INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0095/201x/00–0–000–000
DOI: 10.17957/IJAB/15.1089
http://www.fspublishers.org

Full Length Article

Differential Expression of the MicroRNAs are Responsive to Drought Stress and Exogenous Methyl Jasmonate in Wheat (*Triticum aestivum*)

Chao Ma†, Jun Zhang†, Jiale Yuan, Jingru Guo, Ying Xiong and Yalan Feng*
[1]*College of Agriculture, Henan University of Science and Technology, Luoyang 471023, Henan province, P.R. China*
[2]*Dry-Land Agricultural Engineering Technology Research Center in Henan, Luoyang 471023, Henan province, P.R. China*
*For correspondence: fengyalan@haust.edu.cn; fengyalan2004@163.com
†These authors contributed equally to this work

Abstract

Wheat is an important food crop in the world, and its root system perceives stresses mainly, however, the molecular mechanism of wheat root responses to drought stress are still poorly understood. Jasmonic acid (JA) and its derivative methyl jasmonate (MeJA) participate in multiple biological processes in plant. Exogenous JAs could improve the drought tolerance of various species. The microRNAs (miRNAs) play a significant role in post-transcriptional regulation as well as responding to many adversity stresses. In this study, the sec generation high throughput sequencing technique was employed to identify the drought- and MeJA-response miRNAs, so as to unravel the underlying molecular mechanism. Differentially expressed miRNAs were screened from comparison of treatments and control. These miRNAs could regulate expression of their target genes and might play essential role in the drought tolerance. The screened miRNAs and target genes would be the focus of future study to uncover the molecular mechanism of wheat root responses to drought stress. © 2019 Friends Science Publishers

Keywords: Wheat; Drought; Methyl jasmonate; Deep sequencing; MicroRNAs

Introduction

Jasmonic acid (JA) and its derivative methyl jasmonate (MeJA) are a class of plant endogenous hormones that participate in multiple biological processes, such as germination, senescence, fruit ripening, and response to environmental stress (Creelman and Mullet, 1997; Ma *et al.,* 2014). Previous studies have found that the contents of endogenous JAs would be significantly increased in many plants under drought stress, while exogenous applications of JAs can noticeably improve the drought tolerance of many plants (Anjum *et al.,* 2016). In addition, exogenous applications of JAs had a mitigating effect on salt, heat and cold (Ma *et al.,* 2014), even and copper stresses (Sirhindi *et al.,* 2015). It has been postulated that JAs were involved in many common signal transduction pathways to stress and induced the "self-adaptability" of plants. However, the molecular mechanisms of JAs-induced stress tolerance are poorly understood.

Endogenous miRNAs (usually 20–25 nucleotides in length) are single-stranded RNAs, widely distributed in viruses, animals and plants, and play a vital role in post-transcriptional regulation as well as responding to many adversity stresses (Bartel, 2004). The miRNAs degrade or inhibit transcription by binding to target mRNAs, thereby regulating target genes expression (Zhu *et al.,* 2008). At present, studies on plant miRNAs are mostly concentrated in *Arabidopsis*, rice, *Medicago truncatula*, and maize (D'Ario *et al.,* 2017). Zhang *et al*. (2005) studied the wheat miRNA since 2005. More and more wheat miRNAs have been identified along with the development of high-throughput sequencing technology. Nevertheless, little is known about miRNAs related to wheat drought resistance and exogenous MeJA.

Wheat is an important food crop in the world, and its growth and yield are closely related to the adequacy of water during the growth period. In the environment of plant growth, several stresses are sensed by the root system, as for example, drought, salt, nutrient deficiency and heavy metal. In general, the sensitivity of roots to stress response limits the plant growth (Miao *et al*., 2015; Huang *et al*., 2017; Zhang *et al.,* 2018). Therefore, better understanding of the molecular mechanism of response to drought stress in wheat root would be helpful to develop drought-resistant varieties.

In order to understand the molecular mechanism of exogenous MeJA to enhance the drought tolerance of wheat, the miRNAs of wheat roots in the process of drought and exogenous MeJA were sequenced by the sec generation high throughput sequencing technique. The miRNAs related to drought resistance were identified through bioinformatics analysis, target gene prediction and miRNA expression pattern analysis.

To cite this paper: Ma, C., J. Zhang, J. Yuan, J. Guo, Y. Xiong and Y. Feng, 201x. Differential expression of the micrornas are responsive to drought stress and exogenous methyl jasmonate in wheat (*triticum aestivum*). *Intl. J. Agric. Biol*. 00: 000-000

Materials and Methods

Plant Materials

Zhoumai18, a drought tolerant wheat cultivar, was selected as experimental material,which was bred and provided by Zhoukou Academy of Agricultural Sciences, as well as it is one of the largest wheat varieties in China. Seeds were disinfected with 70% alcohol for 1 min and washed with sterile water 3 times, and then disinfected with 0.1% $HgCl_2$ for 6 min, sterile water rinse 3–4 times. Placed the disinfected seed on the floating net and placed it in distilled water, cultured in the dark 25°C to germinate.

After 3 days the germinated seeds were transferred into the artificial climate chamber, with 12 h photoperiod, 25/20°C day/night temperatures, and relative humidity 70%. The one-leaf stage seedlings were selected and transplanted to the container containing nutrient solution (pH 6.6–6.8, 2 L), 60 seedlings per pot and ventilated for 30 min every day. The nutrient solution was changed every 3 d.

MeJA Treatments on Growth and Drought Tolerance of Wheat Seedling

Seedlings of the two-leaf stage were used in this study, transferred them to the nutrient solution containing different concentrations of MeJA for 6 d, 0 (control), 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8 μM. The nutrient solution was changed once a day. The test set three replicates. After screening, 0.25 μM was determined as the best treatment concentration.

Then four different treatments were carried out at the same time, respectively. Treatment control: nutrient solution; MeJA: nutrient solution contains 0.25 μM MeJA; drought: nutrient solution contains 20% PEG; MeJA + D: nutrient solution contains 20% PEG and 0.25 μM MeJA. The roots of seedlings were collected after 24 h of treatment, rinsed with distilled water and quickly freezed in liquid nitrogen.

Determination of MDA Content

MDA content was determined by thiobarbituric acid (TBA) method described by Dhindsa *et al*. (1981) A 0.5 g of the fresh plant tissue was used to determine the TBA reactive substances (TABARS).

Total RNA Extraction and Reverse Transcription

Total of 100 mg roots from each treatment were harvested for total RNA extraction. Total RNA was isolated in accordance with the manufacture's instruction of RNAiso Plus kit (TaKaRa, China). The integrity of RNA was detected by 1.0% agarose gel electrophoresis. Total RNA was reverse-transcribed into cDNA by PrimeScript RT kits (TaKaRa, China).

Small RNA Isolation, Library Construction, and Sequencing

The genomic DNA was removed with RNase-free DNase I for 30 min at 37°C. Total RNA was separated on 15% denaturing PAGE, and the 18–30 nt strips were gel purified according to the marker for subsequent sequencing. The 18–30 nt small RNA fragments were bonded with adaptor through T4 RNA ligase and then reversely transcribed to construct a sequencing library. HiSeq deep sequencing was used to sequence the sRNAs in the four samples.

Data Processing and Bioinformatics Analysis

The data from HiSeq sequencing were dealt with software developed by the Beijing Genomics Institute (BGI). The 50 nt sequence tags from HiSeq sequencing would go through the data cleaning, summary of length distribution, and standard bioinformatics analysis. The clean tags were annotated into various categories and used to predict the novel miRNAs and their targets.

Classification of gene function was performed by Gene Ontology (GO), which is an international standardized system, with supplying a range of controlled term to comprehensively describe the attribute of genes and their products. Detailed analysis can refer to the method of Feng *et al.* (2016).

qRT-PCR Validation

16 miRNAs were selected to confirm the expression level through qRT-PCR. By using miRNA specific stem-loop reversely transcribed primer and enzyme (TaKaRa, China), mature miRNA was reversely transcribed into cDNAs. A 20 μL reaction system contained 10 μL SYBR® Premix Ex Taq II (TaKaRa, China), 0.4 μM of both forward and reverse primers and 100 ng cDNA template. qRT-PCR was performed in triplicate with Bio-Rad iQ5 instrument (Biorad, USA) following the protocol: initial denaturation at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and finally at 60°C for 20 s. The fold variation of expressed gene was calculated by $2^{-\triangle\triangle}$Ct method (Livak and Schmittgen, 2001). β-actin was taken as an internal control.

