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BnA.JAZ5 Attenuates Drought Tolerance in Rapeseed through Mediation of ABA–JA Crosstalk

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Abstract: Drought stress reduces water availability in plant cells and influences rapeseed yield. Currently, key genetic regulators that contribute to rapeseed response to drought remain largely unexplored, which limits breeding of drought-resistant rapeseed. In this study, we found that *Brassica napus JASMONATE ZIM-DOMAIN 5* (*BnA.JAZ5*), one of the transcriptional repressors functioning in the jasmonate (JA) signaling pathway, was triggered by drought treatment in rapeseed, and drought-susceptibility increased in *BnA.JAZ5*-overexpressing rapeseed plants as compared to wild-type plants, resulting in a lower survival rate after recovery from dehydration. After recovery for 3 days, 22–40% of *p35S::BnA.JAZ5* transgenic plants survived, while approximately 61% of wild-type plants survived. Additionally, seed germination of *BnA.JAZ5*-overexpressing rapeseed was hyposensitive to abscisic acid (ABA). The germination rate of five transgenic lines was 32~42% under 9 μM ABA treatment, while the germination rate of wild-type plants was 14%. We also found that the average stomatal density of five overexpressing lines was 371~446/mm^2, which is higher than that of wild-type (232/mm^2) plants under normal conditions. These results indicate that *BnA.JAZ5* regulated drought response in an ABA-dependent manner, possibly by affecting stomatal density. Interestingly, methyl jasmonate (MeJA) treatment rescued the ABA-hyposensitive seed germination, revealing crosstalk between JAZ5-meidated JA and the ABA signaling pathway. Taken together, our results suggest that *BnA.JAZ5* attenuated drought resistance through the ABA-dependent pathway, which could represent important genetic loci for drought-resistant rapeseed breeding.

Keywords: *BnA.JAZ5*; drought stress; jasmonates; ABA; rapeseed

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1. Introduction

Various environmental stresses affect plant growth and yield. Water availability is the most important abiotic factor contributing to plant evolution [1]. Establishment of seedlings is directly inhibited by drought stress, which reduces plant densities and yields [2]. With the reduction in arable land worldwide, global food production needs to be increased to feed an ever-growing population [3]. Thereby, genetic engineering must be integrated with breeding technologies to develop climate-resilient crops adaptable to environmental changes leading to stress conditions. Rapeseed (*Brassica napus*, AACC, 2n = 36), as an important oilseed crop used for animal fodder and human consumption, is very sensitive to environmental stresses during its growth and reproductive stages [4,5].

Plants have evolved different strategies for protection against drought. A total of 90% of soil water used by plants is lost through transpiration via the opening of stomata [5], and

drought stress causes insufficient uptake of water from soil by roots to meet the requirement of plant transpiration [6,7]. Stomatal density can modulate plant transpiration rates [7,8]. Stomatal density is controlled by both genetic factors and environmental cues [8–10]. Overexpression of *Arabidopsis thaliana GT-2 LIKE 1* (*AtGTL1*), *AtERECTA*, *STOMATAL DENSITY AND DISTRIBUTION1* (*AtSDD1*) and *Oryza sativa stress-induced protein kinase gene 1* (*OsSIK1*) enhances drought tolerance, which is associated with a reduction in stomatal density [7,8,11]. Drought stress induces transcription of dehydration-responsive element-binding proteins (DREBs) and activates genes involved in water movement and chaperone functions, such as late embryogenesis abundant (LEA) proteins [12–15]. RESPONSIVE TO DESICCATION 29A (RD29A), a stress-responsive marker [16], can be used as a control in stress treatments. Overexpression of the gene encoding Δ^1-pyrroline-5-carboxylate synthetase 1 (P5CS1), an enzyme involved in proline biosynthesis, enhances osmotic stress tolerance [17]. Phospholipase C (PLC) works upstream of DREB2 [18].

Jasmonates (JAs) are crucial hormones that regulate plant response to abiotic and biotic stresses [19]. JASMONATE ZIM-DOMAIN (JAZ) proteins are inhibitors of the jasmonic acid signaling pathway [20]. Low jasmonoyl–isoleucine (JA-Ile) levels permit the accumulation of JAZ proteins, which can interact with several bHLH-type transcription factors, including MYC2, MYC3, and MYC4, to activate the transcription of some early JA-responsive genes [21–23]. JAZ proteins can also repress MYC activity to recruit NINJA and TOPLESS [24–26], which participate in JA-Ile perception and induce JAZ degradation [20,27,28]. Some JAZ members interact with other proteins, such as MYB75, that inhibit trichome initiation and anthocyanin accumulation [29]. Some JAZ members play roles in increasing plant tolerance to abiotic stress, while *OsJAZ1* negatively regulates drought resistance by modulating JA and abscisic acid (ABA) signaling in rice [30].

Although JAZ proteins were clearly identified as transcriptional repressors of JA responses in *Arabidopsis* [20,21,31,32], their functions in rapeseed resistance to abiotic stress are unknown. In this study, we explore the function of *BnA.JAZ5*, a rapeseed (*Brassica napus*) homolog of JAZ5, in drought resistance, which could be a potential genetic recourse for stress-resistant plant breeding.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The rapeseed accession K407 was acquired from the Hybrid Rape Research Center of Shaanxi Province [33]. Seeds of K407 and *p35S::BnA.JAZ5* plants were sterilized according to the method of Li et al. (2020) [34] and grown on Murashige and Skoog (MS) plates with 1% sucrose in darkness for 3 days at 4 °C. Then, seedings were transferred to a greenhouse for growth at 22 °C under long-day conditions (16 h light/8 h dark). The seedlings were finally transplanted to Songjiang Farm Station of the Shanghai Institute of Plant Physiology and Ecology, China in early September 2017 and 2018 [35]. To preserve the phenotypic uniformity of individual plants of the wild-type accession, potential aberrant forms were eliminated at several developmental stages.

