the floral stage.

3.4. The Activity of Protective Enzymes and the Expression of Drought-Tolerant-Related Genes Are Changed

In order to reveal the molecular mechanism involved in ZmD11 regulating tobacco drought stress during the floral stage, we tested the activity of protective enzymes and the expression of drought-tolerant-related genes (Figure 5). It was found that, after 7 days of drought treatment, the activity of CAT was about 500 U/g in WT, while it was about 700 U/g in transgenic lines. After 15 days of drought treatment, the activity of CAT was about 600 U/g in WT, while it was about 1000 U/g in transgenic lines (Figure 5A). This indicates that the activity of the protective enzymes in transgenic lines is much higher than that in WT. Similarly, the activities of SOD in transgenic lines were higher than those in WT after both 3 days and 7 days of induced drought stress treatment (Figure 5B). Although there was no difference after 3 days of drought treatment, the activity of POD was higher after 7 days of drought treatment in transgenic lines than in WT (Figure 5C). In contrast to POD, the content of MDA was about 9 nmol/g in WT but about 7 nmol/g in transgenic lines, and this decreased by about 22% in terms of the ZmD11 over-expression of transgenic lines than that in WT (Figure 5D).

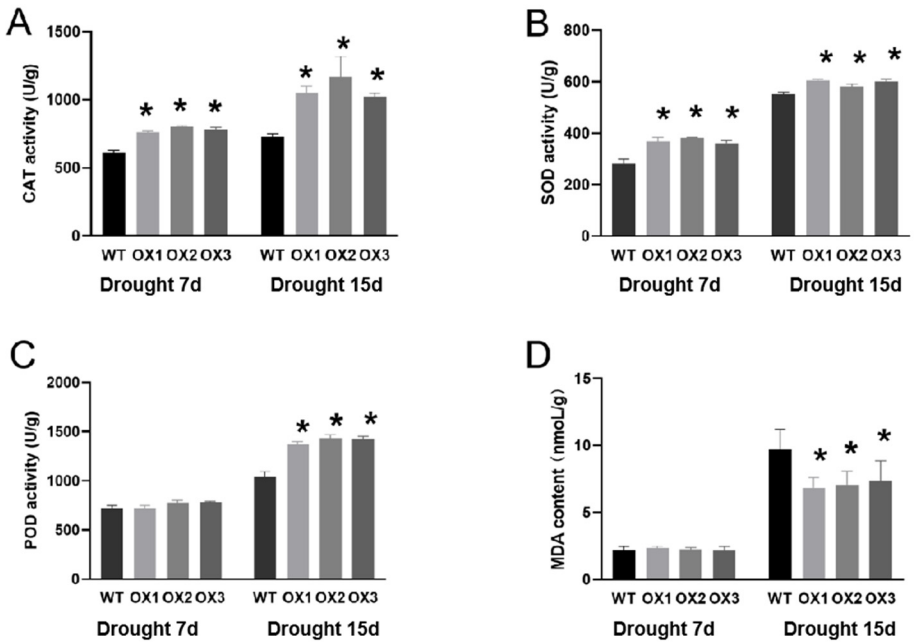

Figure 5. The activity or content of protective enzymes. (**A**) CAT activity, (**B**) SOD Activity, (**C**) POD activity, (**D**) MDA content. Drought 7 d is the 7th day of drought stress treatment, and Drought 15 d is the 15th day of drought stress treatment. WT indicates wild type; OX indicates ZmD11 over-expression of transgenic lines. Three replicates were used. * $p < 0.05$.

Dehydrin genes are important signals in response to drought stress [19]. The transcriptional levels of *NtLEA5* in transgenic lines are about five times higher than in WT after 7 days of drought treatment and are about 10 times higher than in WT after 15 days of drought treatment (Figure 6A). Consistently, the expression levels of *NtERD10* were also increased in transgenic lines after both 3 days and 7 days of drought treatment (Figure 6B). As *Lea5* and *ERD10* are highly similar to *NtLea5* and *NtERD10*, we propose that ZmD11 is

further investigated to improve drought tolerance during the floral stage through regulating the expression of dehydrin genes.