Statistical Analysis

Statistical analysis was carried out by using the *SPSS* software (*version 13*, *SPSS*, Chicago, IL, USA). Differences between means of treatments were performed by the *Duncan*'s test with means considered significantly different at $p<0.05$.

Results

Morphological Changes and Growth Parameters of Wheat Seedlings under Exogenous MeJA

The effects of different concentrations of exogenous MeJA on wheat morphology were significant (Fig. 1). The growth

of wheat seedlings was significantly inhibited from 0.5 μM MeJA with the increase of the application concentration, especially from 1 μM, the length of the new leaf decreased, significantly; even the new leaf did not grow under 8 μM treatment. In addition, the root length, root number and root branch were significantly reduced from 0.5 μM.

Table 1 showed the effect of exogenous MeJA on the growth parameters of wheat seedlings. There was no significant difference in plant height between 0.03–0.25 μM and the control, but under 0.5–8 μM treatments, the plant height compared to the control decreased from 87.1 to 74.4%, respectively. Root length and dry weight (DW) were similar to plant height, under 0.5–8 μM the root length decreased from 82.7 to 75.6% and the DW decreased significantly from 91.4 to 68.8% compared with the control, respectively.

Plant growth could be promoted under low concentrations of exogenous MeJA, although inhibited under high concentrations (Anjum *et al.,* 2016). In order to explore the appropriate concentrations of exogenous MeJA, the content of malondialdehyde (MDA) was detected. As the production of membrane peroxidation, MDA reflects the state of oxidation in cell. Compared with the control, the content of MDA in wheat roots decreased with the increase of MeJA concentration, and decreased to the lowest level at 0.25 μM which was an appropriate concentration for exogenous MeJA (Fig. 2).

Effect of MeJA on Wheat Seedling Drought Tolerance

Three different treatments showed different drought tolerance, including MeJA, drought, and MeJA + D. The difference between MeJA treatment and control was not obvious (Fig. 3). The wheat seedling grown under drought condition showed obvious wilting, decreased plant height, and most of the leaves were yellow and dead, however, the above situations were alleviated under MeJA + D treatment (Fig. 3). Only from the phenotypic point of view, exogenous application of 0.25 μM MeJA significantly improved the drought resistance of wheat.

Global Analysis of sRNAs from Control, MeJA, Drought, and MeJA + D Treatment Groups

The sec generation sequencing technique Solexa was used to construct libraries in order to detect the expression levels of miRNA. Tens of millions of sequences were obtained from the four treatments (Table 2). It was worth noting that the number of total sRNAs and total unique sRNAs were the largest under MeJA + D treatment, followed by MeJA. This suggested that there was a broader regulation of sRNAs at the post-transcriptional level under MeJA + D treatment.

The length of these sequences was distributed at 18–29 nt, accounting for 99.19, 96.7, 98.32 and 98.82% of the total sequencing, respectively (Fig. 4). The proportion of 24 nt was the highest among the lengths. The sequences with a length of 21 nt represented the sec category.

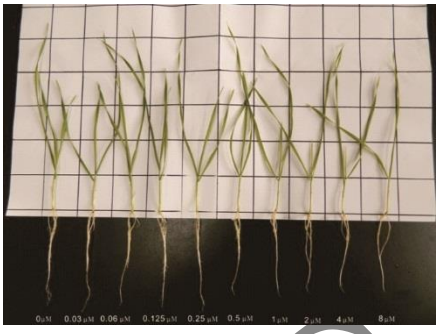

Fig. 1: Morphological changes of wheat under increased concentrations of exogenous MeJA levels

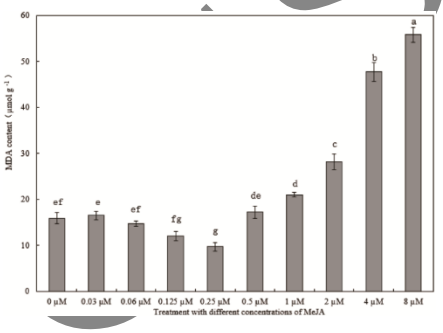

Fig. 2: Effects of different concentrations of MeJA on MDA content in roots of wheat seedling

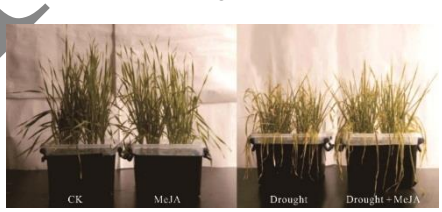

Fig. 3: Morphological changes of wheat under control, MeJA, drought and MeJA + D

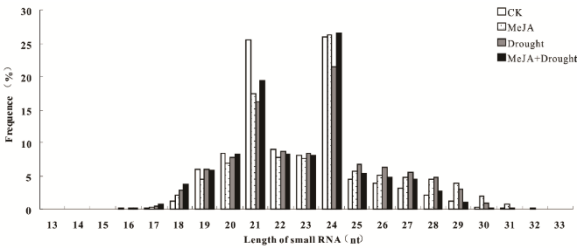

Fig. 4: Length of small RNA in different treatments

However, the percentages of these two lengths under drought treatment were the lowest compared to other treatments.

Comparison of Differentially Expressed miRNAs between Control and MeJA, Control and Drought, Control and MeJA + D Treatments

The differentially expressed miRNAs of control, 0.25 μM MeJA, drought, and MeJA + D treatments were compared,

Table 1: Effect of different concentrations of exogenous MeJA on growth characteristics of wheat seedling

Treatments	Plant height (cm)		Root length (cm)		Dry weight (g plant^{-1})	
	M±SE	Change (%)	M±SE	Change (%)	M±SE	Change (%)
CK	31.7±0.503 a	100	15.6±0.150 a	100	0.0651±0.0005 a	100
0.03 μM	31.6±0.436 a	99.7	15.9±0.196 a	101.9	0.0644±0.0023 a	98.9
0.06 μM	31.3±0.569 a	98.7	15.7±0.093 a	100.6	0.0637±0.0009 a	97.8
0.125 μM	31.4±1.212 a	99.1	15.6±0.135 a	100	0.0644±0.0006 a	98.9
0.25 μM	30.9±1.286 a	97.5	15.6±0.150 a	100	0.0630±0.0011 a	96.8
0.5 μM	27.6±0.265 b	87.1	12.9±0.106 b	82.7	0.0595±0.0007 b	91.4
1 μM	27.3±0.458 b	86.1	12.7±0.153 bc	81.4	0.0546±0.0009 c	83.9
2 μM	26.4±0.451 b	83.3	12.5±0.061 bc	80.1	0.0532±0.0007 c	81.7
4 μM	24.1±0.418 c	76	12.3±0.100 c	78.8	0.0497±0.0012 d	76.3
8 μM	23.6±0.318 c	74.4	11.8±0.160 d	75.6	0.0448±0.0004 e	68.8

and the log2 ratio of normalized expression between the pairwise comparisons was taken as standard, i.e., more than 1 or less than -1. Compared to the control, there were 189, 186, and 228 miRNAs significantly upregulated in MeJA, drought, and MeJA + D treatments, respectively, as well as, 182, 230, and 180 miRNAs significantly downregulated. In addition, 43 and 102 miRNAs showed common up-regulation and downregulation in all three treatments, respectively (Fig. 5).

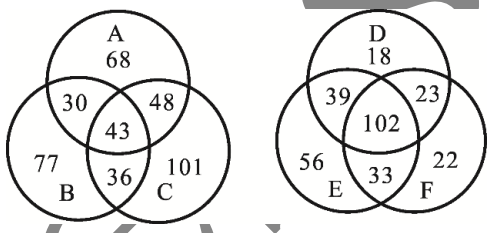

Fig. 5: Differentially expressed miRNA among different treatments. The up-regulated miRNAs in CK and MeJA(A), CK and Drought(B), CK and MeJA + D (C); the downregulated miRNAs in CK and MeJA (D), CK and Drought (E), CK and MeJA + D (F)

Validation of Differentially Expressed miRNAs and GO Analysis of Predicted Target Genes

In order to validate the results of high-throughput sequencing, the expression patterns of 16 miRNAs detected by quantitative real-time PCR (qRT-PCR). The results of qRT-PCR were consistent with the sequenced results (Table 3 and Fig. 6).

