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Original article

The *Brassica napus* MYC2 regulates drought tolerance by monitoring stomatal closure

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Summary

The transcription factor MYC2, an important regulator of the jasmonic acid (JA) signaling pathway, plays a vital role in regulating plant growth and stress tolerance. In this study, the role of MYC2 in regulating the stomatal opening/closure and drought tolerance of rapeseed was studied using *MYC2*-silenced transgenic plants, which were developed previously. Compared with that in the leaves of control plant, the stomatal closure in the lower epidermis of the *MYC2*-silenced leaves was weaker under both light and dark conditions, which results in faster water loss and lower drought tolerance. The transcriptional assays showed that the expression of *MYC2* is induced by JA and abscisic acid (ABA), and that silencing of *MYC2* altered the expression of JA- and ABA-responsive downstream gens. These results are helpful to understand the regulation mechanism of jasmonate signaling pathway in drought tolerance of rapeseed.

Keywords

jasmonic acid, abscisic acid, *MYC2*-silenced transgenic plants, water loss

Significance of this study

What is already known on this subject?

- Hormones play an important role in plant stress tolerance. Jasmonates (JAs) are important endogenous signal molecules in the activation of stress responses in plants, but its function in regulating rapeseed tolerance to drought stress remains unclear.

What are the new findings?

- We found that MYC2, an important regulator of JA signaling pathway, regulates the drought tolerance of rapeseed by modulating stomatal closure in leaves.

What is the expected impact on horticulture?

- Rapeseed is an important oil crop in the world, and its normal growth and yield are seriously affected by drought stress. This study contributes to reveal the mechanism of drought tolerance in rapeseed and to guide the rapeseed breeding for enhanced drought tolerance.

Introduction

Jasmonic acid (JA), an important signaling substance in plants, interacts with other hormones including salicylic acid (SA), ethylene (ET), and abscisic acid (ABA), etc., to form a complicated network that regulates plant growth, development and responses to various external environment stresses (Jones and Dangl, 2006; Pieterse et al., 2012). The MYC2 transcription factor, identified from the jasmine-insensitive *Arabidopsis* mutant *jai1/jin1*, is a critical regulator in the JA signaling pathway (Lorenzo et al., 2004). MYC2 belongs to the basic helix-loop-helix (bHLH) family of transcription factors (Lorenzo et al., 2004), and plays an important role in regulating various processes in plants, such as insect tolerance, flavonoid biosynthesis, and nicotine synthesis (Boter et al., 2004; Dombrecht et al., 2007; Cheng et al., 2011; Zhang et al., 2012).

The JA receptor complex is composed of the CORONATINE INSENSITIVE 1 (COI1), jasmonate ZIM-domain protein (JAZ, a negative regulator of the JA signaling pathway), and inositol pentakisphosphate molecules, and most members of the JAZ protein family interact with MYC2 (Xie et al., 1998; Devoto et al., 2002; Chini et al., 2007; Yan et al., 2009; Wang et al., 2014). Binding of jasmonoyl-isoleucine, a bioactive JA derivative, to the JA receptor initiates the degradation of JAZ protein by the 26S proteasome (i.e., ubiquitination pathway), which releases the transcription factor MYC2 to activate the expression of genes downstream of the JA signaling pathway and to regulate plant growth, development, and stress tolerance (Thines et al., 2007; Chung et al., 2009; Sheard et al., 2010; Zhang et al., 2012).

Rapeseed is an important oil crop across the world. Yet, drought stress seriously affects its normal growth and yield. However, the role of MYC2 in regulating rapeseed tolerance to drought stress is unclear. In this study, the expression of *MYC2* gene in rapeseed leaves was analyzed by treatment with JA and ABA. And, the role of MYC2 in regulating stomatal opening/closure and drought tolerance in rapeseed was studied using *MYC2*-silenced transgenic plants that were established in our previous study (Ding et al., 2017).

Materials and methods

Materials

Brassica napus L. cv. Zhongshuang 11 was used as the plant material for transcriptional and functional assay of MYC2. The *MYC2*-silenced transgenic rapeseed plants were established in our previous study (Ding et al., 2017), and the control plant transformed with empty vector was developed using the same method. The *MYC2*-silenced plants identified by qRT-PCR were used for the physiological and molecular assays, and the hygromycin-resistant plants transformed with empty vector were used as control plants.