2.2. Plasmid Construction and Transformation

The coding sequences of *BnA.JAZ5* genes were amplified from cDNA of *B. napus* using appropriate primers (Supplementary Table S1). The sequences were inserted into a chimeric binary vector. We followed the protocol described by Moloney et al. (1989) and Verma et al. (2012) for rapeseed hypocotyl transformation [30,36], and *Agrobacterium tumefaciens strain* GV3101 pMP90RK was selected [34]. *p35S::BnA.JAZ5* was transferred into *B. napus* accession K407, and transgenic plants were selected on kanamycin after *Agrobacterium*-mediated transformation. Positive seedlings (T0) were transplanted to the greenhouse. T1-generation *p35S::BnA.JAZ5* plants were identified by PCR genotyping using transgene-specific oligos and maintained in a greenhouse as described above.

2.3. RNA Extraction and Real-Time PCR

All plant material was sampled and frozen immediately in liquid nitrogen, and total RNA was extracted with TRIzol (Invitrogen). Total RNA was treated with DNase I (Takara, Shanghai, China), and PrimeScript reverse transcriptase (Takara) was used for cDNA synthesis. Real-time PCR was performed in a 303 MyiQ2 two-color real-time PCR detection system (Bio-Rad, Richmond, CA, USA) [35]. At least three biological replicates per gene were carried out. Transcript levels were normalized relative to those of *UBC21* cDNA [37]. PCR amplifications were performed in 20 mL reaction volumes containing SYBR Green PCR Master Mix (Applied Biosystems), cDNA, and the primers (listed in Supplementary Table S1). Quantification of relative expression levels followed a previously reported method [38].

2.4. MeJA and ABA Treatments

The *B. napus* accession K407 and transgenic lines were subjected to MeJA and ABA treatments. Initial ABA and MeJA concentrations were set according to [39,40]. Seeds were sown on solid MS plates containing MeJA (50 μM), ABA (9 μM), or both MeJA (50 μM) and ABA (9 μM) and treated at 4 °C for 4 d before moving to 22 °C for seed germination. Seed germination was recorded with images taken on the third day after the seed was grown at 22 °C. The emergence of radicle was scored as germination. Cotyledon opening and cotyledon expansion were also recorded [41–43]. The experiment was repeated using different batches of seeds, and at least 150 seeds were used per line. At least three biological replicates were carried out. For every biological replicate, we tested the seeds from the same batch at least three times as technical replicates.

2.5. Stress Treatment at Seedling Stage

For drought-stress treatment of seedlings, the seeds were sown in MS solid medium containing 400 mM mannitol, and then seeds were stratified at 4 °C in the dark for 4 d. The number of germinated seeds was recorded after time intervals of 1, 2, 3, 4, and 5 days. Cotyledon opening and cotyledon expansion were also recorded [41–43]. For determination of root length, the sterilized seeds were first sown on half-strength MS medium for 3 days, transplanted to fresh medium containing 400 mM mannitol and grown vertically. At least three biological replicates were carried out, and at least 150 seeds were used per line. For every biological replicate, we tested the seeds from the same batch at least three times as technical replicates.

For PEG treatment, the seedlings grown on 1/2 MS medium for 7 days were moved to fresh medium with 20% PEG-6000. Seedling samples were harvested after 0, 12, and 48 h according to the methods of Verslues et al. [44,45]. At least three biological replicates were carried out, and at least 150 seeds were used per line. For every biological replicate, we tested the seeds from the same batch at least three times as technical replicates

2.6. Dehydration Treatments

For seedling dehydration, the seedlings grown on half-strength MS medium for 7 days were transplanted to the surface of a Petri dish, maintained in a greenhouse at 22 °C under dark conditions, and then collected for an assay of dehydration effects after 0, 2, 6, and 12 h. At least three biological replicates were carried out, and at least 150 seeds were used per line.

For leaf dehydration, leaves were cut from the five-leaf-stage plants, maintaining the same dehydration conditions as seedlings. Leaves were harvested after time intervals of 0, 6, 12, 24, 36, 48, and 60 h [46]. At least three biological replicates were carried out, and at least 30 seeds were used per line.

For the whole plant dehydration treatment, soil was weighed to ensure that there was equal weight of soil in each pot and the same volume of water was poured into each pot. Seven-day-old seedlings were transplanted into the pots and grown until the five-leaf stage. Water was then withheld for 2 weeks. Plant survival rates were recorded based on the

number of plants surviving 3 days after rewatering [47]. At least three biological replicates were carried out, and at least 30 seeds were used per line.

2.7. Measurement of Leaf Stomatal Density

Plants at the five-leaf stage were treated with drought stress (no watering for 7 days). Third leaves were collected, cut into leaf sections (0.5 cm in length), starting from the middle of a leaf, and fixed overnight in FAA at 25 °C. The leaf sections were rinsed three times using distilled water, dehydrated in ethanol series (30%, 50%, 70%, 80%, and 95%), and rinsed three times in 100% ethanol. The dehydrated samples were sputter-coated with gold, and stomatal observation was performed with a Hitachi S-2300 electron microscope. The number of stomata per mm^2 in middle regions of leaves of five-leaf-stage plants was recorded [48]. At least three biological replicates were carried out, and at least 30 seeds were used per line.

2.8. Water Loss Assay

Leaves from plants at the five-leaf stage were detached and immediately weighed. The leaves were then placed on a laboratory bench and weighed according to the schedule (designated as Wi; Wi represents the weight of leaves at time *i*). Fresh weight (FW) loss was calculated according to the initial weight of the detached leaves. The water-loss rate (WLR) was also calculated: WLR = (FW − Wt)/FW. At least three biological replicates were carried out, and at least 20 seeds were used per line.

2.9. Measurement of Relative Water Content

Relative water content (RWC) was measured as described by Kumar et al. [49], with minor modification. Leaves were detached from plants at the five-leaf stage and immediately weighed to record their fresh weight (FW). The detached leaves were placed in distilled water for 12 h, blotted dry, and weighed to record their turgid weight (TW). To determine the dry weight (DW), the turgid leaves were subjected to oven drying at 70 °C for 36 h. RWC was calculated using the following equation: RWC = ((FW − DW) × 100)/(TW − DW). At least three biological replicates were carried out, and at least 20 seeds were used per line.