We predicted target genes for sequenced miRNAs, and GO classification were used to classify the functions of the predicted target genes which fell into 23 functional sets (Fig. 7). In these categories, "binding", "metabolic process" and "intracellular part" predominate, but fewer targets in the "antioxidant activity", "photosynthesis", and "extracellular region".

Validation of Candidate miRNAs Effects on Potential Target Genes

The predicted target genes were analyzed by Blast, and 8 miRNAs were screened out, which might play primary role in enhancing drought tolerance, the expression of these miRNAs varied among the four treatments. Figs. 6 and 8 showed the expression of these screened miRNAs and predicted targets.

The predicted target gene of miR1166 was calcium-dependent protein kinase 2 (CPK2). Compared with the control, miR1166 was down-regulated in roots treated with MeJA, drought and MeJA + D, and its target gene was up-regulated compared to the control (Figs. 6 and 8), which was consistent with the effect mechanism of miRNA and target gene. The expression patterns of some miRNAs and their target genes were the same as miR1166 and its target gene, including miR163, miR5809, miR3711, miR5565, miR2275,

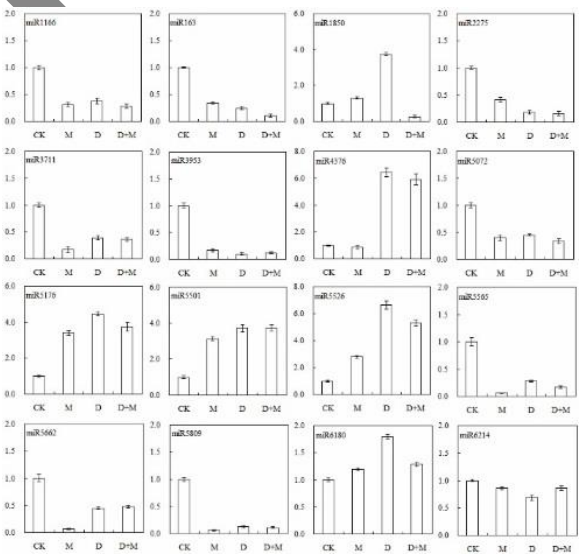

Fig. 6: Validation of the expression patterns of 16 miRNAs in different treatments

and miR6214. However, the expressed patterns of miR6180 and its target gene were opposite to those of miR1166. The expression of miR6180 was up-regulated in roots under different treatments, and its target gene was down-regulated (Figs. 6 and 8). These predicted target genes were mainly involved in signal transduction, hormones, tricarboxylic acid cycle (TCA), transport, stress response, and carbon metabolism in plants.

Table 2: Distribution of small RNA

Treatments	Category	Unique sRNAs	Total sRNAs
CK	Total	4773899(100%)	18433566 (100%)
	miRNA	64253 (1.35%)	3772752 (20.47%)
	rRNA	236117 (4.95%)	2448874 (13.28%)
	Repeat	73790 (1.55%)	344559 (1.87%)
	snRNA	4075 (0.09%)	20564 (0.11%)
	snoRNA	2030 (0.04%)	6402 (0.03%)
	tRNA	80809 (1.69%)	870767 (4.72%)
	Unann	4312825 (90.34%)	10969648 (59.51%)
MeJA	Total	5545535 (100%)	20560147 (100%)
	miRNA	75780 (1.37%)	2121973 (10.32%)
	rRNA	293444 (5.29%)	2875853 (13.99%)
	Repeat	79888 (1.44%)	380271 (1.85%)
	snRNA	5420 (0.1%)	26439 (0.13%)
	snoRNA	2866 (0.05%)	9087 (0.04%)
	tRNA	100067 (1.8%)	1369439 (6.66%)
	Unann	4988070 (89.95%)	13777085 (67.01%)
Drought	Total	427756 (100%)	17891454 (100%)
	miRNA	67277 (1.57%)	2157153 (12.06%)
	rRNA	284088 (6.64%)	3011869 (16.83%)
	Repeat	58587 (1.37%)	280633 (1.57%)
	snRNA	3669 (0.09%)	14404 (0.08%)
	snoRNA	1755 (0.04%)	4761 (0.03%)
	tRNA	85835 (2.01%)	1196047 (6.69%)
	Unann	3776545 (88.28%)	11226587 (62.75%)
MeJA + D	Total	6806738 (100%)	27062892 (100%)
	miRNA	90427 (1.33%)	3172731 (11.72%)
	rRNA	269847 (3.96%)	3624510 (13.39%)
	Repeat	91911 (1.35%)	490772 (1.81%)
	snRNA	5266 (0.08%)	35458 (0.13%)
	snoRNA	2450 (0.04%)	7687 (0.03%)
	tRNA	96267 (1.41%)	1890082 (6.98%)
	Unann	6250570 (91.83%)	17841652 (65.93%)

in different treatments

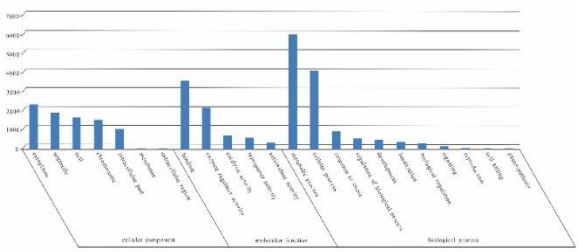

Fig. 7: GO analysis of the predicted targets for sequenced miRNAs in three ontologies: biological process, molecular function and cellular component

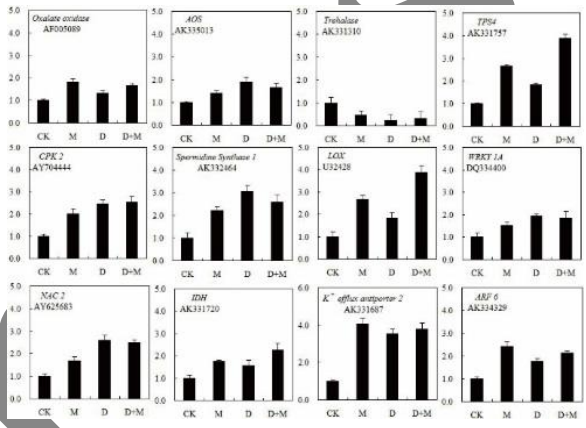

Fig. 8: Expression patterns of screened target genes in different treatments

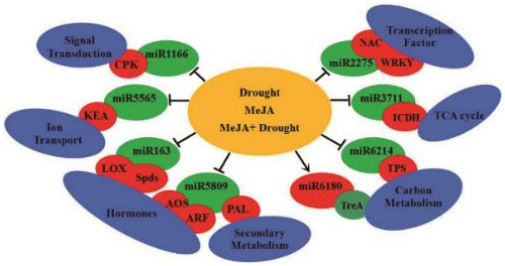

Fig. 9: A supposed regulatory mechanism of wheat root drought tolerance. The screened differentially expressed miRNAs and their predicted target genes. Red indicates up-regulation and green indicates down-regulation. Blue indicates the involved metabolic pathways. For instance, the expression of miR163 and miR5809 was down-regulated, and their predicted target genes were up-regulated, which encode the same or similar class protein, including LOX, Spds, AOS and ARF, they participate in hormones metabolism. In addtion, another predicted target gene of miR5809 also encodes the PAL, which involves in the secondary metabolism

Discussion

The regulation effect of exogenous hormones on plant growth and stress tolerance depends on the concentration and mode of application, as well as the growth situation of plant (Kang *et al.,* 2012). Choosing the appropriate concentration of MeJA is the primary issue, once exceeding the applicable concentration range would impact plants growth (Ma *et al.,* 2014). For example, spray application with 10 μM and 0.5 μM MeJA can significantly enhance the drought resistance of broccoli and wheat (Wu *et al.,* 2012; Anjum *et al.,* 2016). Exogenous MeJA might induce oxidative stress in plants, at relatively low concentrations, the stress response is mild, and the plant cells are in a mildly oxidized situation, which is similar to the process of plant stress adaptation and can activate the cellular antioxidant system. However, high concentrations of exogenous MeJA will cause severe oxidative stress, leading to irreversible oxidative damage (Danaee *et al.,* 2015). In this experiment, phenotypic and growth parameter data indicated that exogenous MeJA would significantly affect the growth of wheat with dose effects. More than 0.5 μM concentration significantly inhibited plant height, root length, and DW. The 0.25 μM concentration produced mild oxidative stress, which resulted in significant decrease in MDA content in wheat roots, and over 0.25 μM would cause excessive membrane lipid peroxidation in wheat root cells. It indicated that 0.25 μM was an optimal application concentration by the application method of nutrient solution addition. Oxidative stress induced by excessively high MeJA concentration (more than 0.25 μM) severely inhibited the growth of wheat, while other lower concentrations (0.03–0.125 μM) did not significantly affect wheat plants.