Phytohormone treatment of rape seedling leaves

The seeds of Zhongshuang 11 cultivar were sown in potting soil and cultured until 3–5 true leaves had developed. MeJA (methyl jasmonate) and ABA were dissolved in ethanol to prepare 500 mM stock solutions, respectively, and stored at -20°C. Upon leaf treatment, solutions of 100 µM MeJA or ABA were prepared to spray the rapeseed leaves, and a set of leaves sprayed with distilled water (control treatment) were used as experimental control. Leaf samples were collected at the indicated time points (i.e., 0 min, 30 min, 1 h, 2 h, 6 h, 12 h, and 24 h) for further analyses. Three replicates were tested for each treatment, totaling 3 (treatment) × 3 (repetition) × 7 (time period) = 63 (samples).

Quantitative RT-PCR analyses

Total leaf RNAs were extracted from each leaf sample and reverse-transcribed into cDNAs. Quantitative PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Company, Foster City, USA) using the transcribed cDNAs as templates. And, *BrActin* gene was used as the internal reference gene. The PCR system consisted of 12.5 µL of 2× SYBR Premix Ex Taq, 0.5 µL of cDNA template, and 0.5 µL each of the gene-specific primer pair, and was made up to 25 µL with double-distilled H_2O. The primers for internal reference gene and *MYC2* gene are as followings: 5'-TGGGTTTGCTGGTGACGAT-3' and 5'-TGCCTAGGACGACCAACAATACT-3' for *BrActin* (GenBank accession: AF111812); 5'-CGACGATAACGCCTCTATGA-3' (BnMYC-Q5) and 5'-CCTTCGTTTGTCCCTTCAAT-3' (BnMYC-Q3) for *BnMYC2*.

The expression of a set of genes downstream JA- and ABA-signaling pathways were also analyzed, and the gene sequences were retrieved from *Brassica* database (BRAD, http://brassicadb.org). Primers for the downstream genes are as followings: 5'-GTCTCCCAAGCTCGCCCTTC-3' and 5'-TGCGGGCACTGTTTGGAAGT-3' for *BrVSP2* (gene ID at BRAD: GSBRNA2T00120292001), 5'-TCGCCTCCATCCTCACCACA-3' and 5'-CATCAGCGGAGGACGGTTGG-3' for *BrLOX3* (gene ID at BRAD: GSBRNA2T00016257001), 5'-TCACCCTTCTCTTCGCTGCT-3' and 5'-GCACTGATTCTTGCAGGCGTT-3' for *BrPDF1.2* (gene ID at BRAD: GSBRNA2T00146665001), 5'-CGCAGCGGCTGGAGTAAAGA-3' and 5'-ACCGCGTAAACGCTCGTCAT-3' for *BrRD22* (gene ID at BRAD: GSBRNA2T00145974001).

Identification of the *MYC2*-silenced transgenic plants

The *MYC2*-silenced transgenic rapeseed plants were developed by *Agrobacterium*-mediated transformation method and identified by PCR as previously described (Ding et al., 2017). In this study, qRT-PCR was used to analyze the *MYC2* expression levels in the *MYC2*-silencing and control plants. The primers used for the qRT-PCR were BnMYC-Q5 and BnMYC-Q3 as mentioned above. And, the qRT-PCR amplifications were performed as above described.

Detection of the water loss rate

The leaves of transgenic and control rapeseed plants were cut quickly at the same position on the plants with scissors. Each sample was set with three replicates of three individual plants, and the leaf weight was measured on an electronic balance and recorded in the desired order. Leaf weight of the first weighing was recorded as 0 h, and those of the subsequent weighings were recorded every 1 h. The water loss for each time point was obtained by subtracting the weight at each time point from the fresh weight at 0 h, and then the water loss at each time point was divided by the fresh weight at 0 h to obtain the water loss rate for each time point. A curve of water loss rate for each sample was plotted taking the values of water loss rate as the ordinate and the time points as the abscissa, and the statistical analysis of the water loss rate for each time point was carried out to determine whether a differential water loss presents between the two samples.