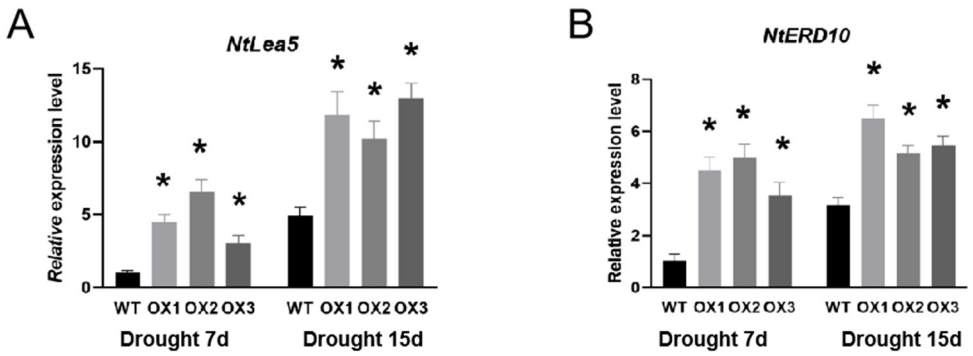

Figure 6. The transcription level of dehydrin genes. (**A**) The relative expression of tobacco dehydrin gene *NtLeat5*. (**B**) The relative expression of tobacco dehydrin gene *NtERD10*. Drought 7 d is the 7th day of drought stress treatment, and Drought 15 d is the 15th day of drought stress treatment. WT indicates wild type; OX indicates ZmD11 over-expression of transgenic tobacco lines. Three replicates were used. * $p < 0.05$.

3.5. The Content of Hormones Have Been Affected by ZmD11

To understand how the hormones content has been affected in transgenic tobacco, we tested the content of BR, SA, and ABA. We found that the content of BR was about 20 ng/g in the wild type tobacco and was about 22 ng/g in the ZmD11 over-expression tobacco lines, showing an increase of about 10% (Figure 7A). The content of SA was about 120 pmol/g in the wild type tobacco, and it was increased in the ZmD11 over-expression tobacco lines (Figure 7B). The content of ABA was about 51 μg/g in the wild type tobacco, and it was also increased in the ZmD11 over-expression tobacco lines (Figure 7C).

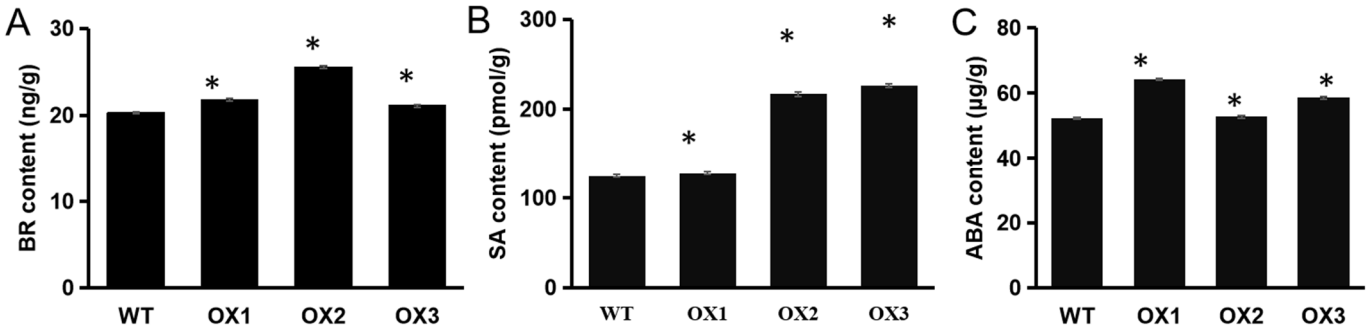

Figure 7. Hormone content in tobacco. (**A**) The content of BR. (**B**) The content of SA. (**C**) The content of ABA. WT indicates wild type; OX indicates over-expression of transgenic tobacco lines. Three biological replicates were used. * $p < 0.05$.