Table 3: Expression results of sequenced miRNA in different treatments

miRNAs ID	fold-change Log 2			Predicted target
	MeJA/CK	Drought/CK	MeJA+D/CK	
miR157	-0.42	-1.18	-0.43	calcium-dependent protein kinase 9
miR163	-14.68	-8.39	-2.31	spermidine synthase 1-like protein
				photosystem II polypeptide
				lipoxygenase
miR444	-0.07	-0.04	-0.06	MADS-box transcription factor
miR854	1.15	-1.3	-0.06	ABA-responsive protein
miR1048	0.93	-1.02	-1.64	ammonium transporter
miR1166	-13.55	-13.55	-9.38	calcium-dependent protein kinase
miR2275	-5.35	-5.16	-5.19	senescence-associated protein
				pyruvate dehydrogenase E1 component subunit
				Na^+/H^+ antiporter precursor
				calmodulin TaCaM4-1
				WRKY transcription factor
miR2594	0.09	1.25	0.19	auxin transporter-like protein
				protein WAX2-like
				nucleobase-ascorbate transporter
				S-type anion channel SLAH3-like
				BURP domain-containing protein
				chlorophyllase-1-like
				ethylene constitutive triple response protein
miR3440	2.46	3.45	3.16	DREB transcription factor 4C
				stress-associated endoplasmic
				phenylalanine ammonia-lyase-like
				DREB transcription factor 4A
				heat shock protein
				heat shock protein HSP26
miR3441	-4.01	-15.94	-15.94	AP2-like ethylene-responsive transcription factor
miR3516	-15.67	-15.67	-11.46	pyruvate dehydrogenase E1 component subunit alpha-2
miR3711	-16.04	-9.98	-11.58	isocitrate dehydrogenase
miR3953	-14.78	-14.78	-14.78	ABA responsive element binding factor 2
miR4376	-4.46	14.98	12.48	sucrose-phosphate synthase
				polygalacturonase-inhibiting protein
				MAP kinase homolog
				ascorbate peroxidase
miR5053	-2.3	7.11	-7.39	cytosolic glutathione reductase
				calcium-dependent protein kinase 24
miR5054	-1.04	-1.52	-0.63	ethylene-responsive transcription factor RAP2-12-like
				desiccation-related protein PCC13
miR5059	-1.33	-1.51	-0.7	heat shock protein 70
miR5072	-2.95	-3.08	-2.78	ethylene-responsive transcription factor RAP2-12-like
				L-ascorbate oxidase-like
miR5137	1.97	1.92	1.83	NADP-dependent malic enzyme
				2-oxoglutarate/malate translocator
				galactinol synthasecalcium/calmodulin-dependent serine/threonine-protein kinase 1-like
miR5176	2.65	3.63	0.39	lipoxygenase
				heat shock protein
miR5287	-0.41	2.34	-0.11	Zn-finger transcription factor
				NADP-dependent malic enzyme-like
				gibberellin 3-oxidase 2-3
miR5501	1.13	1.49	0.12	ACC synthase
miR5526	0.69	2.16	0.19	Dof transcription factor
				heat shock protein
miR5565	-12.22	-1.71	-0.76	K^+ efflux antiporter
miR5662	2.75	5.47	4.03	calcium-dependent protein kinase
				auxin response factor
				allene oxide synthase
				polyamine oxidase
				calcium-dependent protein kinase
				ethylene-responsive element binding protein
				sucrose-phosphate synthase
				WRKY transcription factor
				homeodomain-leucine zipper transcription factor
				MADS-box transcription factor

Table 3: Continued

Table 3: Continued

miR5644	-1.37	6.86	-2.18	potassium channel AKT2 chloroplast ribulose-1,5-bisphosphate carboxylase activase WRKY2 transcription factor calcium-dependent protein kinase 3-like ethylene-responsive element binding protein 2 sucrose-phosphate synthase WRKY20 transcription factor MADS-box transcription factor TaAGL29 calcium-dependent protein kinase 16 calcium-dependent protein kinase 13
miR5809	-10.19	-2.24	-10.19	auxin response factor allene oxide synthase polyamine oxidase calcium-dependent protein kinase ethylene-responsive element binding protein sucrose-phosphate synthase WRKY transcription factor homeodomain-leucine zipper transcription factor MADS-box transcription factor
miR6180	7.57	12.05	8.68	trehalase-like protein K^+ efflux antiporter succinate dehydrogenase anthocyanin 5-aromatic acyltransferase-like protein NADH-cytochrome b5 reductase 1-like protein AP2-like ethylene-responsive transcription factor MADS-box transcription factor apoptosis-inducing factor homolog A-like protein WRKY transcription factor
miR6214	-0.46	-1.29	-0.45	alcohol dehydrogenase-like protein MADS-box transcription factor trehalose-6-phosphate synthase eukaryotic translation initiation factor eukaryotic translation initiation factor cryptochrome-1-like protein NADP-dependent malic enzyme anthocyanidin 5,3-O-glucosyltransferase cryptochrome-1-like protein dehydration-responsive element-binding protein

In this study, we screened out 8 miRNAs related to drought tolerance according to the functional analysis of the predicted target genes. These predicted target genes mainly took part in signal transduction, hormones, TCA, transport, stress response, and carbon metabolism in plants.

The target gene of the down-regulated miR1166 encodes CPK2, widely distributed in various plant tissues. CPK2 plays a noticeable role in the signal transduction of calcium (Batistič and Kudla, 2012). The concentration of calcium ions in the plant cells will change instantaneously, persistently or oscillate under the biotic or abiotic stresses in the external environment (Görlach *et al.,* 2015). While plant converts these stimuli into a sec signal by means of a change in intracellular calcium concentration, which mediates a series of changes in plant that respond to environmental changes (Kudla *et al.,* 2010). CPK2 is a crucial regulator involved in signal transduction or transcriptional regulation under different stress responses and numerous environmental stimuli (Asrar *et al.,* 2014). Previous studies have reported that *CPK* genes involved in enhancing drought tolerance. Over-expressed *AtCPK6* in *Arabidopsis* caused enhanced drought tolerance (Xu *et al.,* 2010). Similarly, *AtCPK10* regulated the stomatal movement under drought stress by interacting with *HSP1* (heat shock protein 1) in *Arabidopsis* (Zou *et al.,* 2010). Evidences showed that *OsCPK9* participated in abscisic acid (ABA)-responsive drought tolerance (Wei *et al.,* 2014). *OsCPK10* enhanced the drought stress tolerance by eliminating reactive oxygen species (ROS) to defend cellular membranes (Yin *et al.,* 2015). Overall, *CPK* involved with drought stress tolerance in various plants, as its regulator, miR1166 was down-regulated in this research, it was speculated that miR1166 also took part in diversified processes related to drought in wheat.

One of the target genes for miR163 is a lipoxygenase (*LOX*) gene, which encodes LOX that is the first enzyme in synthesis of JAs for the plant (Hwang and Hwang, 2010). JAs are widely involved in response and regulation of various biotic and abiotic stresses (Yan *et al.,* 2013). Over-expression of *LOX* in tomato increased JAs synthesis and enhanced resistance to pathogenic fungi, high temperature, insect and mechanical damage (Yan *et al.,* 2013; Hu *et al.,* 2013). In addition, the activity of LOX increased significantly in plants, such as rice (Mostofa *et al.,* 2015) and tomato (Shalata and Tal, 1998) under salt stress, as well as, olive (Sofo *et al.,* 2004)

and *Brassica juncea* (Alam *et al.,* 2014) under drought stress. The other target gene of miR163 is a spermidine synthase (*SpdSyn*) gene, which plays a critical role in plant embryo development (Imai *et al.,* 2004). Moreover, expression of *SpdSyn* could delay the onset of ripening, transgenic tomato fruits with yeast spermidine synthase gene (*ySpdSyn*) have a longer shelf life and delayed decay symptoms during storage, such as shriveling (Nambeesan *et al.,* 2010). In this study, the expression of miR163 was down-regulated, as a negative regulator of defense response (Chow *et al.,* 2017), its decrease induced the up-regulated expression of its target genes, which in turn enhanced the drought tolerance of wheat seedlings.