Stomatal aperture observation

To determine the stomatal opening and closer, transgenic and control rapeseed plants were exposed to light for 3 h at 25°C to promote stomatal opening. Then, the leaves were detached from the same position of different plants and washed with distilled water for lower epidermis collection by tearing from the edge. Following, the epidermis was cut into 1 cm × 1 cm pieces and the stomata was observed and photographed under a light microscope (Olympus AX70). The stomatal aperture was also measured. On the other hand, a set of plants were kept in the dark for 3 h, and the leaves were then taken for lower epidermis collection as described above. And, the stomata was observed and photographed under the light microscope for stomatal aperture measurement. Six epider-

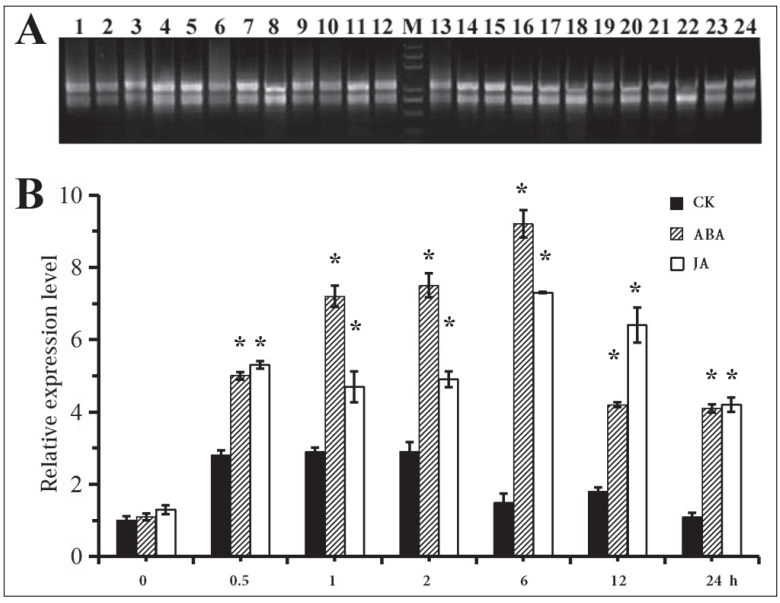


Figure 1. Effects of plant hormones JA and ABA on *MYC2* gene expression. A: RNA extraction of rape samples; M indicates molecular marker; numbers indicate different samples. B: qRT-PCR expression analysis of *MYC2* gene in rape seedlings which applied exogenous JA/ABA/ water (CK). The expression level in CK at time point "0" was set as "1". Asterisks indicate significance (Student's *t*-test, $P<0.05$) to the CK at the same time point.

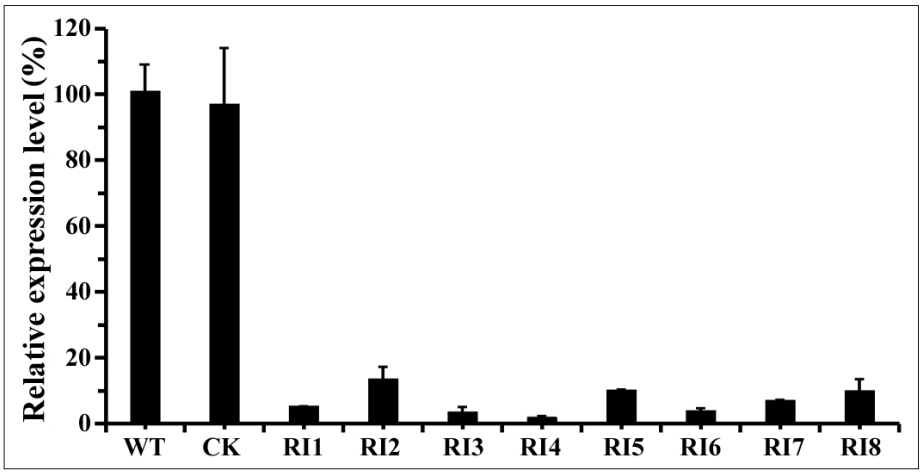


Figure 2. qRT-PCR validation of transgenic rapeseed plants. WT indicates the non-transformed control of wild type plant. CK indicates control plant transformed with empty vector; RI1-8 indicates different *MYC2*-silencing lines. The expression level in WT was set as 100%.

mal strips collected from three plant lines were observed for each experimental treatment, and each experiment was repeated three times.