2.10. Sequence Alignment and Phylogenetic Analysis

Arabidopsis JAZ protein sequences were obtained from the Arabidopsis Information Resource (https://www.arabidopsis.org/ (accessed on 3 November 2021). BnA.JAZ5 and BnC.JAZ5 were selected based on their high similarity with AtJAZ5. Full-length amino-acid sequence multiple alignments were performed in ClustalW. Unrooted phylogenetic trees were constructed from the aligned amino-acid sequences in MEGA 6.0, and bootstrapping was carried out with 1000 iterations [50].

2.11. Statistical Analysis

Student's *t*-tests were used for statistical analyses, and statistical significance was determined with p value < 0.05.

3. Results

3.1. Expression Patterns of JA Signaling Regulator BnA.JAZ5 Revealed Its Potential Function in Rapeseed Response to Drought Stress

JAs are involved in plant development, reproduction, and defense. Within JA signaling cascades, JAZ proteins play a central role. *BnA.JAZ5* and *BnC.JAZ5* are two of the *JAZ5* homoeologous genes present in polyploid rapeseed (Figures 1A and S1). To investigate whether JAZ5 in rapeseed is involved in drought stress response, we checked the expression profiles of *BnA.JAZ5* and *BnC.JAZ5* in 18-day-old rapeseed treated with 20% high-molecular-weight polyethylene glycol (PEG-6000) for 48 h using real-time PCR. We found that *BnA.JAZ5* was strongly induced by 20% PEG-6000, whereas *BnC.JAZ5* was not (Figures 1B and S4E). In addition, the expression level of *BnA.JAZ5* was further increased

under continued dehydration stress (Figures 1C and S4E). Taken together, these results suggest that *BnA.JAZ5* may be involved in plant drought response.

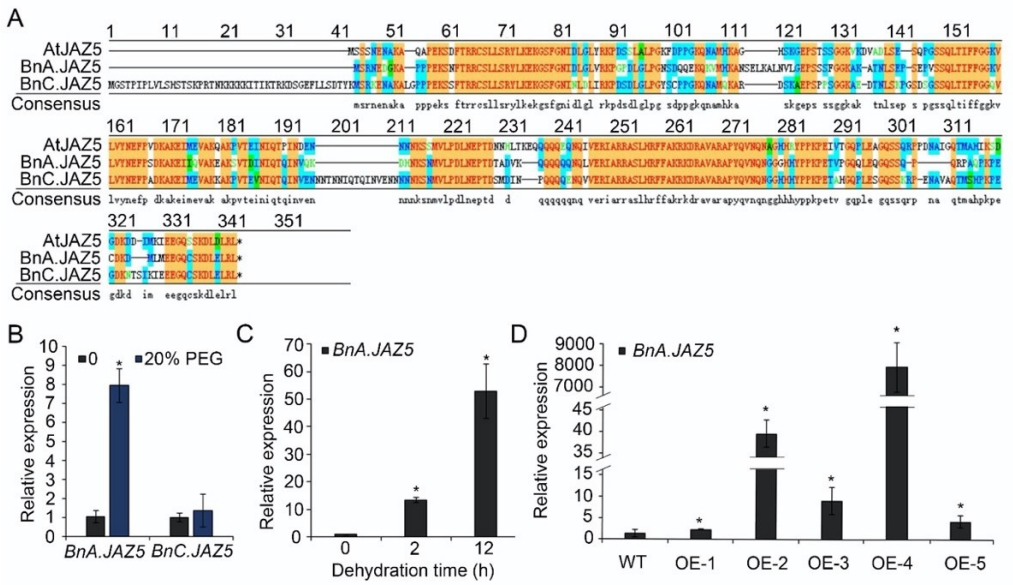

Figure 1. Homologous alignment of AtJAZ5 in rapeseed and expression levels of *BnA.JAZ5* and *BnC.JAZ5*. (**A**) Alignment of the protein sequences of AtJAZ5 in *Arabidopsis* and BnA.JAZ5 and BnC.JAZ5 in *B. napus*. (**B**) Expression levels of *BnA.JAZ5* and *BnC.JAZ5* under 20% PEG-6000. (**C**) Expression levels of *BnA.JAZ5* under dehydration stress. Detached leaves of five-leaf-stage plants were subjected to dehydration treatment for 2 and 12 h. (**D**) The expression levels of *BnA.JAZ5* in *p35S::BnA.JAZ5* plants (OE-1, OE-2, OE-3, OE-4 and OE-5) and wild-type plants. Error bars indicate standard errors. Asterisks indicate significant differences ($p < 0.05$). WT, accession K407, OE-1, OE-2, OE-3, OE-4, OE-5, and *p35S::BnA.JAZ5* lines.

3.2. Overexpression of BnA.JAZ5 Reduced Drought Tolerance

To study the functions of *BnA.JAZ5* in response to drought, we constructed *BnA.JAZ5*-overexpressing transgenic plants by introducing a *p35S::BnA.JAZ5* construct into the K407 cultivar of rapeseed (designated as *p35S::BnA.JAZ5*), which was self-fertilized and further selected to obtain homozygous lines for subsequent experiments. In total, five independent lines (OE-1, OE-2, OE-3 OE-4, and OE-5) were generated, and we found that *BnA.JAZ5* was overexpressed from 3-fold to 7000-fold compared to the wild-type plants (Figure 1D).

To study the tolerance of *p35S::BnA.JAZ5*-overexpressing plants to drought stress, seeds of the transgenic plants and K407 were sown on MS medium containing 400 mM mannitol (Figure 2). Compared with the control (0 mM mannitol), germination rates of all five of these transgenic lines were significantly reduced under 400 mM mannitol (Figures 2A,B and S4A) and exhibited much lower percentages of expanded cotyledons on the fifth day after the seed was sown (Figure 2C). Root elongation of *p35S::BnA.JAZ5* plants was inhibited compared with wild-type plants (Figure 2D,E). These results indicate that *BnA.JAZ5* decreased drought tolerance of *B. napus*.

