Article

Identification of elite rapeseed drought-tolerant germplasm and candidate genes in a natural population of 265 accessions

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Abstract: Rapeseed (*Brassica napus*) is one of the most important oil crops in the world; however, drought significantly curtails its growth and productivity. Identifying drought-tolerant germplasm is an efficient and low-cost strategy for addressing water shortages. Using water loss ratio (WLR) as an index of drought tolerance, we screened a panel of 265 *B. napus* lines. We identified eight low-WLR and six high-WLR accessions, which were regarded as drought-tolerant and drought-sensitive, respectively. Further validated these selected accessions at the seedling stage under drought-stress conditions. The drought-tolerant accessions had significantly greater fresh and dry weights under drought stress than the drought sensitive accessions. Using RT-qPCR, we showed that a set of previously reported drought-adaptive marker genes were expressed at higher levels in the drought-tolerant lines than in the drought-sensitive lines. These results indicated that the drought-tolerant genotypes could be identified from natural populations using WLR. Then, we performed a genome-wide association study to identify loci harboring single nucleotide polymorphisms (SNPs). A total of 139 SNPs were significantly associated with the WLR, of which chromosome A10 harbored the largest number. Furthermore, four putative candidate genes were selected by combining the SNP–WLR association results and transcriptional expression data with the changes in drought tolerance. Thus, we have identified two drought-tolerant *B. napus* cultivars and uncovered genome-wide variation differentiating *B. napus* lines related to WLR, in addition to providing insights for further research into WLR-related drought mechanisms.

Keywords: GWAS, drought, rapeseed, water loss ratio

1. Introduction

Drought stress is one of the most prevalent and limiting abiotic factors impacting plants in many regions of the world, especially in arid and semiarid areas, where it acutely restricts plant distribution and crop production [1]. Drought has many destructive effects on plants, from the cellular to whole-organism level, impacting cell membrane stability, hormone metabolism, enzyme activity, stomatal regulation, transpiration efficiency, and the growth of various plant tissues [2–4]. Plants have evolved many molecular and biochemical adaptive strategies that allow them to withstand the deleterious effects of drought stress by regulating their metabolism and physiology. These strategies involve stress signal transduction networks, stress-responsive gene expression, elevated abscisic acid (ABA) levels, altered stomatal physiology, and the increased accumulation of osmoprotectants and antioxidants [5]. An elevated ABA level under drought stress induces several molecular and cellular responses, such as triggering stomatal closure and upregulating the expression of stress-related genes, which collectively reduce transpirational water loss [6,7].

Transpirational water loss through the stomata is a key indicator of the level of drought tolerance for a particular plant [8,9]. As water loss conservation patterns are conferred by variations in stomatal density [10], leaf-level water management processes in plants under well-watered conditions have been proposed as a useful indicator of drought tolerance [11]. Drought stress induces ABA biosynthesis, which stimulates the generation of H_2O_2 in guard cells via NADPH oxidase. The generated H_2O_2 plays a key role in stomatal closure by activating the plasma membrane calcium channels [12,13]. ABA-induced stomatal closure and the concomitant reduction in transpiration rate are influenced by many genes, such as *translationally controlled tumor protein* (*AtTCTP*), the overexpression of which confers drought tolerance by enhancing ABA-mediated stomatal closure via an interaction with microtubules that is enhanced by calcium binding [14]. Moreover, overexpression of *abscisic acid, stress and ripening 5* (*ASR5*) in rice (*Oryza sativa*) improved drought tolerance by regulating leaf water status. In addition to promoting ABA biosynthesis and H_2O_2 accumulation, ASR5 functions as chaperone-like protein that helps activate drought-related proteins [15].

Natural variation is a sustainable and beneficial source of genotypic and phenotypic diversity within plant species, and can offer useful traits for breeding. Natural variation is mostly quantitative and is delineated by molecular polymorphisms at multiple loci and genes (multigenic), which can be described as quantitative trait loci (QTLs) and quantitative trait genes, respectively. Genome-wide association studies (GWAS), based on genetic linkage disequilibrium, are an efficient approach for detecting important QTLs or genes underlying complex trait variations in a natural population [16], taking advantage of both natural variation and ancient recombination events [17]. GWAS have been successfully used in many crops, including in rice, wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*), to dissect the complex genetic basis of drought tolerance [18–20]. Rapeseed (*Brassica napus*) is a globally important source of vegetable oil, with 27.67 million metric tons produced worldwide annually [21]. Drought stress results in a severe reduction of *B. napus* biomass and seed yield [22]. In *B. napus*, various important genetic loci underlying different agronomic traits were identified using a GWAS [23]; however, few investigations have explored drought tolerance QTLs in this crop [24,25].

In this study, we aimed to address this shortcoming by identifying drought-tolerant lines and drought-tolerant QTLs related to the water loss ratio (WLR) trait in *B. napus*. We phenotyped a panel of 265 *B. napus* lines at the full-bloom stage under normal conditions by measuring the water loss ratio (WLR) of their detached leaves. As a low leaf WLR is associated with increased drought tolerance of the plant [11], we selected a set of drought-tolerant and drought-sensitive lines on the basis of WLRs and further validated their performances at the seedling stage under water-deficit

conditions. Furthermore, we performed a GWAS to uncover the genetic basis of WLR, and identified candidate genes by combining SNP–WLR associations and transcriptional expression data. In addition to identifying two drought-tolerant genotypes, we dissected the genetic structure of the WLR trait and identified candidate genes associated with drought tolerance, laying the foundation for improving WLR-associated drought tolerance in rapeseed.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

A total of 265 *B. napus* accessions from Zhejiang University, Zhejiang, China, were used in this study. Detailed information about these lines can be found in a previous publication [26]. The detailed field cultivation protocol for these *B. napus* accessions was described previously [27]. The association panel was planted in an experimental field at Beibei, Chongqing, China (29° 45' N, 106° 22' E, 238.57 m above sea level) under natural conditions in 2019–2020. The seeds were sown in triplicate in a randomized block design. Each plot contained three rows, with 10 plants per row, 20 cm between plants within a row, and 30 cm between rows. Two replicates of each accession were phenotyped.

2.2 WLR Measurement

The WLR trait was investigated in the 265 accessions. The fifth fresh leaves above ground were picked from stems at the full-bloom stage and then leaves were put on the shelf in a room with temperature of about 25 °C. Leaves were weighed at 0, 24, and 48 h after being removed from the plants. The WLR was calculated as follows: weight loss / initial leaf weight. A frequency distribution plot was drawn to demonstrate the WLR phenotypic data using GraphPad Prism 8 (GraphPad, San Diego, California, USA).