Results

Induction of *MYC2* expression by the phytohormone ABA and JA

The induced expression of *MYC2* in *B. napus* cv. Zhongshuang 11 was tested by qRT-PCR with cDNAs from the leaf RNAs (Figure 1A) of ABA, MeJA, or control treatment in the indicated time course. The results showed that the expression of *MYC2* gene was enhanced by the ABA-treatment. As shown in Figure 1B, an increase in the expression level of *MYC2* was observed after 30 min of ABA treatment, and the expression level was higher than that of the control treatment through the whole time course of hormone treatment. The expression of *MYC2* gene was also inducible by JA. Its expression level was increased to a higher level after 30 min of MeJA treatment, and kept at higher expression levels than that of the control treatment till after 24 h of treatment (Figure 1B). The results showed that the expression of *MYC2* was induced by both JA or ABA in rapeseed plants.

Identification of *MYC2*-silenced transgenic plants by qRT-PCR amplification

The *MYC2*-silencing transgenic plants were developed previously (Ding et al., 2017). To identify the *MYC2*-silenced plants, total RNAs were extracted from the leaves of transgenic plants to determine the expression levels of *MYC2* in these plants by qRT-PCR with the primers BnMYC-Q5 and BnMYC-Q3 as described above. After qRT-PCR amplification, the data were analyzed with software provided for the CFX96 Touch™ Real-Time PCR Detection System, and the plants whose *MYC2* expression was suppressed by over 80% of that in the control plants were selected for further assays. The results showed that the expression of *MYC2* was successfully suppressed in 8 of the transgenic lines (Figure 2). And, line

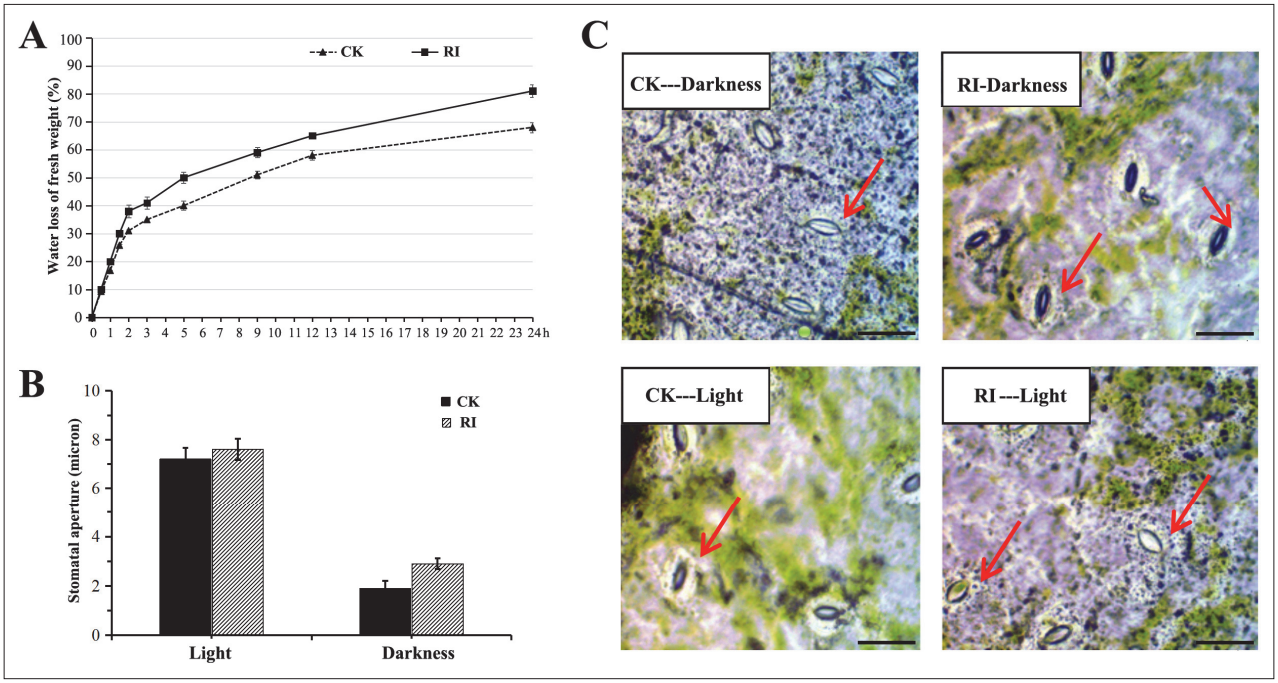


Figure 3. Drought tolerance of *MYC2*-silenced oilseed rape. A. Water preserving capability in transgenic plants and control plants. B. Stomatal aperture of control (CK) and *MYC2*-silenced (RI) rape plants at different light conditions. C. Representative stomatal images of CK and RI rape plants at different light conditions. Scale bar indicates 50 μm.