For the down-regulated miR5809, target genes encode oxalate oxidase and auxin response factor (ARF). Oxalate oxidase catalyzes the degradation of oxalate to hydrogen peroxide and carbon dioxide in plant, which plays a pivotal role in disease and salt stress resistance, as well as regulation of development (Zhang *et al.,* 1995). The product hydrogen peroxide, derived from oxalate, involved in lots of defensive responses. It inhibits the growth of pathogens and insects through the induction of membrane lipid peroxidation (Ilyas *et al.,* 2016), or induces cell wall enhancement or pathogenesis related protein expression (Davidson *et al.,* 2009). The other target gene for miR5809 is not annotated in the database, but the similarity between this gene and the allene oxide synthase (*AOS*) gene in rice is 80%. AOS is also a crucial enzyme in synthesis of JAs (Song *et al.,* 1993). ARF could active or repress genes expression which were involved with auxin signaling (Chandler, 2016). ARF-mediated auxin responses were vital in some abiotic stress responses, the majority of *ARFs* families in soybean were up-regulated or down-regulated transcriptionally in shoots or roots by dehydration (Ha *et al.,* 2013). Furthermore, the expression of many *ARF* genes varied following salt and drought stress in *Sorghum bicolor* (Wang *et al.,* 2010). *ARF* respond to abiotic stress might mainly via miRNAs. For example, *ARF* was the target gene of miR160 under salt stress in radish (Sun *et al.,* 2015), and miR167, whose target genes were *ARF6* and *ARF8*, was induced through drought in *Arabidopsis thaliana* (Liu *et al.,* 2008). Therefore, *ARFs* as key regulator, responds to environmental stress in plants.

The target gene for miR3711 was not annotated in the wheat database, but it was annotated as isocitrate dehydrogenase (*ICDH*) gene in the *Brachypodium distachyon*, which is a model plant for many important grass family crops, such as wheat and barley (Lv *et al.,* 2014). Its genome sequencing and annotation have been completed (Initiative 2010), and it is of great significance for the analysis of molecular evolution, gene composition and gene regulation in Poaceae family crops. ICDH is ubiquitous in aerobic organisms, including bacteria and humans. Talbi *et al*. (2015) reported that NADP-depend ICDH contributed to increase the efficiency of ascorbic acid reduced glutathione (ASC–GSH) cycle in *Oudneya africana*, which is involved in the drought tolerance regulation. ICDH is a rate-limiting enzyme in TCA cycle, reversible phosphorylation of ICDH can regulate the activity of TCA cycle (Sazanov and Jackson, 1994). TCA cycle is the ultimate metabolic pathway for the three major nutrients (carbohydrates, lipids and amino acids), and is the junction of metabolism of carbohydrates, lipids and amino acids (Cronan and Laporte, 2005). In addition, another important function of the TCA cycle is to provide small molecular precursors for other anabolism, for example, α-ketoglutarate and oxaloacetate are precursors for the synthesis of glutamic acid and aspartic acid (Mailloux *et al.,* 2007). α-ketoglutaric acid is produced by ICDH catalyzing the isocitric acid in TCA, which is the intermediate substance that connects the carbon and nitrogen metabolism in plant (Wise *et al.,* 2011). Exogenous α-ketoglutaric acid could regulate plant nitrogen metabolism obviously, while moderate nitrogen nutrition would increase proline content and enhence drought tolerance in plant (Yuan *et al.,* 2007).

For miR5565, its target gene was not annotated in the wheat database, but in the *B. distachyon*, it was annotated as a potassium (K^+) efflux antiporter (*KEA*) gene. In plants, the KEA protein acts as a cation/proton exchanger in chloroplasts and maintains pH homeostasis (Aranda-Sicilia *et al.,* 2012). Sheng *et al*. (2014) isolated a KEA gene *AM1* from rice, highly expressed in the leaves. The expression of *AM1* was up-regulated under salt stress. Moreover, the wild type was more sensitive to KCl than the *am1* mutant, suggesting that *AM1* plays a critical role in regulating cation transport and maintaining plant cell ion homeostasis. Furthermore, *am1* exhibited abnormal expression of chloroplast gene as well as accumulation of protein, and these findings provided genetic evidence for the involvement of KEA in chloroplast development (Sheng *et al.,* 2014). Studies have shown that improvement of the KEA could be beneficial to enhance the stress tolerance in rice (Obata *et al.,* 2007).

The miR2275 has two target genes, annotated as WRKY transcription factor (TF) and NAC TF in the database, respectively. WRKY is a plant specific TF, containing zinc finger structure. Total of 74 and 105 family members have been found in *A. thaliana* and *Oryza sativa*, respectively (Wu *et al.,* 2005; Li *et al.,* 2016). It was reported that WRKY TFs would play a significant role in defending abiotic as well as biotic stresses, and regulate plant growth and development (Rushton *et al.,* 2010). Overexpression of *TaWRKY1* and *TaWRKY33* activated several downstream genes in *A. thaliana*, which were stress-related, then the germination rates and root growth could be improved under stresses (He *et al.,* 2016). Jiang *et al*. (2016) showed that heterologous expression of *AtWRKY57* conferred drought tolerance in *O. sativa*. Overexpressing *TaWRKY10* enhanced the resistance of transgenic tobacco against drought stress was reported by Wang *et al*. (2013). Nevertheless, the Arabidopsis plants which were knocked out *AtWRKY63* showed less drought resistance than the wild type (Ren *et al.,* 2010).

NAC TFs were first discovered in Petunia (Nuruzzaman *et al.,* 2010). There are 170 and 138 family members in rice and *Arabidopsis* (http://planttfdb.cbi.pku.edu.cn/). Various studies have presented that NAC TFs play a significant role in growth (Olsen *et al.,* 2005), development (Xie *et al.,* 2000), hormone regulation (Pei *et al.,* 2013), organ differentiationand formation (Souer *et al.,* 1996) in plant, besides, defense of a variety of biotic and abiotic stresses (Nuruzzaman *et al.,* 2013). Evidence has shown that NAC modulated the reactive oxygen species and then conferred heat and drought tolerance in rice (Fang *et al.,* 2015). Over-expression of *Populus euphratica NAC036* (*PeNAC036*) in *A. thaliana* wild type showed intensive tolerance to salt and drought, while over-expression of *PeNAC034* exhibited enhanced salt and drought sensitivity (Lu *et al.,* 2018). Transgenic plants overexpressing *Artemisia annua NAC1* (*AaNAC1*) seem to be more tolerant to drought and increased disease resistance than wild type (Lv *et al.,* 2016).

The target gene of miR6214 encodes trehalose-6-phosphate synthase (TPS). TPS is an important enzyme for trehalose biosynthesis in plants. The trehalose biosynthetic pathway is ubiquitous in plants and is crucial for carbohydrate use in plant growth and development (Figueroa *et al.,* 2016). Furthermore, trehalose plays a vital role in improving plant resistance, which is determined not only by the physical and chemical properties of trehalose, but also related to its biological activity, which helps in osmotic adjustment (Garg *et al.,* 2002). It can also regulate the osmotic potential of cells under stress conditions, on the other hand its unique molecular structure can stabilize many intracellular biological macromolecules, thereby enhancing plant stress resistance (Fernandez *et al.,* 2010). It has been revealed that *TPS* genes may play a key role in response to various stresses (Mu *et al.,* 2016). Overexpression of the *OsTPS1* could improve tolerance to low temperature, salt, and drought in rice (Li *et al.,* 2011). Previous research indicated that over-expression of a trehalose-6-phosphate synthase/phosphatase fusion gene in tomato enhanced photosynthesis and tolerance to drought and salt stress (Lyu *et al.,* 2013). In addition, transgenic plants overexpressing *TPSs* from bacteria (Jang *et al.,* 2003), yeast (Romero *et al.,* 1997), and *Arabidopsis* (Avonce *et al.,* 2004) showed increased drought tolerance.