2.3 Drought Treatment and Sampling

To understand the relationship between the WLR trait and drought tolerance in *B. napus*, six accessions with a relatively high WLR (HWLR) and eight with a relatively low WLR (LWLR) were subjected to drought treatments. The seeds were germinated in the experimental field using a Jiffy pellet (JIFFY, Pokemouche, Canada), and 10 to 15 seedlings at the five-leaf stage (four-week-old seedlings) were subjected to rainfed (dry) treatment, with five seedlings per accession being well-watered as a control. The treatment lasted for seven days, after which the soil moisture content decreased to a physiological limit at which the plants were not viable. The third youngest leaves were sampled throughout the treatment period and immediately frozen at –80°C for the verification of gene expression. The fresh and dry weights of the aerial tissues were determined seven days after the start of the drought treatment.

2.4 RT-qPCR Validation of Marker Gene Expression

To validate the performance of the selected 14 accessions in response to drought stress, two relatively high-WLR accessions and two relatively low-WLR accessions (HWLR4, HWLR6, LWLR5, and LWLR6) were selected for the verification of the expression patterns of four marker genes, which were previously reported to specifically respond to drought [28,29]. The third youngest leaves were sampled at four growth stages (0, 3, 4, and 7 days after the drought stress treatment) and analyzed using RT-qPCR, which was performed as previously described [23]. The primer pairs are listed in

Table S1.

2.5 GWAS

The efficient mixed-model association expedited (EMMAX) cloud computing procedure (http://121.41.229.126:3838/gwas/) was used to perform a GWAS. The WLR data from the panel of 265 accessions at 0–24 h and 0–48 h under normal conditions were imported into a Bna-GWAS-cloud online pipeline, and the EMMAX model was chosen to perform the GWAS. The *p*-value of each SNP was calculated; $-\log_{10}(p\text{-value}) > 5$ was set as the suggestive threshold, and $-\log_{10}(p\text{-value}) > 6$ was set as the significant threshold to screen for significant SNP–WLR associations.

2.6 Identification and Validation of Candidate Genes

The 75-kbp flanking sequences upstream and downstream of the significant SNPs were considered to be confidence intervals for the identification of the candidate genes responsible for the WLR effect. To confirm the function of these candidate genes in maintaining the water content of the leaves, their expression levels were quantified from the transcriptomes of 14 leaves at different growth stages, using transcriptome data acquired from BrassicaEDB (https://biodb.swu.edu.cn/brassica/). Genes with a FPKM (fragment per kilobase of transcript per million mapped reads) of > 1 were selected for further analysis. The expression levels of the candidate genes under drought and heat treatments were also obtained from BrassicaEDB. The candidate genes were then further verified among two LWLR and two HWLR accessions using RT-qPCR analysis, which was performed across four timepoints using the same procedure as used for the marker gene validation.

2.7 Statistical Analysis

Descriptive statistical analyses, normality tests, one-way analyses of variance (ANOVAs), and correlation analyses were all performed in SPSS 20.0 (IBM, Armonk, New York, USA). The means, standard deviations, minimum values, maximum values, and coefficients of variation were included in the descriptive statistical analyses. Kolmogorov–Smirnova and Shapiro–Wilk analyses of the phenotypic traits were performed for the normality tests. Student's *t*-tests were used to analyze differences between the treatment and control conditions for each individual accession.

3. Results

3.1 Phenotypic Analysis of the WLR in a Natural Population

The WLR of leaves taken over two periods (0–24 h and 0–48 h) was investigated in a *B. napus* population comprising 265 accessions. As expected, the WLR over 48 h was higher than it was over 24 h. Further, the WLR values followed the same trend across the two data sets, and were close to normally distributed (Figure 1 and Table S2). The WLRs of individual accessions ranged from 4% to 63% (WLR data set 1; WLR1) and 2% to 62% (WLR2) in 0–24 h, while the WLRs for 0–48 h ranged from 10% to 75% (WLR3) and 7% to 77% (WLR4) (Table 1). No significant differences were detected using a one-way ANOVA, demonstrating the high level of similarity between biological repeats (Tables 2 and S3). In addition, a clear positive correlation was observed between the WLR values in the four data sets (WLR1, WLR2, WLR3, and WLR4) (Table 3); for example, the correlation coefficient (r^2) was 0.94 when comparing phenotypic values of WLR1 (0–24 h) and WLR3 (0–48 h), and 0.95 when

comparing the WLR2 (0–24 h) and WLR4 (0–48 h) data sets. Together, these results show that our WLR data were characteristic of a quantitative trait, and thus suitable for performing a GWAS.

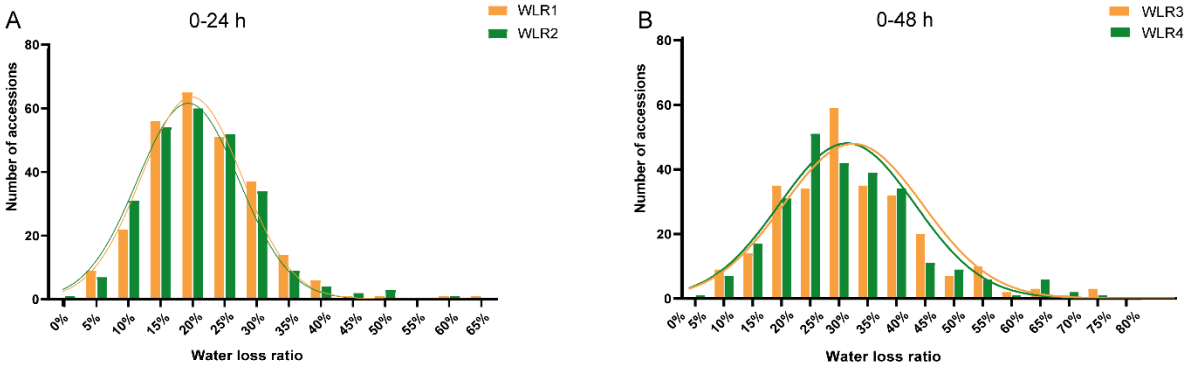

Figure 1. Frequency distribution of WLR in a 265-accession panel. The leaf water loss ratio during two time periods (0–24 and 0–48 h after the onset of drought treatment) were measured for 265 accessions.

Table 1. Statistical analysis of WLR in rapeseed

Trait	Mean ±SD	Min	Median	Max	CV
WLR1	21.52%±0.50%	4.0%	21.07%	63.0%	0.80%
WLR2	21.17%±0.50%	2.0%	20.46%	62.0%	0.80%
WLR3	32.06%±0.70%	10.0%	30.90%	75.0%	1.50%
WLR4	31.51%±0.70%	7.0%	29.32%	77.0%	1.50%

WLR1 and WLR2: two WLR replicates for the 0–24 h period. WLR3 and WLR4: two WLR replicates for the 0–48 h period.