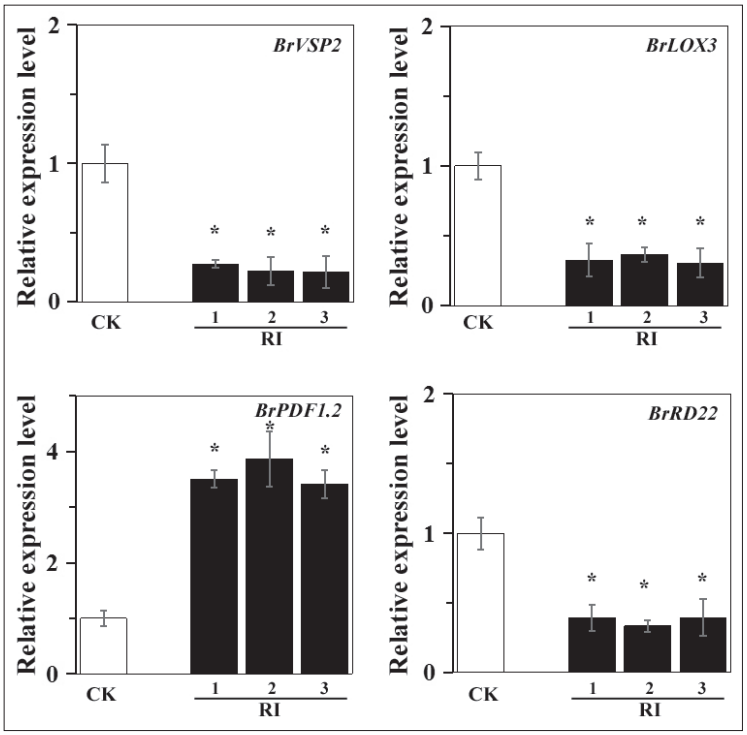


Figure 4. qRT-PCR expression analyses of JA and ABA responsive genes in the *MYC2*-silenced rapeseed plants. CK indicates control plant transformed with empty vector. RI indicates *MYC2*-silenced plants, and the numbers indicate the transgenic lines. The expression level of each genes in CK was set as "1". Asterisks indicate significance difference from the CK (Student's *t*-test, $P < 0.05$).

RI1, RI2 and RI3 were used for the following experiments. Three hygromycin-resistant plants transformed with empty vector were selected as the control plants.

Silencing of *MYC2* affected the water loss rate and stomatal opening/closure of the rapeseed leaves

To investigate the effect of *MYC2*-silencing on the drought tolerance of rapeseed, the difference in water loss rate between the transgenic and control plants was determined by water loss assay with detached leaves as described in the Materials and methods section. The results showed that the water loss rate of *MYC2*-silenced transgenic plants was higher than that of the control plants under the same condition (Figure 3A). The difference in water loss rate became obvious after 2 h of experimental time, and subsequently turned larger till after 24 h or experiment (Figure 3A). The increased water loss rate in *MYC2*-silenced rapeseed leaves suggested that MYC2 functions as a regulator of the water loss in the rapeseed leaves.

Since the water loss of plant leaves is closely correlated with the leaf stomata, we further determined the effects of *MYC2*-silencing on the stomata aperture of rapeseed leaves. After exposure to light condition, the average stomatal opening was about ~7.5 micron in the lower epidermis of rapeseed leaves from both *MYC2*-silenced plants and control plants, but the stomatal opening diameter was slightly larger in the leaves of *MYC2*-silenced plants. After dark treatment, the average stomatal opening was approximately 3.8 micron in the leaves of *MYC2*-silenced plants, and that in the leaves of control plants was only 1.6 micron (Figures 3B and 3C), indicating a faster water loss in the leaves of *MYC2*-silenced plants than in that of the control plants. Taken together, the stomatal opening in the leaves of *MYC2*-silenced plants is larger than in that of the control plants under both light and dark conditions, which supports the higher water loss rate in the leaves of *MYC2*-silenced plants. Thus, silencing of *MYC2* resulted in a decrease in the drought tolerance of rapeseed plants.