Different from the expression patterns of miRNAs and their target genes mentioned above, miR6180 was up-regulated in the different treatments compared with the control, while the expression of target gene was down-regulated. The target gene for miR6180 was not annotated in the wheat database, but it was annotated as trehalase in *B. distachyon*. Trehalase catalyzes the hydrolysis of trehalose into glucose, therefore, the decrease of trehalase activity is beneficial to maintain the stability of trehalose and improve the stress resistance in plant (Goddijn and Dun, 1999). Trehalase maintains a low trehalose concentration in plant cells, so it is difficult to detect trehalose in plants and usually due to the higher trehalase activity (Houtte *et al.,* 2013). El-Bashiti *et al*. (2005) reported that trehalase activity reduced in wheat under salt and drought stresses, especially in the resistant cultivars. However, in a study by Houtte *et al*. (2013), over-expression of *AtTre1* (a gene encoding the trehalase), can increase tolerance to drought stress in *Arabidopsis* by regulating stomatal closure. Differences in the activity of these enzymes (decreased or increased) may be relevant to the distinct characteristics of wheat and *Arabidopsis*, the former is resistant and the latter is sensitive. Therefore, the effects of trehalase activities on stress tolerance are diverse in different plants.

Plants can only grow in sprouting areas and face a variety of environmental stresses, which are different from animals. In order to maintain a relatively normal state and adapt to a sequence of environmental stresses, plants have evolved a series of complex and highly ordered adaptive mechanisms to resist various environmental stresses. Post-transcriptional regulation is one of these complex mechanisms. Each miRNA might have multiple target genes, and one gene may be co-regulated by multiple miRNAs. This sophisticated regulatory network would regulate the expression of multiple genes through a miRNA, and/or the combination of several miRNAs (Fig. 9). For example, the predicted target genes of miR163 and miR5809 are *LOX*, *SpdSyn*, and *AOS*, *ARF* genes, respectively (Fig. 9). They all participate in the hormones metabolism, which is one of the critical pathways during stress response in plants. In addition, another predicted target gene of miR5809 is *PAL* gene, whose product is a vital connection of the primary and secary metabolism. Consequently, small RNA analysis based on high-throughput sequencing is beneficial to understand the influence of drought stress and MeJA on the growth of wheat. The present results showed that exogenous application of MeJA alleviated the adverse effects of drought stress on wheat growth through signal transduction, hormones, stress response, etc.

Conclusion

Through concentration gradient screening, the 0.25 μM was the best appropriate concentration for exogenous MeJA. Moreover, 67,277, 75,780, 90,427 and 64,253 unique miRNAs were obtained from drought, MeJA, MeJA + D, and control through the sec generation high throughput sequencing technique, respectively. Differentially expressed miRNAs were screened from comparison of treatments and control. A total of 416 mRNAs, 371 and 408 miRNAs were differentially expressed in drought, MeJA and MeJA + D, respectively. These miRNAs could regulate expression of their target genes and might play essential role in the drought tolerance. The screened miRNAs and target genes would be the focus of future study to uncover the molecular mechanism of wheat root responses to drought stress.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (Grant Nos. 31401323), the Natural Science Foundation of Henan Province (Grant Nos. 182300410040), the Key Research Projects of Higher Education in Henan Province (Grant Nos. 18B210002), the Fund of Henan University of Science and Technology (Grant Nos. 13480072 and 09001814), and HAUST discipline improvement and promotion plan A (Grant Nos. 13660002)

References

Alam, M.M., K. Nahar, M. Hasanuzzaman and M. Fujita, 2014. Exogenous jasmonic acid modulates the physiology, antioxidant defense and glyoxalase systems in imparting drought stress tolerance in different *Brassica* species. *Plant Biotechnol. Rep.*, 8: 279–293

Anjum, S.A., M. Tanveer, S. Hussain, S.A. Tung, R.A. Samad, L.C. Wang, I. Khan, N. Rehman, A.N. Shah and B. Shahzad, 2016. Exogenously applied methyl jasmonate improves the drought tolerance in wheat imposed at early and late developmental stages. *Acta Physiol. Plant.,* 38: 25

Aranda-Sicilia, M.N., O. Cagnac, S. Chanroj, H. Sze, M.P. Rodríguez-Rosales and K. Venema, 2012. *Arabidopsis* KEA2, a homolog of bacterial KefC, encodes a K^+/H^+ antiporter with a chloroplast transit peptide. *Biochim. Biophys. Acta*, 1818: 2362–2371

Asrar, Z., H. Mozafari, F. Rezanejad, S. Pourseyedi and M.M. Yaghoobi, 2014. Calcium and L-histidine effects on ascorbate-glutathione cycle components under nickel-induced oxidative stress in tomato plants. *Biol. Plant.*, 58: 709–716

Avonce, N., B. Leyman, J.O. Mascorro-Gallardo, P.V. Dijck, J.M. Thevelein and G. Iturriaga, 2004. The Arabidopsis trehalose-6-P synthase AtTPS1 gene is a regulator of glucose, abscisic acid, and stress signaling. *Plant Physiol.*, 136: 3649–3659

Bartel, D.P., 2004. MicroNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116: 281–297

Batistič, O. and J. Kudla, 2012. Analysis of calcium signaling pathways in plants. *Biochim. Biophys. Acta*, 1820: 1283–1293

Chandler, J.W., 2016. Auxin response factors. *Plant Cell Environ.*, 39: 1014–1028

Chow, H.T. and D.W.K. Ng, 2017. Regulation of miR163 and its targets in defense against *Pseudomonas syringae* in *Arabidopsis thaliana*. *Sci. Rep.*, 7: 46433

Creelman, R.A. and J.E. Mullet, 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol.*, 48: 355–381

Cronan, J.E. and D. Laporte, 2005. Tricarboxylic acid cycle and glyoxylate bypass. *EcoSal Plus,* 1: 206–216

Danaee, M., R. Farzinebrahimi, M.A. Kadir, U.R. Sinniah, R. Mohamad and R.M. Taha, 2015. Effects of MeJA and SA elicitation on secary metabolic activity, antioxidant content and callogenesis in *Phyllanthus pulcher*. *Braz. J. Bot.*, 38: 265–272

D'Ario, M., S. Griffiths-Jones, M. Kim, 2017. Small RNAs: big impact on plant development. *Trends Plant Sci*., 22: 1056–1068

Davidson, R.M., P.A. Reeves, P.M. Manosalva and J.E. Leach, 2009. Germins: a diverse protein family important for crop improvement. *Plant Sci.*, 177: 499–510

Dhindsa, R.S., P. Plumb-Dhindsa and T.A. Thorpe, 1981. Leaf senescence:correlated with increase leaves of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J. Exp. Bot.*, 32: 93–101

El-Bashiti, T., H. Hamamcı, H.A. Öktem and M. Yücel, 2005. Biochemical analysis of trehalose and its metabolizing enzymes in wheat under abiotic stress conditions. *Plant Sci.*, 169: 47–54

Fang, Y.J., K.F. Liao, H. Du, Y. Xu, H.Z. Song, X.H. Li and L.Z. Xiong, 2015. A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. *J. Exp. Bot.*, 66: 6803–6817

Feng, Y.L., Y.Y. Zhao, K.T. Wang, Y.C. Li, X. Wang and J. Yin, 2016. Identification of vernalization responsive genes in the winter wheat cultivar Jing841 by transcriptome sequencing. *J. Genet.*, 95: 957–964

Fernandez, O., L. Béthencourt, A. Quero, R.S. Sangwan and C. Clément, 2010. Trehalose and plant stress responses: friend or foe?. *Trends Plant Sci.*, 15: 409–417

Figueroa, C.M., R. Feil, H. Ishihara, M. Watanabe, K. Kölling, U. Krause, M. Höhne, B. Encke, W.C. Plaxton, S.C. Zeeman, Z. Li, W.X. Schulze, R. Hoefgen, M. Stitt and J.E. Lunn, 2016. Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon availability. *Plant J.*, 85: 410–423

Garg, A.K., J.K. Kim, T.G. Owens, A.P. Ranwala, Y.D. Choi, L.V. Kochian and R.J. Wu, 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *P. Natl. Acad. Sci. USA*, 99: 15898–15903