Table 2. ANOVA of WLR in different replicates

Phenotypic data		Sum of squares	Degrees of freedom	Mean square	F	Significance
0–24 h	Between groups	0.004	1	0.004	0.529	0.467
	Within groups	3.997	520	0.008		
	Total	4.002	521			
0–48 h	Between groups	0.009	1	0.009	0.604	0.437
	Within groups	7.732	520	0.015		
	Total	7.741	521			

Table 3. Pearson correlation analysis of the four WLR data sets

		WLR1	WLR2	WLR3	WLR4
WLR1	Pearson correlation	1	0.222**	0.941**	0.216**
	Significance (two-tailed)		0	0	0
	Number of data points	264	258	264	258
WLR2	Pearson correlation	0.222**	1	0.165**	0.948**
	Significance (two-tailed)	0		0.008	0
	Number of data points	258	258	258	258
WLR3	Pearson correlation	0.941**	0.165**	1	0.176**
	Significance (two-tailed)	0	0.008		0.005
	Number of data points	264	258	264	258
WLR4	Pearson correlation	0.216**	0.948**	0.176**	1
	Significance (two-tailed)	0	0	0.005	
	Number of data points	258	258	258	258

**, p-value < 0.01.

3.2 Identification of Drought-tolerant Germplasm

To identify germplasm with a high tolerance to drought stress, we selected eight accessions with a relatively low WLR (LWLR) and six with a relatively high WLR (HWLR) from the 265 accessions (Table S4), and investigated their performance under drought stress. For 8 LWLR accessions, the WLR of the LWLR leaves was less than 10% after 24 h and no more than 16% after 48 h (Figure 2A), which implied that these cultivars were probably tolerant of drought stress due to their reduced evaporation from the leaves. By contrast, the WLR of the excised HWLR leaves exceeded 35% after 24 h and over 50% after 48 h (Figure 2B), indicating that these varieties lose much more water than most of the accessions comprising the association panel. Hence, these six HWLR accessions are likely to be susceptible to drought or heat stress because of the rapid evaporation of water from their leaves.

Reduced water loss from leaves is a vital indicator that could reflect water-deficit tolerance when plants are subjected to drought stress [30]. Here, we assume that the LWLR accessions are drought tolerant and the HWLR accessions are drought susceptible. We subjected the LWLR and HWLR accessions to drought stress at the seedling stage to examine their performance. After seven days without watering, a variety of phenotypic changes could be observed in the HWLR and LWLR accessions. Four of the eight LWLR accessions (LWLR2, LWLR4, and LWLR6) showed no or only small differences in fresh weight between the drought-treated and well-watered plants (Figure 3A).

By contrast, only two of the six HWLR cultivars (HWLR1 and HWLR2) displayed no significant difference in fresh weight between the drought-treated and well-watered plants (Figure 3C). This result illustrated that cultivars with a low leaf WLR maintained their growth better than cultivars with a relatively high leaf WLR under water-deficit conditions.

Five of the eight LWLR accessions (LWLR1, LWLR2, LWLR4, LWLR6, and LWLR8) displayed no significant difference in dry weight between the treatment and control conditions (Figure 3B). Interestingly, the dry weights of two LWLR accessions (LWLR2 and LWLR4) were higher in the drought-stressed plants than the well-watered plants (but not statistically significant), indicating that drought stress promoted the accumulation of dry matter in these two LWLR accessions. Three of the six HWLR cultivars showed no apparent difference in the dry weights of the stressed and well-watered plants, indicating that the HWLR accessions were able to accumulate dry matter when subjected to drought stress (Figure 3D).

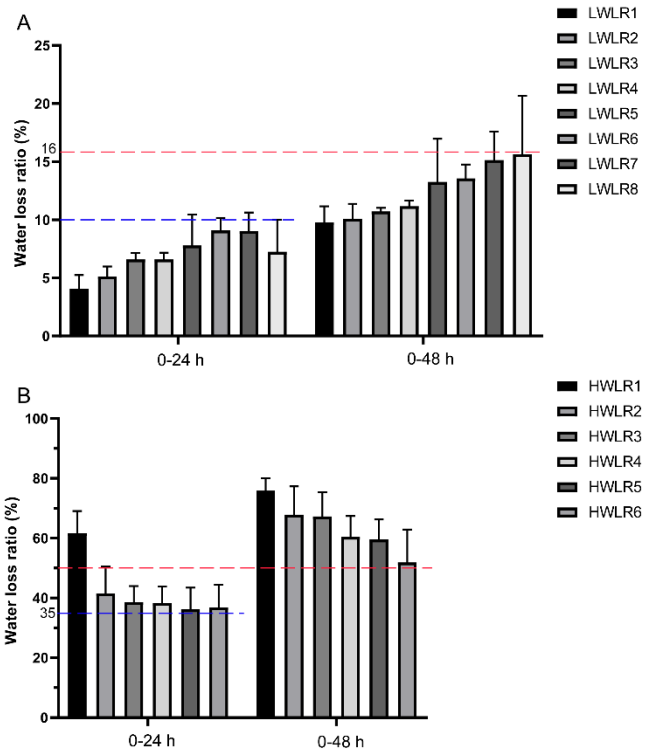

Figure 2. WLRs of 14 selected accessions at the flowering stage. Bars represent means ± SD of three samples of individual accessions. In A, the red dotted line indicates the maximum WLR value after 48 h, while the blue dotted line indicates the maximum WLR value after 24 h. By contrast, in B, the red dotted line represents the minimum WLR value after 48 h, and the blue dotted line represents the minimum WLR value after 24 h. LWLR: low-WLR accession; HWLR: high-WLR accession.