Silencing of *MYC2* altered the expression of JA and ABA responsive downstream genes

MYC2 is a regulator of both JA and ABA signaling pathways, and several downstream genes downstream MYC2 in these pathways were previously reported (Abe et al., 1997; Lorenzo et al., 2004). To determine the underlying molecular mechanism of *MYC2*-silencing caused decrease in drought tolerance of rapeseed, we analyzed the expression of four JA and ABA responsive genes, which were suggested to contain *MYC2*-binding elements in promoters, including *BrVSP2*, *BrLOX3*, *BrPDF1.2* and *BrRD22* (Abe et al., 1997; Lorenzo et al., 2004). As shown in Figure 4, the gene expression was quantified by qRT-PCR in the control plants and three lines of *MYC2*-silenced plants. The results showed that the JA-inducing gene *BrVSP2* and *BrLOX3* were suppressed over 60% in the *MYC2*-silenced plant comparing to that in the control plants, while the JA-suppressing gene *BrPDF1.2* was enhanced over three times higher than in the control plants. Furthermore, the ABA responsive gene *BrRD22* was also suppressed by 60% in the *MYC2*-silenced plant comparing to that in the control plants. These results show that silencing of *MYC2* has altered the expression of JA and ABA responsive genes in rapeseed.

Discussion

Plant stomatal development is regulated by many plant hormones, such as ABA, JA, gibberellin, auxin, and brassinosteroids (Seki et al., 2007). Previous studies showed that a class of bHLH transcription factors, including SPEECHLESS, MUTE, and FAMA, are involved in the regulation of stomatal development (MacAlister and Bergmann, 2011). On the other hand, the phytohormone JA also plays important roles in this process, and both JA and its precursor 12-oxophytodienoic acid (12-OPDA) can act as regulators of stomatal closure and the correlated drought responses (Gehring et al., 1997; Savchenko et al., 2014). MYC2 is an important regulator of the JA signaling pathway, however, studies on the regulation of stomatal opening/closure by MYC2 in rapeseed have not

been reported. Here, our study shows that MYC2 could regulate the stomatal opening and closure in the rapeseed leaves, thus to mediate the water loss and drought tolerance. Our study has provided an evidence that the bHLH transcription factor in JA signaling pathway could function in the controlling of stomatal opening/closure.

The stomatal opening/closure in plant leaves depends on the regulation of ABA signaling pathway (Kim et al., 2010; Schroeder et al., 2001). This process is mainly regulated by the Ca^{2+}-dependent signaling pathway, but also depends on the regulation of Ca^{2+}-independent pathways (Hamilton et al., 2000; Jammes et al., 2009; Li et al., 2006; Vahisalu et al., 2008). ABA could induce the alkalization of cytoplasm in Ca^{2+} independent manner to change the intracellular ion concentration, and subsequently induce the stomatal closer (Zhang et al., 2009; Kim et al., 2010; Li et al., 2006). MYC2 is a key transcriptional regulator in the JA signaling pathway, and some researches also indicated that MYC2 may have a close relationship with the ABA signaling pathway (Abe et al., 1997, 2003; Suhita et al., 2004; Aleman et al., 2016). Our results showed that the expression of *MYC2* is induced by ABA treatment, supporting a role of MYC2 in the ABA mediated responses in plants. The roles of MYC2 in regulating the stomatal opening/closure as well as the water loss in the leaves of rapeseed implicated a molecular link of JA and ABA signaling pathways. This hypothesis is further supported by the transcriptional analyses which showed that MYC2 could regulate the expression of downstream genes responsive to both JA and ABA signaling pathways. Therefore, MYC2 may act as a central hub in the regulatory network of multiple signaling pathways that mediates the stomatal opening/closure and stresses tolerance in plants. Drought tolerance has an important effect on the yield and quality of rapeseed, and findings in this work are of importance for the stress tolerance breeding of rapeseed.

Acknowledgments

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