Compared to the wild-type plants, the leaves of *p35S::BnA.JAZ5* plants rolled and wilted earlier (Figure 3A). Relative water content (RWC) and water-loss rate (WLR) are two indicators of cell and tissue hydration. Therefore, we tested the WLR and found the WLR of *BnA.JAZ5*-overexpressing rapeseed plants was significantly higher than that of wild-type (Figures 3B and S4B). Besides, the RWC of *BnA.JAZ5*-overexpressing rapeseed plants was significantly lower than that of K407 under drought conditions (Figures 3C and S4C). These results suggest that *BnA.JAZ5* played a negative role in drought resistance.

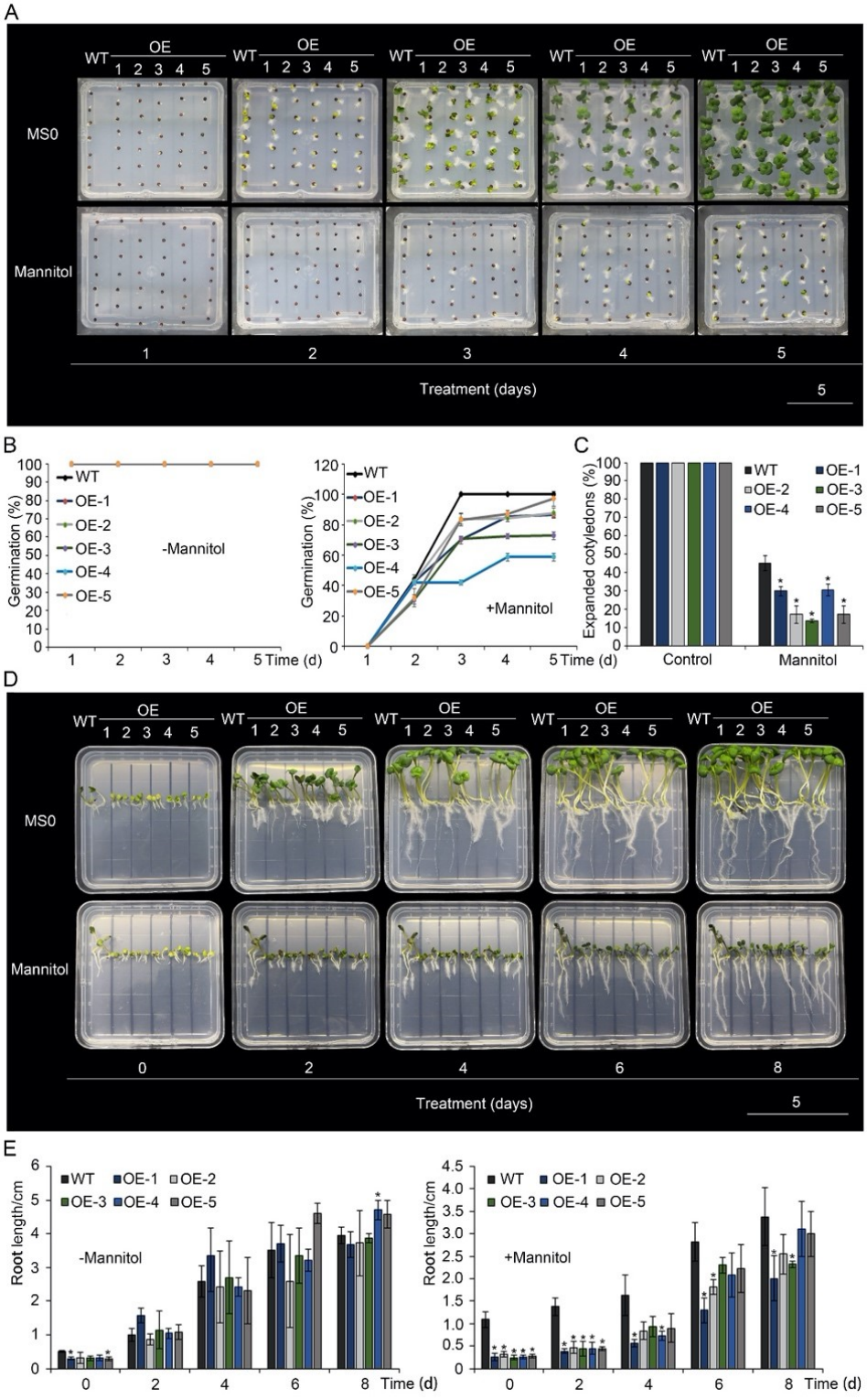

Figure 2. Drought-stress responses of *p35S::BnA.JAZ5* rapeseed plants. (**A**) Phenotypes of germinated six-day-old overexpressing lines under 400 mM mannitol treatment. (**B**) Seed germination rate of the five overexpressing lines under 400 mM mannitol treatment. (**C**) The percentage of wild-type and *p35S::BnA.JAZ5* rapeseed seedlings with expanded cotyledons was scored 5 d after stratification on MS medium supplemented with 400 mM mannitol. (**D**) Phenotypes of root elongation of five overexpressing lines under 400 mM mannitol treatment. (**E**) Root length of five overexpressing lines under 400 mM mannitol treatment. Error bars represent standard errors. Asterisks indicate significant differences between wild-type and *p35S::BnA.JAZ5* lines ($p < 0.05$).