Goddijn, O.J.M. and K. Dun, 1999. Trehalose metabolism in plants. *Trends Plant Sci.*, 4: 315–319

Görlach, A., K. Bertram, S. Hudecova and O. Krizanova, 2015. Calcium and ROS: a mutual interplay. *Redox Biol.*, 6: 260–271

Ha, C.V., D.T. Le, R. Nishiyama, Y. Watanabe, S. Sulieman, U.T. Tran, K. Mochida, N.V. Dong, K. Yamaguchi-Shinozaki, K. Shinozaki and L.S.P. Tran, 2013. The auxin response factor transcription factor family in soybean: genome-wide identification and expression analyses during development and water stress. *DNA Res.,* 20: 511–524

He, G.H., J.Y. Xu, Y.X. Wang, J.M. Liu, P.S. Li, M. Chen, Y.Z. Ma and Z.S. Xu, 2016. Drought-responsive WRKY transcription factor genes *TaWRKY1* and *TaWRKY33* from wheat confer drought and/or heat resistance in *Arabidopsis*. *BMC Plant Biol.*, 16: 116

Houtte, H.V., L. Vandesteene, L. López-Galvis, L. Lemmens, E. Kissel, S. Carpentier, R. Feil, N. Avonce, T. Beeckman, J.E. Lunn and P.V. Dijck, 2013. Overexpression of the trehalase gene *AtTRE1* leads to increased drought stress tolerance in Arabidopsis and is involved in abscisic acid-induced stomatal closure. *Plant Physiol.*, 161: 1158–1171

Huang, M., Z.H. Wang, L.C. Luo, S. Wang, X.L. Hui, G. He, H.B. Cao, X.L. Ma, T.M. Huang, Y. Zhao, C.P. Diao, X.F. Zheng, H.B. Zhao, J.S. Liu, and S.S. Malhi, 2017. Soil testing at harvest to enhance productivity and reduce nitrate residues in dryland wheat production. *Field Crop Res*., 212: 153–164

Hu, T.Z., H. Zeng, Z.L. Hu, X.X. Qv and G.P. Chen, 2013. Overexpression of the tomato 13-lipoxygenase gene *TomloxD* increases generation of endogenous jasmonic acid and resistance to cladosporium fulvum and high temperature. *Plant Mol. Biol. Rep.*, 31: 1141–1149

Hwang, I.S. and B.K. Hwang, 2010. The pepper 9-lipoxygenase gene *CaLOX1* functions in defense and cell death responses to microbial pathogens. *Plant Physiol.*, 152: 948–967

Imai, A., T. Matsuyama, Y. Hanzawa, T. Akiyama, M. Tamaoki, H. Saji, Y. Shirano, T. Kato, H. Hayashi, D. Shibata, S. Tabata, Y. Komeda and T. Takahashi, 2004. Spermidine synthase genes are essential for survival of Arabidopsis. *Plant Physiol.*, 135: 1565–1573

Initiative, T.I.B., 2010. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature*, 463: 763–768

Jang, I.C., S.J. Oh, J.S. Seo, W.B. Choi, S.I. Song, C.H. Kim, Y.S. Kim, H.S. Seo, Y.D. Choi, B.H. Nahm and J.K. Kim, 2003. Expression of bifunctional fusion of the Escherichia coli genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiol.*, 131: 516–524

Jiang, Y.J., Y.P. Qiu, Y.R. Hu and D.Q. Yu, 2016. Heterologous expression of *AtWRKY57* confers drought tolerance in *Oryza sativa*. *Front Plant Sci.*, 7: 145

Kang, G.Z., G.Z. Li, W. Xu, X.Q. Peng, Q.X. Han, Y.J. Zhu and T.C. Guo, 2012. Proteomics reveals the effects of salicylic acid on growth and tolerance to subsequent drought stress in wheat. *J. Proteome Res.*, 11: 6066–6079

Kudla, J., O. Batistič and K. Hashimoto, 2010. Calcium signals: the lead currency of plant information processing. *Plant Cell*, 22: 541–563

Li, H.W., B.S. Zang, X.W. Deng and X.P. Wang, 2011. Overexpression of the trehalose-6-phosphate synthase gene OsTPS1 enhances abiotic stress tolerance in rice. *Planta*, 234: 1007–1018

Liu, H.H., X. Tian, Y.J. Li, C.G. Wu and C.C. Zheng, 2008. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*, 14: 836–846

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25: 402–408

Li, W., H.P. Wang and D.Q. Yu, 2016. *Arabidopsis* WRKY transcription factors WRKY12 and WRKY13 oppositely regulate flowering under short-day conditions. *Mol. Plant*, 9: 1492–1503

Ilyas, M., A. Rasheed and T. Mahmood, 2016. Functional characterization of germin and germin-like protein genes in various plant species using transgenic approaches. *Biotechnol. Lett.*, 38: 1405–1421

Lu, X., X.L. Zhang, H. Duan, C.L. Lian, C. Liu, W.L. Yin and X.L. Xia, 2018. Three stress-responsive NAC transcription factors from *Populus euphratica* differentially regulate salt and drought tolerance in transgenic plants. *Physiol. Plant.*, 162: 73–97

Lv, D.W., S. Subburaj, M. Cao, X. Yan, X.H. Li, R. Appels, D.F. Sun, W.J. Ma and Y.M. Yan, 2014. Proteome and phosphoproteome characterization reveals new response and defense mechanisms of *Brachypodium distachyon* leaves under salt stress. *Mol. Cell. Proteom.*, 13: 632–652

Lv, Z.Y., S. Wang, F.Y. Zhang, L.X. Chen, X.L. Hao, Q.F. Pan, X.Q. Fu, L. Li, X.F. Sun and K.X. Tang, 2016. Overexpression of a novel NAC domain-containing transcription factor gene (*AaNAC1*) enhances the content of artemisinin and increases tolerance to drought and *Botrytis cinerea* in *Artemisia annua*. *Plant Cell Physiol.*, 57: 1961–1971

Lyu, J.I., S.R. Min, J.H. Lee, Y.H. Lim, J.K. Kim, C.H. Bae and J.R. Liu, 2013. Overexpression of a trehalose-6-phosphate synthase/phosphatase fusion gene enhances tolerance and photosynthesis during drought and salt stress without growth aberrations in tomato. *Plant Cell Tiss. Org. Cult.*, 112: 257–262

Ma, C., Z.Q. Wang, L.T. Zhang, M.M. Sun and T.B. Lin, 2014. Photosynthetic responses of wheat (*Triticum aestivum L.*) to combined effects of drought and exogenous methyl jasmonate. *Photosynthetica*, 52: 377–385

Mailloux, R.J., R. Bériault, J. Lemire, R. Singh, D.R. Chénier, R.D. Hamel and V.D. Appanna, 2007. The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. *PLoS One*, 2: e690

Miao, Y.F., Z.H. Wang and S.X. Li, 2015. Relation of nitrate N accumulation in dryland soil with wheat response to N fertilizer. *Field Crop Res.*, 170: 119–130

Mostofa, M.G., M.A. Hossain and M. Fujita, 2015. Trehalose pretreatment induces salt tolerance in rice (*Oryza sativa L.*) seedlings: oxidative damage and co-induction of antioxidant defense and glyoxalase systems. *Protoplasma*, 252: 461–475

Mu, M., X.K. Lu, J.J. Wang, D.L. Wang, Z.J. Yin, S. Wang, W.L. Fan and W.W. Ye, 2016. Genome-wide Identification and analysis of the stress-resistance function of the TPS (Trehalose-6-Phosphate Synthase) gene family in cotton. *BMC Genet.*, 17: 54

Nambeesan, S., T. Datsenka, M.G. Ferruzzi, A. Malladi, A.K. Mattoo and A.K. Handa, 2010. Overexpression of yeast spermidine synthase impacts ripening, senescence and decay symptoms in tomato. *Plant J.*, 63: 836–847

Nuruzzaman, M., A.M. Sharoni and S. Kikuchi, 2013. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Front Microbiol.*, 4: 248

Nuruzzaman, M., R. Manimekalai, A.M. Sharoni, K. Satoh, H. Kondoh, H. Ooka and S. Kikuchi, 2010. Genome-wide analysis of NAC transcription factor family in rice. *Gene*, 465: 30–44