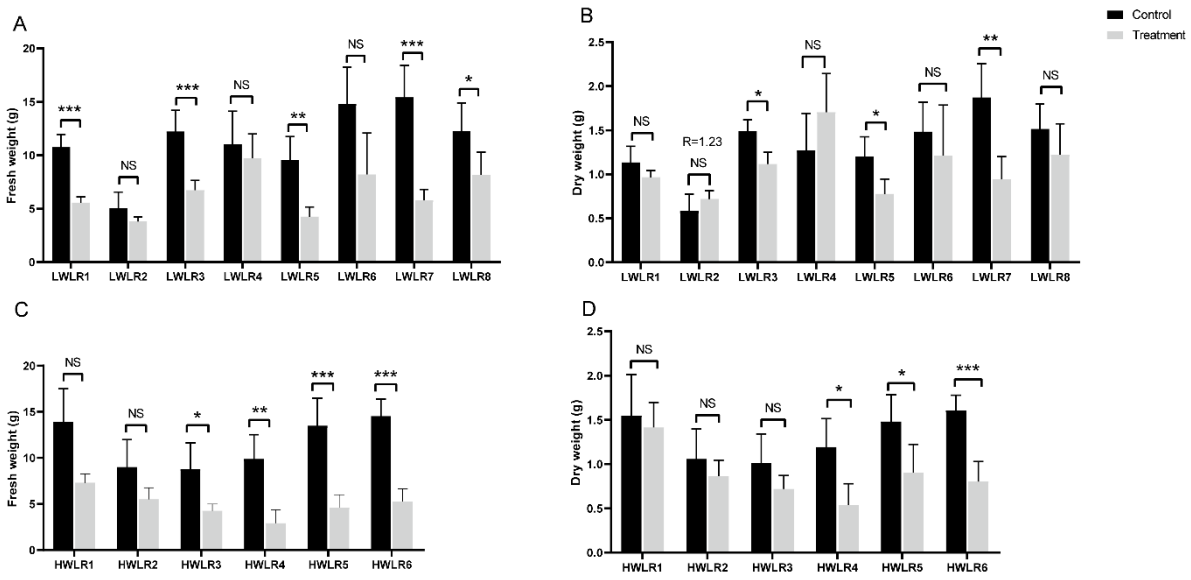

Figure 3. Fresh and dry weights of HWLR and LWLR accessions after seven days without watering. Bars represent means ± SD of three to five samples of individual accessions. NS: no significant difference. *, $p < 0.05$; **$p < 0.01$; ***, $p < 0.001$, Student's *t*-test.

3.3 Comparison of the Expression Patterns of Marker Genes Related to Drought Tolerance Between the LWLR and HWLR Accessions

To further validate degrees of drought stress tolerance in the LWLR and HWLR accessions, we assessed the expression patterns of four marker genes, previously shown to function in the response to drought stress, in two HWLR accessions (HWLR4 and HWLR6) and two LWLR accessions (LWLR5 and LWLR6).

B*etaine aldehyde dehydrogenase* (*BADH*) is a member of the *Acetaldehyde dehydrogenase* (*ALDH*) family, and catalyzes the conversion of betaine aldehyde to betaine under drought stress [31]. Betaine is an important osmotic regulator in plants, and previous studies showed that overexpression of *BADH* improves plant stress tolerance [32]. Here, we showed that *BADH* expression began to increase by three days after the drought stress treatment in all four accessions; however, *BADH* expression remained relatively low after seven days (Figure 4). This result indicated that *BADH* expression may not correspond with the WLR values of extremely different accessions, and thus drought resistance cannot be predicted from the *BADH* expression among the two pairs of accessions.

Brassica turgor gene 26 (*btg*-26) also belongs to the *ALDH* family and has been reported to be induced rapidly by water loss from the leaves [28]. Here, we showed that, in LWLR4 and LWLR6, *btg*-26 expression began to increase four days after the drought treatment and the expression further escalated by seven days after the stress treatment (Figure 4A,B). In the two HWLR accessions, *btg*-26 expression was increased at three days after the drought treatment but declined the following day, before becoming slightly elevated seven days after the onset of drought treatment (Figure 4C,D). The rapid increase in *btg-26* expression in LWLR4 and LWLR6 indicated that this gene was involved in the drought tolerance mechanism once the seedlings had become damaged.

Proline is an important factor that confers osmotic stress tolerance to plants. *Delta 1-pyrroline-5-carboxylate synthetase* 2 (*P5CS2*) encodes an enzyme with both gamma-glutamyl kinase and glutamic-gamma-semialdehyde dehydrogenase activities, which catalyzes the first two steps in proline biosynthesis [33]. Here, we showed that *P5CS2* expression began to rise three days after the onset of drought treatment, peaked after four days, and fell after 7 days in both LWLR4 and LWLR6 (Figure

4A,B). In the HWLR5 and HWLR6 accessions, *P5CS2* expression also increased from three days after the onset of the drought treatment, but declined from four days after the onset of the drought treatment (Figure 4C,D). Therefore, proline could be biosynthesized more rapidly in LWLR4 and LWLR6, enabling these plants to adjust their osmotic pressure in the seedlings to protect against the water deficit.

In addition to proline and betaine, putrescine also plays an important role in the osmotic adjustment process. In plants, arginine decarboxylase (ADC) is an essential compound in biosynthesis of putrescine [34]. *ADC* expression was previously shown to be upregulated in *A. thaliana* under drought stress, while the overexpression of *ADC* can effectively improve drought resistance [35]. In our field experiment, *ADC* expression increased from three days after the start of the stress treatment but dropped the following day in LWLR6, HWLR5, and HWLR6 (Figure 4B,C,D). The *ADC* expression level remained relatively high at seven days after the treatment in LWLR4 in comparison with the HWLR lines. In the LWLR6 accession, however, *ADC* expression was higher than in the other accessions at three days after the onset of the drought stress treatment, and then remained stable at a relatively high level from four to seven days after the start of treatment (Figure 4B). These findings suggest that putrescine accumulates to much higher levels in LWLR4 and LWLR6 than in the HWLR lines because the relatively high *ADC* expression levels lasted longer in the LWLR accessions, which could assist the leaves in adjusting their osmotic pressure.

Thus, the marker genes showed better performance in the two LWLR accessions than in the two HWLR accessions, which corresponds to the phenotypic traits of these lines under drought treatment. This result indicated that WLR could be a useful indicator of drought-tolerant accessions.

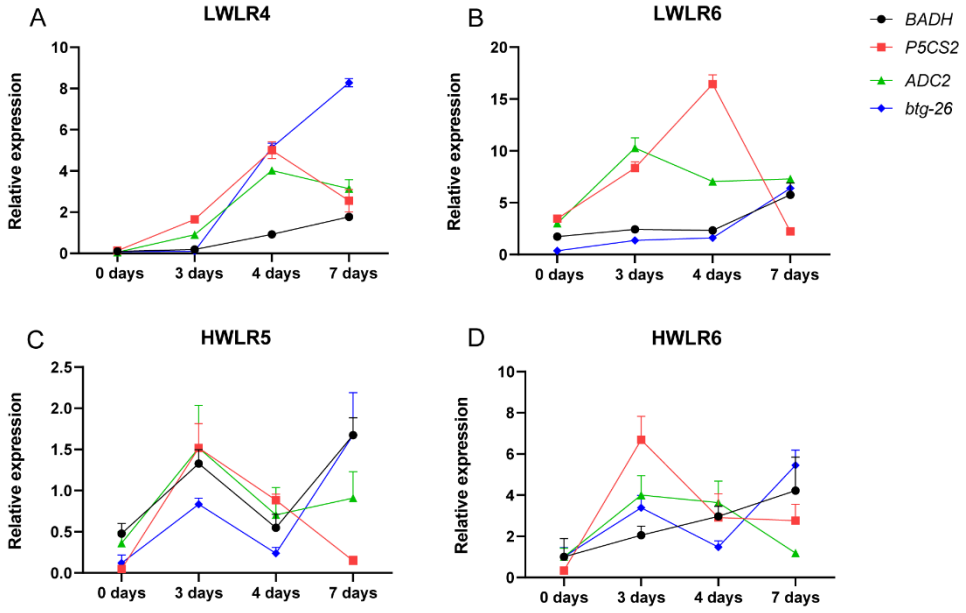

Figure 4. Expression patterns of drought-tolerant marker genes in two LWLR accessions and two HWLR accessions. The treatment panel was subjected to no watering and the control panel was well-watered. Error bars indicate the standard deviation of three technical replicates. 0, 3, 4, 7 days indicate the length of time after the onset of drought stress treatment. *BADH, Betaine aldehyde dehydrogenase*; *P5CS2, Delta 1-pyrroline-5-carboxylate synthetase*; *ADC2, Arginine decarboxylase*; *btg-26, Brassica turgor gene 26*.