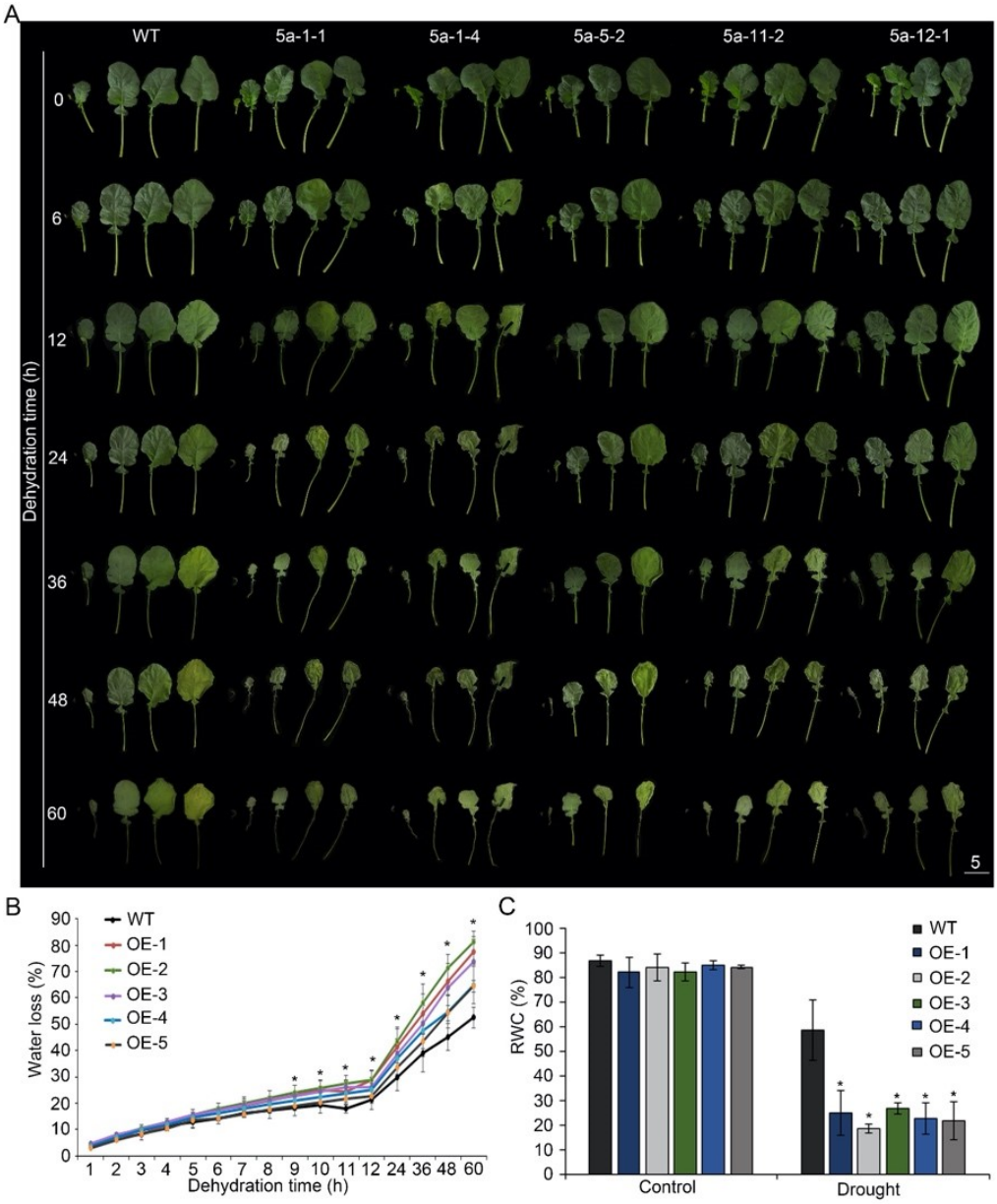

Figure 3. Dehydration test of *p35S::BnA.JAZ5* plants at the five-leaf stage. (**A**) Phenotypes of detached leaves of *p35S::BnA.JAZ5* plants. (**B**) WLR of five overexpressing lines. Water loss was measured at the indicated time points. Asterisks indicate significant differences between wild-type and *p35S::BnA.JAZ5* lines ($p < 0.05$, Student's *t*-test). (**C**) RWC of five overexpressing lines under drought-stress treatment. Error bars represent standard errors. Asterisks indicate significant differences between control and drought stress ($p < 0.05$, Student's *t*-test). WT, accession K407. OE-1, OE-2, OE-3, OE-4, OE-5, and *p35S::BnA.JAZ5* lines. Scale bar, 5 cm.

To further investigate the response of plants under drought conditions, five overexpressing lines were planted in the greenhouse for 5 weeks and then subjected to drought stress by withholding water for 14 days (Figure 4A). Drought-associated symptoms, such as leaf rolling and wilting, appeared earlier in *p35S::BnA.JAZ5* plants than in the wild-type during drought treatment. After recovery for 3 days, only 22–40% of *p35S::BnA.JAZ5* transgenic plants survived, when approximately 61% of wild-type ones survived (Figures 4B and S4D). Overall, these results revealed that *BnA.JAZ5* was a negative regulator of drought resistance.