4. Discussion

A large number of experiments have been conducted to understand the relationship between BRs and plant drought stress at the seedling stage, but only a few have been performed at the floral stage [28–31]. Studies found that suitable concentrated exogenous BRs can improve maize drought stress resistance [32]. However, it seems that improving the activity of BRs biosynthesis enzymes cannot always improve seedling stage drought stress resistance. For example, under maize seedling stage drought stress, there is no clear difference between ZmD11 over-expression maize transgenic lines and the wide type control [22]. We analyzed the tobacco seedling root and shoot using ZmD11 over-expression tobacco transgenic lines. Consistent with the previous study, we did not observe a clear difference in the morphological traits of tobacco during the seedling stage. This might

be because changing the endogenous BR biosynthesis enzyme activity is not enough to improve drought resistance at the seeding stage.

Maize and other crops are most sensitive to drought stress at the floral stage [32]. Improving drought stress resistance at the maize floral stage is critical for maize yield [33]. Interestingly, at the floral stage of drought stress, it was found that the heterologous expression of ZmD11 increased tobacco floral size. Another research paper reported that when rice was grown under moderate drought stress, the over-expression of OsD11 enhanced the growth of inflorescence meristem [23]. Our finding that ZmD11 reduces stress-induced flower size reduction is consistent with their results. It might be related with other hormones which are controlling cell growth [34]. The mechanism of why ZmD11 reduces the stress-induced reduction needs to be further studied.

Antioxidant enzymes, such as CAT, SOD, and POD, and the lipid peroxidation marker, such as MDA, are parameters for plant drought stress resistance [15–19]. It was found that the activity of antioxidant enzymes increased in ZmD11 over-expression tobacco transgenic lines, while the MDA content reduced in the same lines. The relative expression of dehydrin genes further demonstrated that ZmD11 over-expression tobacco transgenic lines have higher drought stress resistance [20,30]. Although it is still unclear whether overexpressing ZmD11 has the same function in maize, it is valuable to carry out more research using ZmD11 over-expression maize transgenic lines [21,22,35].

The trade-off between plant growth and stress defense has been deeply studied [10]. It was reported that BRs play critical roles in regulating growth and influencing defense balance [36]. The function of BRs in the plant growth and stress defense trade-off is often related to SA and ABA content. We found that the SA and ABA content is increased in ZmD11 over-expression tobacco transgenic lines, further supporting previous reports. In rice, Zhang et al. (2019) reported that relatively high BRs promote the growth of inflorescence meristem [23]. Under normal conditions, both rice and maize *d11* mutant show dwarf phenotype and reduced kernel size, over-expressing ZmD11 increased kernel yield in maize [22]. This study found that under normal conditions, the heterologous expression of ZmD11 promoted the growth of both shoot and root in tobacco. Since there is a similar phenotype between monocot rice, maize, and eudicot tobacco, our data indicate the highly conserved function of D11 between the monocot and eudicot. Thus, the ZmD11 enzyme's activity in transgenic plants might be considered to enhance plant adaptation to drought, and the knowledge of ZmD11 can be extended to more plants.

5. Conclusions

Under normal growth conditions, the heterologous over-expression of ZmD11 significantly enhanced the vegetative growth of tobacco. Under drought stress, overexpressing ZmD11 reduced drought-stress-induced flower size reduction. After drought treatment, the activities of protective enzymes, including CAT (Catalase), SOD (Superoxide Dismutase), and POD (Peroxidase), were higher, while the content of MDA (Malondialdehyde) was lower in ZmD11 over-expression lines than that in the wild type control. The relative expression of dehydrin-related genes *NtLeat5* and *NtERD10* was increased in ZmD11 over-expression lines compared to that in the control. BR, SA, and ABA were also increased in ZmD11 over-expression lines compared to that in the wild type control.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14071381/s1, Table S1: Primers used in this research.

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