3.4 Significant WLR-associated SNPs

To uncover SNPs with a significant association to the WLR trait and the quantitative loci associated with drought-stress tolerance, we performed a GWAS using EMMAX online software. A total of 78 SNPs associated with the WLR in the 0–24-h experiment ($-\log_{10}(p\text{-value}) > 5$) were screened, and 7 SNP–WLR associations were found to be over the significance threshold ($-\log_{10}(p\text{-value}) > 6$; Figure 5A,B; Table S5). These SNPs were unevenly distributed on chromosomes A01, A06, A03, A09, A10, C01, C02, C03, C04, C05, C07, C08, and C09, with more than half (41 of 78) located on chromosome A10. A total of 61 SNPs associated with the WLR in the 0–48-h experiment ($-\log_{10}(p\text{-value}) > 5$) were identified, and 5 SNP–WLR associations over the significance level ($-\log_{10}(p\text{-value}) > 6$) were identified (Figure 5C,D; Table S5). These SNPs were located on chromosomes A01, A02, A03, A06, A07, A08, A09, A10, C01, C02, C03, C04, C07, and C09, with almost half of them (26 of 61) also located on chromosome A10.

A total of 139 SNPs were above the suggestive threshold in both SNP–WLR association data sets, and 13 SNPs were above the significant threshold in both screens (Tables 4 and S5). A wide range of WLR-associated genetic loci were identified offering abundant basis for improving WLR-related agronomy trait.

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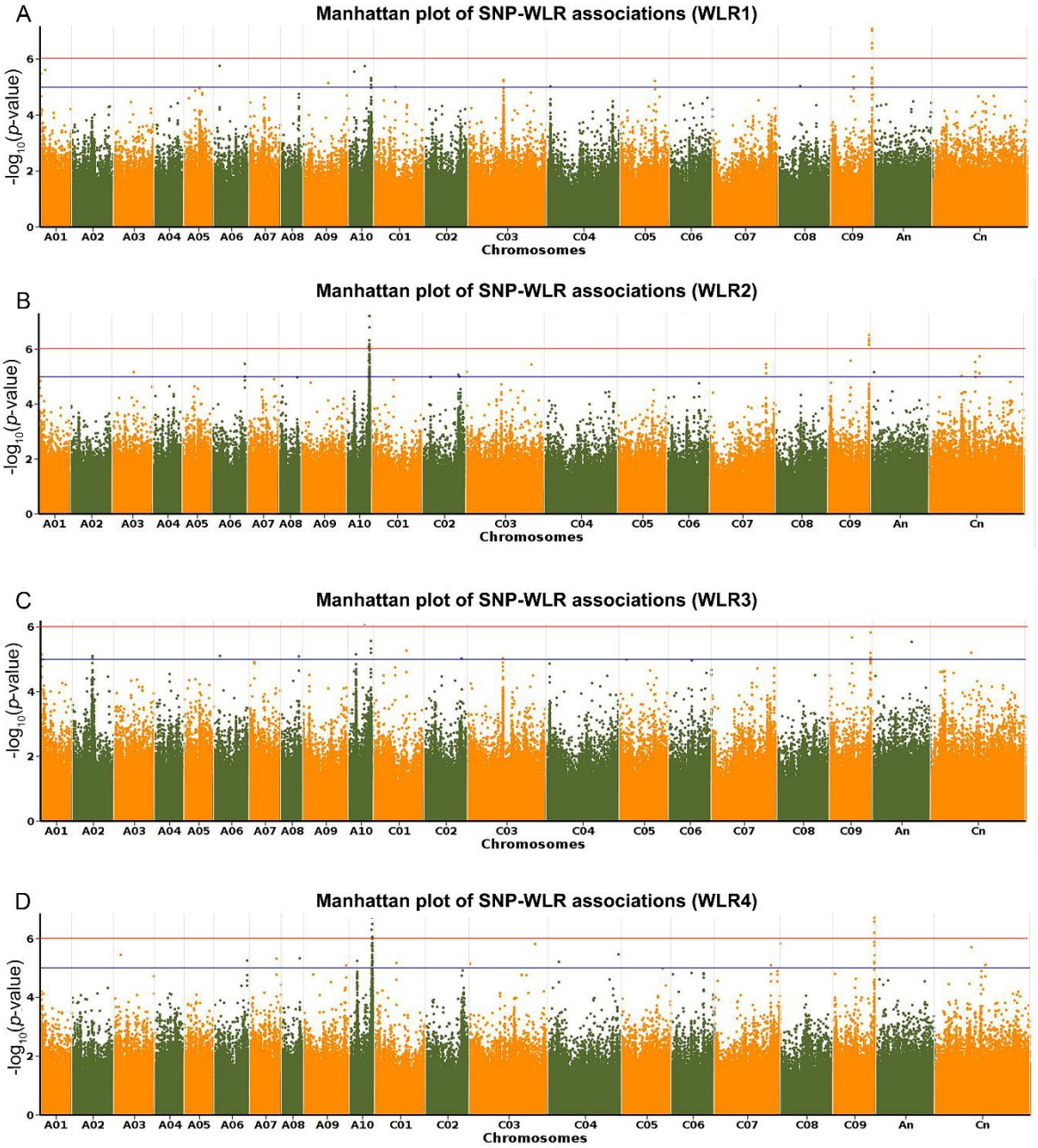

290 **Figure 5.** SNPs associated with the WLR identified using the EMMAX model. Manhattan plot
291 displaying significant SNPs associated with the WLR trait that were identified by performing GWAS
292 using the EMMAX model. Error bars indicate the standard deviation of three technical replicates. The
293 blue horizontal line indicates $-\log_{10}(p\text{-value}) > 5$ and the red horizontal line indicates $-\log_{10}(p\text{-value}) >$
294 6. WLR1 and WLR2: two WLR replicates for the 0–24 h period. WLR3 and WLR4: two WLR replicates
295 for the 0–48 h period.