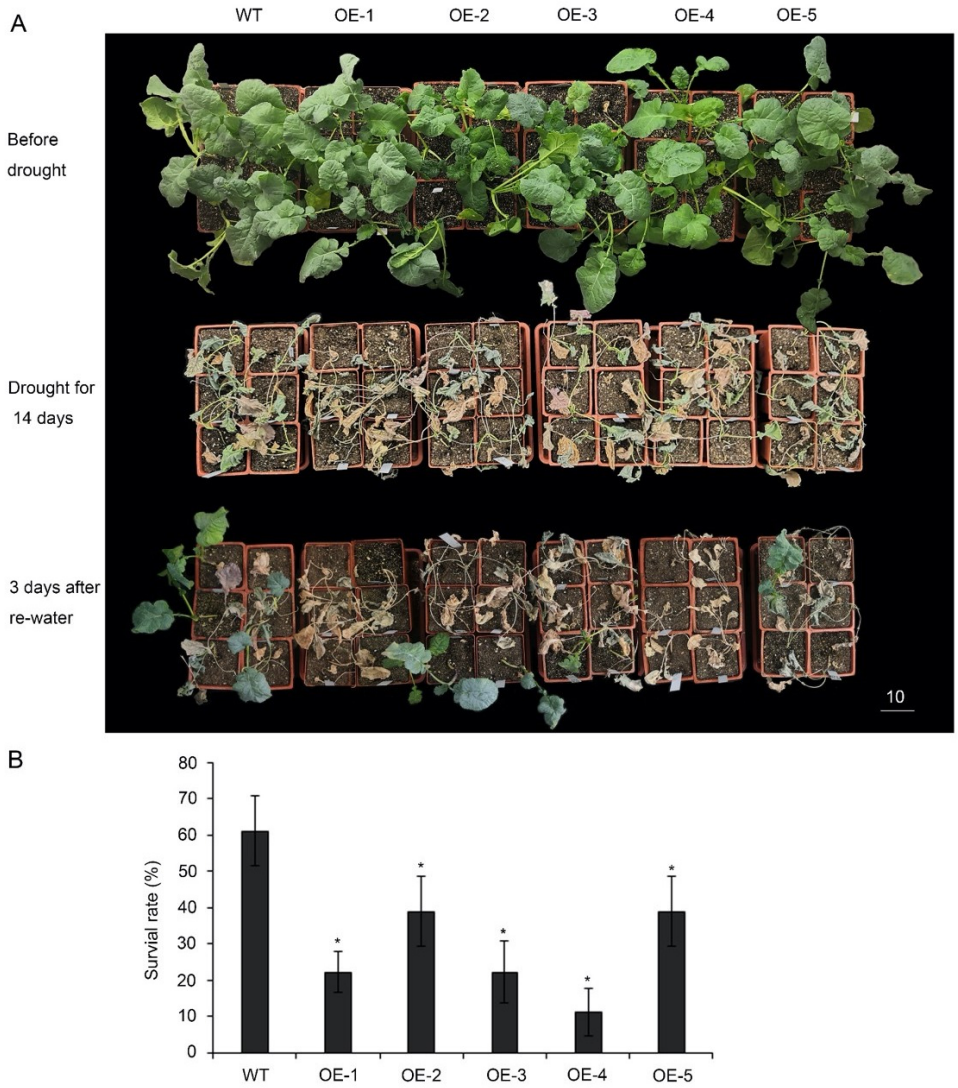

Figure 4. Drought resistance testing of *p35S::BnA.JAZ5* plants at the five-leaf stage. (**A**) Drought resistance assay of five overexpressing lines. (**B**) Survival rates of five overexpressing lines after 3-day recovery from drought stress treatment. WT, accession K407. OE-1, OE-2, OE-3, OE-4 and OE-5, *p35S::BnA.JAZ5* plants. Asterisks indicate significant differences between wild-type and *p35S::BnA.JAZ5* lines ($p < 0.05$, Student's *t*-test). Scale bar, 10 cm.

3.3. BnA.JAZ5 Overexpression Altered Plant Responses to JA and ABA

Given that *JAZ5* belongs to the *JAZ* family, whose members are key inhibitors of the JA signaling pathway, we examined the function of JA on seed germination of *BnA.JAZ5*-overexpressing rapeseed. Overall, methyl jasmonate (MeJA) inhibited the seed germination of both wide-type and *BnA.JAZ5*-overexpressing rapeseed (Figure 5A–E). However, the germination rate of these five overexpressing lines showed no significant change under treatment with 50 μM MeJA (Figures 5A,B and S4A), and the cotyledon expansion of the overexpressing lines was no significant difference on media containing 50 μM MeJA on the 4th day after seed was sown (Figure 5A,C). In fact, we found that, in wide-type plants, with 50 μM MeJA treatment, *BnA.JAZ5* expression increased up to 46-fold as compared with the control (Figure 5E). These results suggested that *BnA.JAZ5* overexpression did not affect the seed germination sensitivity to JA.

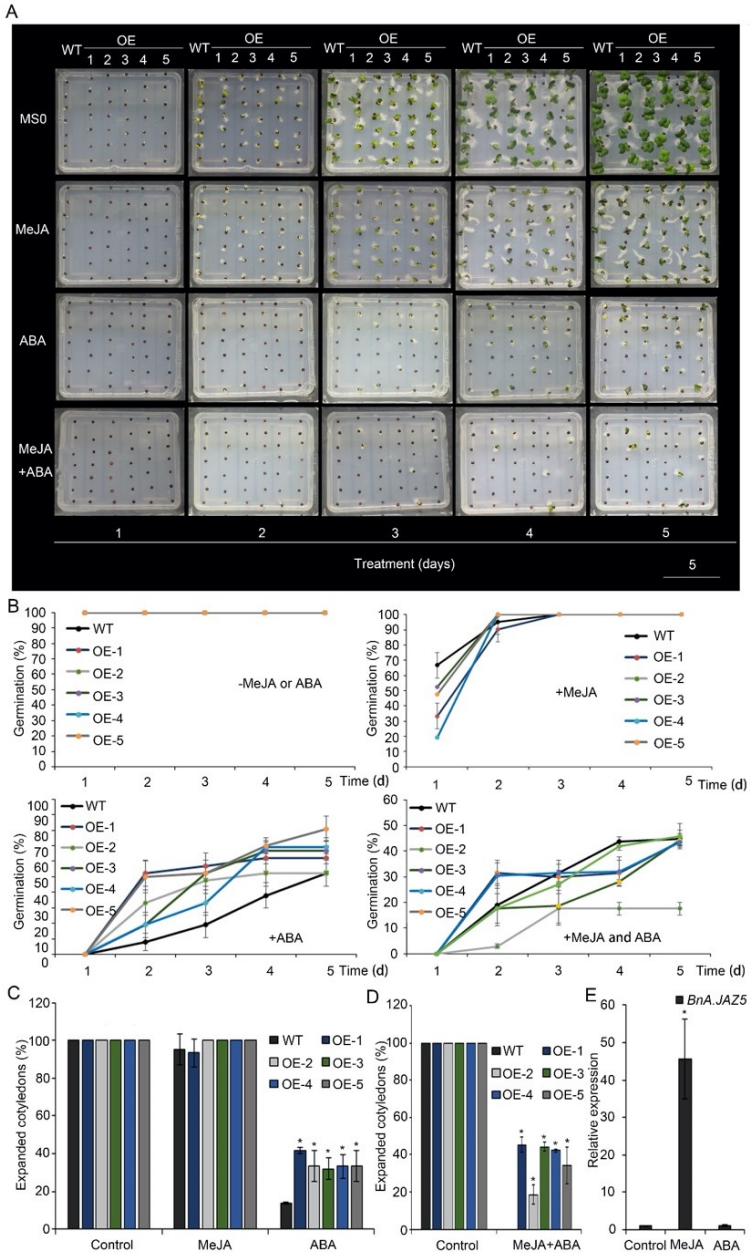

Figure 5. Seed germination of *p35S::BnA.JAZ5* plants on MS medium supplemented with 50 μM MeJA, 9 μM ABA or both 50 μM MeJA and 9 μM ABA, respectively. (**A**) Phenotypes of seed germination of five overexpressing lines under 50 μM MeJA, 9 μM ABA or both 50 μM MeJA and 9 μM ABA treatments. (**B**) Seed germination rate of five overexpressing lines under 50 μM MeJA, 9 μM ABA or both 50 μM MeJA and 9 μM ABA treatments. (**C**) The percentage of wild-type and *p35S::BnA.JAZ5* rapeseed seedlings with expanded cotyledons was scored 4 d after stratification on MS medium supplemented with 50 μM MeJA or 9 μM ABA. (**D**) The percentage of wild-type and *p35S::BnA.JAZ5* rapeseed seedlings with expanded cotyledons was scored 9 d after stratification on MS medium supplemented with both 50 μM MeJA and 9 μM ABA. (**E**) Expression levels of *BnA.JAZ5* under 50 μM MeJA or 9 μM ABA treatments. Error bars represent standard errors. Asterisks indicate significant differences between wild-type and *p35S::BnA.JAZ5* lines ($p < 0.05$). WT, accession K407. OE-1, OE-2, OE-3, OE-4 and OE-5, *p35S::BnA.JAZ5* lines. Scale bar, 5 cm.

In rapeseed seeds, exogenous application of ABA inhibits germination [51]. To test the possible roles of *BnA.JAZ5* in ABA signaling, we treated the wild-type and five overexpressing lines with 9 μM ABA, as suggested by previous studies [52,53]. From this analysis,

we found the seed germination of five overexpressing lines exhibited hyposensitivity to ABA as compared to wild-type plants (Figures 5A,B and S4A). Additionally, compared with the wild-type line, the five overexpressing lines exhibited much higher percentages of expanded cotyledons on media containing 9 μM ABA on the 5th day after the seed was sown (Figure 5A,C). In addition, we found no change in the expression levels of *BnA.JAZ5* as compared to the control with 9 μM ABA treatment (Figure 5E). These findings indicate that *BnA.JAZ5* may regulate drought response in an ABA-dependent manner.

Next, to test the JAZ5 function in JA–ABA crosstalk, we treated the plants with a combination of 50 μM MeJA and 9 μM ABA. As expected, the seed germination of wild-type and *p35S::BnA.JAZ5*-overexpressing plants was severely inhibited (Figures 5A,B,D and S4A). Interestingly, while the seed germination rate of *p35S::BnA.JAZ5*-overexpressing plants was higher as compared to wild-type plants under ABA treatment, the seed germination rate showed no difference with the combination of ABA and JA treatment, suggesting that JAZ5 plays a role in crosstalk between JA and ABA. Taken together, these results suggest that *BnA.JAZ5* regulated rapeseed plant responses to drought stress through ABA and JA signaling.

3.4. BnA.JAZ5 Regulates ABA-Dependent Stress-Responsive Genes

To explore the role of *BnA.JAZ5* in drought stress through the ABA signaling pathway, we examined JA- and ABA-responsive marker genes in plants under 400 mM mannitol treatment (Figure 6A). Our real-time PCR results revealed that *BnA.JAZ5* overexpression led to downregulation of the JA-responsive gene *BnMYC2* and upregulation of the ABA-responsive gene *BnABF3* under 400 mM mannitol treatment (Figure 6A). In addition, we examined stress-related marker genes in plants under dehydration treatment (Figure S2A). *BnA.JAZ5* overexpression led to downregulation of the stress-related genes *BnP5CS*, *BnCT-STM1*, and *BnLEA76* (Figure S2B), and the expression of *BnPLC* was repressed in *BnA.JAZ5*-overexpressing lines after 6 h and 12 h drought treatment as compared to wide-types. This finding suggests that *BnA.JAZ5* attenuated the drought tolerance of the transgenic plants through downregulation of ABA-dependent stress-responsive genes.

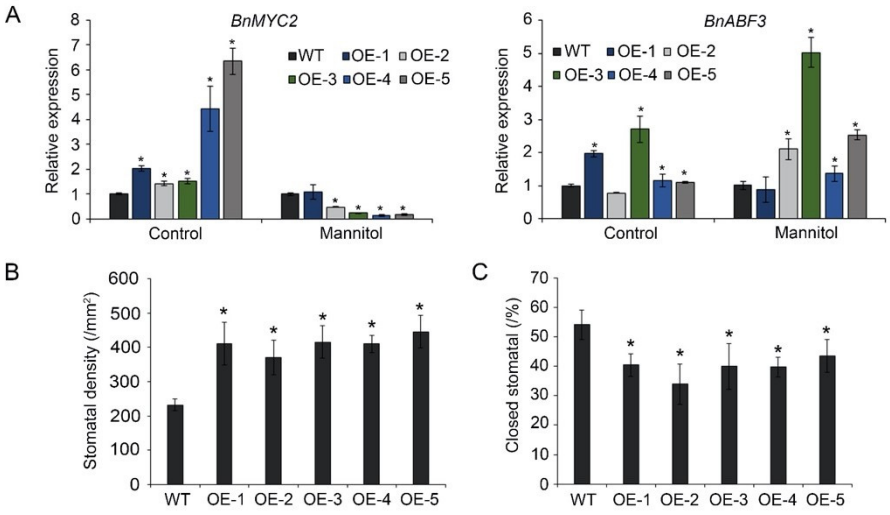

Figure 6. Changes in stomatal density and expression levels of stress-related marker genes. (**A**) Expression patterns of JA- and ABA-responsive genes under 400 mM mannitol stress. Error bars represent standard errors. Asterisks indicate significant differences ($p < 0.05$). (**B**) Stomatal density of OE-1, OE-2, OE-3, OE-4, and OE-5 lines. (**C**) Percentage of closed stomata under drought stress. Asterisks indicate significant differences between WT and *p35S::BnA.JAZ5* lines ($p < 0.05$, Student's *t*-test). WT, accession K407; OE-1, OE-2, OE-3, OE-4, OE-5, and *p35S::BnA.JAZ5* transgenic lines.