296 **Table 4.** Summary of SNPs significantly associated with WLR

Phenotypic data	Chromosome	SNP location	$-\log_{10}(p\text{-value})$	Candidate genes	Orthologous gene in *Arabidopsis*	Literature
WLR1	C09	46268738	6.58			
	A10	14518988	6.08			
				BnaA10g21040D	AT5G11450	[51]
	A10	14797339	6.12			
	A10	14863060	7.21	*BnaA10g21880D*	AT5G65360	[72]
	A10	14865349	6.33			
WLR2	A10	14967680	6.19			
	A10	15040248	6.08			
	A10	15046760	7.21			
	C09	46221003	6.19	*BnaC09g46240D*	AT5G44050	[67]
				BnaC09g46300D	AT5G10360	[59]
	C09	46268778	6.38			
	C09	46270880	6.3			
WLR3	A10	10510902	6.1			
	A10	14518988	6.3			
				BnaA10g21040D	AT5G11450	[51]
	A10	14863060	6.72	*BnaA10g21880D*	AT5G65360	[72]
WLR4	A10	14865349	6.75			
	A10	15043924	6.06			
	C09	46221003	6.2	*BnaC09g46240D*	AT5G44050	[67]
				BnaC09g46300D	AT5G10360	[59]
	C09	46270880	6.71			

297

298

299 *3.5 Identification and Validation of the Candidate Genes Associated with WLR*

300 To identify the WLR-associated genes that may have an important role in improving plant
301 drought resistance, all the genes within the 75-kbp regions flanking each of the 13 SNPs
302 significantly associated with WLR were investigated. The expression patterns of the 180 genes
303 identified from the SNP-flanking regions were analyzed with the transcriptional expression
304 data from leaves of the 'ZS11' cultivar at 14 different growth stages, resulting in 36 genes with
305 a FPKM > 1 that were analyzed further (Figure S1). These 36 putative WLR genes were studied
306 in terms of their function in the response to drought stress. Using the BrassicaEDB database,
307 we established that four of the genes improved the capacity of drought resistance in Brassica
308 species, while two genes were negatively associated with drought stress (Table S6 and Figure
309 S2). These six genes were selected for further analysis.
310 To verify the function of the six putative candidate genes, we validated their expression
311 patterns in the two LWLR and two HWLR accessions across four timepoints (0, 3, 4, and 7 days
312 after the onset of the drought treatment) using RT-qPCR (Figures 6 and 7). The expression level
313 of *BnaC09.MATE* rose at three days after the start of the drought treatment in all four accessions,
314 but fell from four days post-drought stress, and was not detected at seven days after the start
315 of the drought treatment (Figure 6). The expression of *BnaA10.PPD5* was upregulated at three
316 days after the stress treatment started but declined the following day in LWLR6 and HWLR5.
317 By contrast, in HWLR6, *BnaA10.PPD5* expression increased until four days after the drought
318 treatment before declining at seven days after the treatment (Figure 6). These results indicate
319 that *BnaC09.MATE* and *BnaA10.PPD5* are induced early in the drought response and exhibit
320 peak expression levels for only one or two days, suggesting that they could provide early
321 protection against water scarcity.
322 The expression pattern of *BnaC09.EMB3010* in the two LWLR accessions clearly differed
323 from that in the two HWLR accessions. In LWLR4 and LWLR6, *BnaC09.EMB3010* expression
324 fell from a peak immediately after the drought treatment throughout the following seven days
325 (Figure 6A,B). In HWLR5, *BnaC09.EMB3010* expression was stable for the first four days after
326 the drought-stress treatment, decreasing slightly at seven days after the treatment (Figure 6C).
327 By contrast, *BnaC09.EMB3010* expression in HWLR6 rose continuously throughout the seven
328 days following the onset of drought treatment (Figure 6D). This phenomenon strongly suggests
329 that *BnaC09.EMB3010* was negatively regulated in the LWLR accessions following exposure to
330 drought stress, but either responded tardily or was positively regulated in the HWLR
331 accessions. *BnaC09.EMB3010* expression may therefore be closely related to drought tolerance.
332 The *BnaA10.PNTH* expression varied between the four accessions, implying that this gene
333 might respond to other stimuli and is therefore not a crucial candidate gene associated with
334 drought tolerance (Figure 6).
335 Similarly, the *BnaC09.Histone* expression pattern also showed an apparent difference
336 between the LWLR accessions and the HWLR accessions (Figure 7). In the two LWLR accessions,
337 *BnaC09.Histone* expression decreased at three days after the onset of drought treatment, but
338 subsequently increased. By contrast, *BnaC09.Histone* expression fluctuated for the first four
339 days after the onset of drought treatment then rose to peak at seven days after the onset of
340 treatment. These results demonstrated that *BnaC09.Histone* expression has a complex response

to drought stress, and that this gene may play an important role when *B. napus* is subjected to severe water deficiency.

BnaA10.Histone, which is a homolog of *BnaC09.Histone*, is expressed at very low levels in all four accessions, illustrating that it may not respond to drought stress in the selected genotypes (Figure 7).

In summary, we identified four candidate genes (*BnaC09.EMB3010*, *BnaC09.MATE*, *BnaA10.PPD5*, and *BnaC09.Histone*) that could confer drought tolerance to *B. napus* (Table 4).

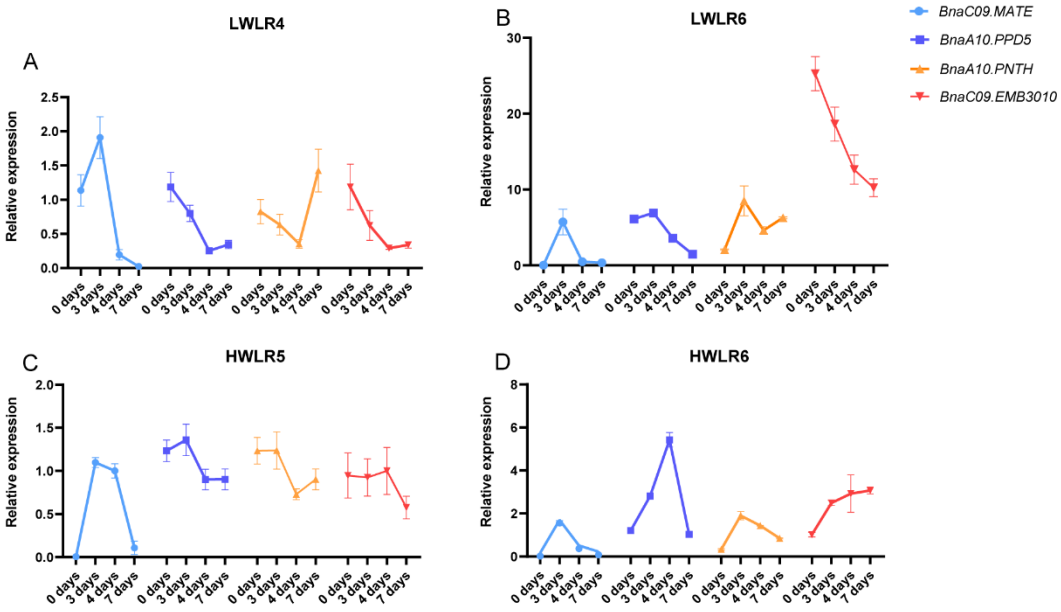

Figure 6. Expression patterns of four drought-upregulated candidate genes in the LWLR and HWLR accessions. Error bars indicate standard deviation of three technical replicates.