3.5. BnA.JAZ5 Overexpression Increased Stomatal Density and Reduced Stomatal Closure

Guard-cell signaling plays a critical role in plant drought response, which is regulated by ABA [1]. We measured stomatal density under normal and drought-stress conditions. Under normal conditions, the average stomatal density of five overexpressing lines was higher than that of wild-type plants (Figures 6B and S3). After drought stress, the percentage of closed stomata of five overexpressing lines was lower than that of the wild-type plants (Figures 6C and S3). These results suggest that *BnA.JAZ5* overexpression increased stomatal density under normal conditions and reduced stomatal closure when transgenic plants were subjected to drought stress.

4. Discussion

Rapeseed (*B. napus*), soybean, and oil palm are the most extensively grown oil crop species worldwide. Rapeseed, however, is very sensitive to water stress, a main factor of crop failure in rapeseed [5]. Identifying genes related to dehydration stress or markers linked to these genes is a key step in genomics-assisted breeding in rapeseed [54]. JAZ proteins are key components of the JA signaling pathway. The *OsJAZ* genes were induced upon abiotic stress in *OsbHLH148*-overexpressing plants [55]. *OsJAZ9* can increase salt and cold tolerance in rice by inhibiting the expression of *OsbHLH062* and *OsMYB30* [56,57]. *GsJAZ2* overexpression in *Arabidopsis* reduces plant sensitivity to salt stress [58]. In rice, interaction of OsJAZ proteins with a basic helix–loop–helix protein leads to drought tolerance [55–57], whereas *OsJAZ1* negatively regulates drought resistance by modulating JA and ABA signaling [52]. The varied roles of JAZ members indicate that JAZ protein family members regulate abiotic stresses differentially. However, little is known about JAZ functional roles and their mechanisms in rapeseed. We collected cotyledon, first true leaf, root (five-leaf stage), third leaf (five-leaf stage), bud, flower, and young silique samples from the rapeseed accession K407 for real-time PCR analysis. We found that *BnA.JAZ5* was generally expressed in all these tissues of rapeseed plants (Figure S2C). Furthermore, both 20% PEG-6000 treatment and dehydration stress significantly induced the expression of *BnA.JAZ5*. The germination rate of *p35S::BnA.JAZ5* transgenic seeds was weaker than that of wild-type seeds on medium containing 400 M mannitol. Fewer *p35S::BnA.JAZ5* plants survived than wild-type plants following withholding of water for 14 days and recovery for 3 days. Thus, we thought that *BnA.JAZ5* may have a negative role in drought resistance, and this conclusion is in agreement with those concerning *OsJAZ1* [52].

Most plant transpiration occurs in stomata, and stomatal density and/or movement can adjust the transpiration rate [59,60]. Leaves can respond to water status by changing stomata density and guard-cell size under water stress [61]. We found that stomatal density was significantly increased in *p35S::BnA.JAZ5* plants, and a lower proportion of closed stomata was observed in *p35S::BnA.JAZ5* plants under drought stress. This change caused the *p35S::BnA.JAZ5* plants to lose water more rapidly under drought stress, thereby accelerating death and reducing drought resistance. Consistently, *p35S::BnA.JAZ5* plants had lower RWC under drought stress and higher WLR than wild-type plants. Stomatal closure is one of the ABA-regulated pathways activated by water-deficit conditions [62,63]. A smaller stomatal aperture and stomatal density contribute to reduced water loss from plant cells, thereby enhancing drought or osmotic stress tolerance [8–10,64]. *BnA.JAZ5* gene did not respond to exogenous ABA treatment, but *p35S::BnA.JAZ5* plants were ABA-hyposensitive, and the germination rate of *p35S::BnA.JAZ5* seeds on MS medium containing ABA was higher than that of wild-type seeds. Studies have shown that JAZ proteins interact with ABA-responsive transcription factors in *Arabidopsis* [15,41]. The expression of JA-responsive gene *BnMYC2* was downregulated, and the expression of ABA-responsive gene *BnABF3* was upregulated in *p35S::BnA.JAZ5* plants under 400 mM mannitol treatment. At the same time, *BnA.JAZ5* could be induced by MeJA. In addition, the proline synthesis gene *BnP5CS* and stress tolerance-related genes *BnRD29A*, *BnLEA*, and *BnCYSTM1* tend to be downregulated in *p35S::BnA.JAZ5* plants under drought-stress conditions. These

results suggest that *BnA.JAZ5* negatively regulates drought resistance in rapeseed through JA–ABA crosstalk, and this process affects stomatal development. *BnA.JAZ5* can be used as a candidate gene for improving drought resistance of rapeseed. We can design sgRNA to edit these *BnA.JAZ5* homologs using the CRISPR/Cas9 system to obtain rape germplasm with higher drought resistance.

5. Conclusions

In this study, the function of rapeseed *BnA.JAZ5* in drought response was characterized. *BnA.JAZ5*-overexpressing rapeseed plants were further investigated under drought conditions and plant hormone (JA and ABA) treatment, revealing the role of *BnA.JAZ5* in attenuating rapeseed drought resistance in an ABA-dependent manner. Molecular study showed that *BnA.JAZ5* regulated ABA-mediated stress-responsive genes *MYC2* and *ABF3*. Finally, significantly increased stomatal density and reduced stomatal closure were observed in *BnA.JAZ5*-overexpressing rapeseed plants. Taken together, these results suggest that *BnA.JAZ5*-mediated crosstalk between JA and ABA signaling pathways contributed to the rapeseed response to drought stress. *BnA.JAZ5* could be used as CRISPR-editing genetic loci for drought-resistant rapeseed breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8020131/s1, Table S1. List of primers used in this study. Figure S1. Phylogeny tree of rapeseed BnA.JAZ5 (GSBRNA2T00103000001) and BnC.JAZ5 (GSBRNA2T00089260001) with *Arabidopsis* JAZ genes. Figure S2. Changes in plant phenotype and gene expression under dehydration stress. Figure S3. SEM images showing adaxial epidermal cells of OE-1, OE-2, OE-3, OE-4, and OE-5 lines at 350× magnification. Figure S4. Molecular data of another two biological replicates per experiment.

Author Contributions: Y.H. and S.H. designed the project. B.C., X.W., J.B. and Y.Z. performed the experiments. B.C., X.Y., S.H. and Y.H. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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