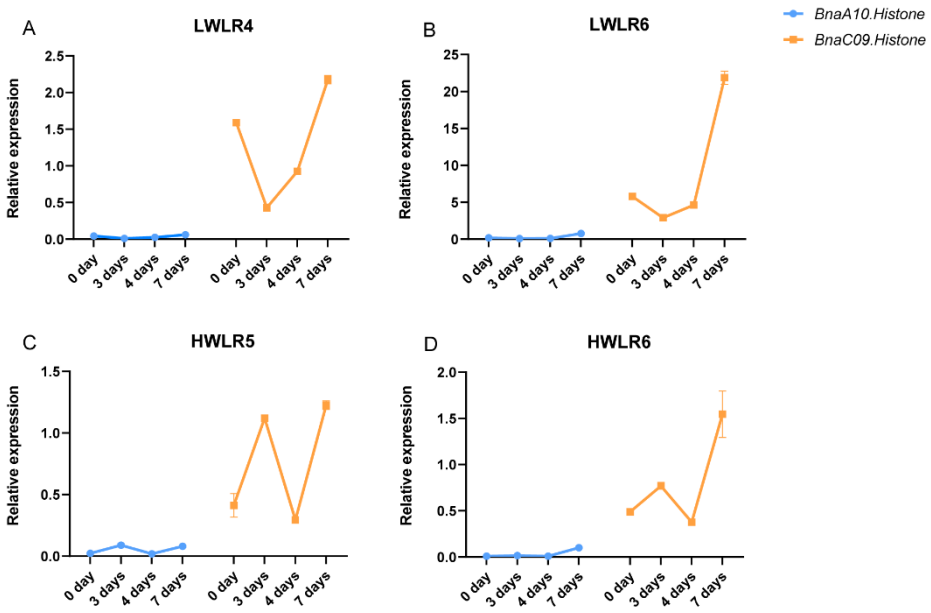

Figure 7. Expression patterns of two drought-downregulated candidate genes in the LWLR and HWLR accessions. Error bars indicate the standard deviations of three technical replicates.

4. Discussion

4.1 WLR is a Useful Index for Screening Potential Drought-tolerant Cultivars

Drought stress is a prevalent abiotic stress and causes severe reductions in crop yields [36,37]. *Brassica napus* is sensitive to water deficiency throughout its entire growth period [38], and breeders have searched for drought-tolerant germplasm with the ability to withstand water deficiency in the field. Drought-tolerant indices, such as water-use efficiency, drought susceptibility index, relative-vigor index, and the leaf-wilting index (LWI), facilitate the efficient identification of drought-tolerant accessions, and are widely used [39]. The LWI has a strong positive correlation with eight physiological indicators, which are crucial aspects of the physiological trait response to drought stress in *B. napus.* Four elite drought-tolerant *B. napus* accessions were identified using the LWI [40]. Due to the importance of preserving leaf water in dehydration, many indicators that reflect the leaf water loss have been employed to identify drought-tolerant species, such as the relative water content and leaf water content [41,42]. Drought-tolerant plants tend to have a good ability to preserve water in their leaves or ground cover, which can help the plants withstand drought conditions.

In our study, we used the leaf WLR as an indicator of drought tolerance to assess 265 accessions collected from around the world. Eight accessions with relatively low WLRs and six with relatively high WLRs were selected from this natural population and subjected to drought stress at the four-week-old seedling stage to validate their tolerance of drought. After seven days of drought stress, the eight LWLR accessions had higher fresh and dry weights than the six HWLR accessions. Three of the eight LWLR accessions showed no significant differences in fresh weight between the treatment and control groups, while five of the LWLR accessions showed no significant difference in dry weight under the treatment and control conditions (Figure 3A, B). By contrast, two of the six HWLR cultivars displayed no obvious difference in fresh weight between the drought-treated and control plants (Figure 3C). In addition, three HWLR accessions exhibited no difference in dry weight when subjected to drought stress (Figure 3D). Furthermore, four known drought-response genes were more highly expressed in the two LWLR accessions than in the two HWLR accessions (Figure 4). Overall, our results showed that the WLR can be used to identify drought-tolerant germplasms from a natural population, and that it is therefore a useful physiological indicator for selecting for drought tolerance during breeding.

4.2 Identification of SNP–WLR Associations using GWAS

The dehydration imposed by drought stress results in a severe reduction of the *B. napus* biomass and seed yield [22]. Drought-tolerant cultivars have been selected in breeding programs over the past few decades; however, conventional advances in the identification of drought-tolerant germplasm have not been sufficient to meet the current demand for oilseed production. Dissecting drought-tolerant QTLs and unraveling water deficit-responsive genes has thus become a new objective for enhancing drought tolerance in *B. napus*.

In this study, we performed a GWAS to dissect the SNP–WLR associations. We identified 13 significant SNPs located on chromosomes A10 and C09 of the *B. napus* genome. In a previous study, nine, eleven, and nine QTLs were respectively found to be associated with the drought-tolerant index, shoot biomass accumulation, and flowering time under rainfed conditions [43]. Of these, three QTLs were located on chromosome A10 and two on chromosome C09. One QTL responsible for shoot

biomass, 4108375, is located 21.4 kbp away from the Bna-A10-4948281 SNP site identified here, which suggests that the QTLs of WLR and shoot biomass may partially overlap. Previously, the stress tolerance index and stress susceptibility index were employed to identify the SNPs associated with drought stress in *B. napus* using a GWAS [24]. In that study, 577 SNPs associated with the stress tolerance index and the stress susceptibility index were screened, resulting in the identification of 17 SNPs located on chromosome A10 and 57 SNPs located on chromosome C09. None of these SNPs were found to overlap with those identified in the present study. The SNP loci identified here could therefore enhance our understanding of the genetic mechanisms underpinning drought tolerance in *B. napus*, but further validation of these data is needed.

4.3 Candidate Genes Contributing to Stomatal Closure via the ABA-dependent or ABA-independent Drought-response Pathways

Plants must regulate their stomatal opening in response to stress because, besides from allowing CO_2 into leaf cells, open stomata facilitate rapid water loss; thus, the reduction in water loss through stomatal closure is a critical physiological response of plants to drought stress [44,45]. Chloroplasts are considered to be an important intracellular site for abiotic stress responses in plants, since a significant amount of reactive oxygen species (ROS) are produced in these organelles [46]. ROS and ABA are signaling molecules that can mediate stomatal closure under stress conditions such as drought [47]. The ABA signaling pathway involves three core components: the intracellular ABA receptors (PYLs), type-2C protein phosphatases (PP_2Cs), and SNF1-related protein kinase 2 ($SnRK_2$) [48]. ABA interacts with an intracellular PYL receptor, resulting in the inhibition of PP2C activity and the depression of SnRK2s to activate the downstream proteins that mediate stomatal closure and other ABA responses [49]. OST1 is one of the SnRK2s that is stimulated by ABA, and controls stomatal movement by phosphorylating various substrates [50]. Here, we reported that a candidate gene, *BnaA10g21040D*, which encodes an ortholog of *A. thaliana* PPD5, displays different expression patterns in the drought-tolerant and drought-sensitive *B. napus* lines under drought stress, with a significant downregulation observed in the drought-tolerant lines. Hong et al. [51] observed that PPD5 in *A. thaliana* negatively regulates drought resistance by modulating H_2O_2 accumulation in the guard cells via an OST1-dependent pathway. The *ppd5* mutants had improved H_2O_2 accumulation in the guard cells and enhanced stomatal closure under drought stress. PPD5 possesses a PsbP domain, N-terminal transit peptide domain for plastid localization, and a C-terminal domain. OST1 functions near the chloroplast (potentially the cytoplasmic side of the chloroplast membrane) to phosphorylate PPD5 at the C-terminal domain and increase its protein stability [51]. Protein phosphorylation in the chloroplast rather than the regulation of the photosynthetic light reaction was also suggested to be the strategy by which the chloroplast enables plants to withstand environmental stresses [52]. OST1-mediated PPD5 phosphorylation could be a stress acclimation mechanism by which the drought stress signals are transduced into chloroplast actions for stomatal regulation. The stronger interaction of the phosphorylated PPD5 with OST1 may sequestrate OST1 in the chloroplast membrane [51], thus preventing it from phosphorylating its other substrates, including the plasma membrane-localized RbohF (NADPH oxidase), which leads to the production of apoplastic O_2^- and H_2O_2 [53]. The higher accumulation of ABA in *ppd5* mutants under drought conditions suggests that PPD5 may be involved in regulating ABA metabolism [51]; however, further experiments are required to investigate this role.

The other three candidate genes we identified encode a ribosomal protein (RP), a multidrug and toxic compound extrusion (MATE) protein, and a histone superfamily protein, respectively. RPs are involved in many crucial functions, including ribosome assembly, protein translation, and other basic

cellular functions that significantly affect cellular energy homeostasis [54]. The role of RPs in plants has also been studied in the response to environmental stresses [55]. RPs comprise two subunit proteins, known as small (RPS) and large subunit (RPL) proteins. RIBOSOMAL PROTEIN SMALL SUBUNIT 6 (RPS6) is located in the 40S subunit of cytosolic ribosomes, and is the main target of the TOR signaling pathway. Under favorable conditions, TOR promotes the activation of RIBOSOMAL S6 KINASE (S6K), which phosphorylates RPS6 [56]. Various environmental stimuli affect the phosphoprotein RPS6; for example, heat shock and oxygen deprivation promote the dephosphorylation of RPS6 [57,58]. Under unfavorable conditions, ABA induces the SnRKs, which inactivate the TOR kinase and ultimately reduce RSP6 phosphorylation [56]. Cell size reduction, delayed growth, and delayed flowering were observed in the *Arabidopsis rps6* mutant [59]. In the current study, the expression level of *RPS6B,* also referred to as *EMB3010,* a putative *B. napus* homolog of one of the two *Arabidopsis RPS6* paralogs, was reduced in the drought-tolerant lines under drought stress.

MATE transporter proteins, also called detoxification efflux carriers (DTX), are involved in the metabolism of the toxic compounds and organic acids. Topologically, MATE transporters contain 12 transmembrane helices arranged in two bundles, with long C- and N-terminal extensions [60,61]. MATE proteins generally exist in two conformations, straight or bent, which are determined by the protonation state of the acidic residues [62]. Hydrophobic residues are often found surrounding the substrate-binding cavity, and might provide the appropriate level of affinity for the association and dissociation of a substrate [63]. Different MATE transporters were reported to play a role in drought tolerance by regulating stomatal closure through different pathways, including AtDTX56 [64], AtDTX33, AtDTX35 [65], and AtDTX50. Mutation of *DTX50* promotes the accumulation of ABA in the guard cells, resulting in rapid stomatal closure [66]. AtDTX28 is a putative flavonol transporter [67]; a role for flavonols in suppressing H_2O_2 accumulation and stomatal closure has been explored. Ethylene-induced accumulation of flavonols in guard cells suppressed ROS accumulation and reduced ABA-dependent stomatal closure [68].

Gene expression is influenced by chromatin structure, which is controlled by processes often linked with epigenetic regulation, including DNA methylation and post-translational histone modifications [69]. Histone proteins are modified through N-terminal tails after translation and these modifications delineate the gene expression level by altering the strength of DNA histone interaction or recruitment of non-histone proteins. Drought-induced regulation of gene expression is linked with alterations in histone modification pattern [70,71]. In our current study, we detected variations in expression pattern of histone family protein under drought stress. Further investigation is required to identify the specific roles of this protein in the plant's response to drought.

Despite these advances, no study has directly addressed the roles of these RPs, MATE protein, and histone superfamily protein in drought stress. In the current study, a preliminary RT-qPCR validation was performed; however, future studies should further validate the roles of these candidate genes and explore their functions in the drought stress response. Overall, this research provides a valuable source for the study of a drought tolerance mechanism related to the WLR trait in *B. napus*. The detected SNP loci and candidate genes will be useful for future investigations of drought tolerance in rapeseed. Furthermore, an integrative approach using WLR as a drought tolerance index appears to be a viable strategy for detecting drought-tolerant germplasm.

Supplementary Materials:

Supplementary materials can be found at www.mdpi.com/xxx/s1. Table S1: Primer pairs of drought-tolerant maker genes for RT-qPCR analysis; Table S2: Normal distribution test of four WLR data sets; Table S3: Variance homogeneity test of two time period WLR data; Table S4: Information of 14 selected accessions for drought stress treatment; Table S5: Summary of SNPs significant associated with WLR trait; Table S6: Transcriptional expression data of 4 candidate genes in drought stress treatment; Figure S1: Transcriptional expression of 36 genes screened by combing GWAS results and transcriptome profile; Figure S2 Transcriptional expression of six putative candidate gene under drought treatment.

Author Contributions:

K.L. and C.Q. conceived the experiments and methodology; A.S., M.Q., B.S., U.M., S.T. and Y.H. performed the experiments, measurements and analyses; W.C., L.D. and H.Z. curated and analyzed the data; A.S., M.Q. and K.L. wrote the manuscript draft; J.L. and C.Q. reviewed, edited and completed the manuscript; K.L. supervised the whole work. All authors have read and agreed to the published version of the manuscript.

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