Obata, T., H.K. Kitamoto, A. Nakamura, A. Fukuda and Y. Tanaka, 2007. Rice shaker potassium channel oskat1 confers tolerance to salinity stress on yeast and rice cells. *Plant Physiol.*, 144: 1978–1985

Olsen, A.N., H.A. Ernst, L.L. Leggio and K. Skriver, 2005. NAC transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci.*, 10: 79–87

Pei, H.X., N. Ma, J. Tian, J. Luo, J.W. Chen, J. Li, Y. Zheng, X. Chen, Z.J. Fei and J.P. Gao, 2013. An NAC transcription factor controls ethylene-regulated cell expansion in flower petals. *Plant Physiol.*, 163: 775–791

Ren, X.Z., Z.Z. Chen, Y. Liu, H.R. Zhang, M. Zhang, Q. Liu, X.H. Hong, J.K. Zhu and Z.Z. Gong, 2010. ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis. *Plant J.*, 63: 417–429

Romero, C., J.M. Bellés, J.L. Vayá, R. Serrano and F.A. Culiáñez-Macià, 1997. Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta*, 201: 293–297

Rushton, P., I.E. Somssich, PatriciaRingler and Q.J. Shen, 2010. WRKY transcription factors. *Trends Plant Sci.*, 15: 247–258

Sazanov, L.A. and J.B. Jackson, 1994. Proton-translocating transhydrogenase and NAD- and NADP- linked isocitrate dehydrogenases operate in a substrate cycle which contributes to fine regulation of the tricarboxylic acid cycle activity in mitochondria. *FEBS J.*, 344: 109–116

Shalata, A. and M. Tal, 1998. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salttolerant relative Lycopersicon pennellii. *Physiol. Plant.*, 104: 169–174

Sheng, P.K., J.J. Tan, M.N. Jin, F.Q. Wu, K.N. Zhou, W.W. Ma, Y.Q. Heng, J.L. Wang, X.P. Guo, X. Zhang, Z.J. Cheng, L.L. Liu, C.M. Wang, X.M. Liu and J.M. Wan, 2014. *Albino midrib 1*, encoding a putative potassium efflux antiporter, affects chloroplast development and drought tolerance in rice. *Plant Cell Rep.*, 33: 1581–1594

Sirhindi, G., P. Sharma, A. Singh, H. Kaur and M. Mir, 2015. Alteration in photosynthetic pigments, osmolytes and antioxidants in imparting copper stress tolerance by exogenous jasmonic acid treatment in *Cajanus cajan*. *Intl. J. Plant Physiol. Biochem.*, 7: 30–39

Sofo, A., B. Dichio, C. Xiloyannis and A. Masia, 2004. Lipoxygenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. *Physiol. Plant.*, 121: 58–65

Song, W.C., C.D. Funk and A.R. Brash, 1993. Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. *P. Natl. Acad. Sci. USA*, 90: 8519–8523

Souer, E., A.V. Houwelingen, D. Kloos, J. Mol and R. Koes, 1996. *The No Apical Meristem* gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell*, 85: 159–170

Sun, X.C., L. Xu, Y. Wang, R.G. Yu, X.W. Zhu, X.B. Luo, Y.Q. Gong, R.H. Wang, C.L. Limera, K.Y. Zhang and L.W. Liu, 2015. Identification of novel and salt-responsive miRNAs to explore miRNA-mediated regulatory network of salt stress response in radish (*Raphanus sativus L.*). *BMC Genom.*, 16: 197

Talbi, S., M.C. Romero-Puertas, A. Hernández, L. Terrón, A. Ferchichi and L.M. Sandalio, 2015. Drought tolerance in a Saharian plant *Oudneya africana*: Role of antioxidant defences. *Environ. Exp. Bot.*, 111: 114–126

Wang, C., P.Y. Deng, L.L. Chen, X.T. Wang, H. Ma, W. Hu, N.C. Yao, Y. Feng, R.H. Chai, G.X. Yang and G.Y. He, 2013. A wheat WRKY transcription factor TaWRKY10 confers tolerance to multiple abiotic stresses in transgenic tobacco. *PLoS One*, 8: e65120

Wang, S.K., Y.H. Bai, C.J. Shen, Y.R. Wu, S.N. Zhang, D. Jiang, T.J. Guilfoyle, M. Chen and Y.H. Qi, 2010. Auxin-related gene families in abiotic stress response in *Sorghum bicolor*. *Funct. Integr. Genom.*, 10: 533–546

Wei, S.Y., W. Hu, X.M. Deng, Y.Y. Zhang, X.D. Liu, X.D. Zhao, Q.C. Luo, Z.Y. Jin, Y. Li, S.Y. Zhou, T. Sun, L.Z. Wang, G.X. Yang and G.Y. He, 2014. A rice calcium-dependent protein kinase OsCPK9 positively regulates drought stress tolerance and spikelet fertility. *BMC Plant Biol.*, 14: 133

Wise, D.R., P.S. Ward, J.E.S. Shay, J.R. Cross, J.J. Gruber, U.M. Sachdeva, J.M. Platt, R.G. DeMatteo, M.C. Simon and C.B. Thompson, 2011. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α-ketoglutarate to citrate to support cell growth and viability. *P. Natl. Acad Sci. USA*, 108: 19611–19616

Wu, H.L., X.L. Wu, Z.H. Li, L.S. Duan and M.C. Zhang, 2012. Physiological evaluation of drought stress tolerance and recovery in cauliflower (*Brassica oleracea L.*) seedlings treated with methyl jasmonate and coronatine. *J. Plant Growth Regul.*, 31: 113–123

Wu, K.L., Z.J. Guo, H.H. Wang and J. Li, 2005. The WRKY family of transcription factors in rice and Arabidopsis and their origins. *DNA Res.*, 12: 9–26

Xu, J., Y.S. Tian, R.H. Peng, A.S. Xiong, B. Zhu, X.F. Jin, F. Gao, X.Y. Fu, X.L. Hou and Q.H. Yao, 2010. AtCPK6, a functionally redundant and positive regulator involved in salt/drought stress tolerance in Arabidopsis. *Planta*, 31: 1251–1260

Xie, Q., G. Frugis, D. Colgan and N.H. Chua, 2000. Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Gene Dev.*, 14: 3024–3036

Yan, L.H., Q.Z. Zhai, J.N. Wei, S.Y. Li, B. Wang, T.T. Huang, M.M. Du, J.Q. Sun, L. Kang, C.B. Li and C.Y. Li, 2013. Role of tomato lipoxygenase D in wound-induced jasmonate biosynthesis and plant immunity to insect herbivores. *PLoS Genet.*, 9: e1003964

Yin, X.M., L.F. Huang, X. Zhang, M.L. Wang, G.Y. Xu and X.J. Xia, 2015. *OsCML4* improves drought tolerance through scavenging of reactive oxygen species in rice. *J. Plant Biol.*, 58: 68–73

Yuan, Y.Z., J.Q. Ou, Z.Q. Wang, C.F. Zhang, Z.P. Zhou and Q.H. Lin, 2007. Regulation of carbon and nitrogen metabolisms in rice roots by 2-oxoglutarate at the level of hexokinase. *Physiol. Plant.*, 129: 296–306

Zhang, B.H., X.P. Pan, Q.L. Wang, G.P. Cobb and T.A. Anderson, 2005. Identification and characterization of new plant microRNAs using EST analysis. *Cell Res.*, 15: 336–360

Zhang, L., J. Wang, G.Z. Fu, Y.G. Zhao, 2018. Rotary tillage in rotation with plowing tillage improves soil properties and crop yield in a wheat-maize cropping system. *PLoS One*, 13: e0198193

Zhang, Z.G., D.B. Collinge and H. Thordal-Christensen, 1995. Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *Plant J.*, 8: 139–145

Zhu, Q.H., A. Spriggs, L. Matthew, L.J. Fan, G. Kennedy, F. Gubler and C. Helliwell, 2008. A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. *Genom. Res.*, 18: 1456–1465

Zou, J.J., F.J. Wei, C. Wang, J.J. Wu, D. Ratnasekera, W.X. Liu and W.H. Wu, 2010. Arabidopsis calcium-dependent protein kinase CPK10 functions in abscisic acid- and Ca^{2+}-mediated stomatal regulation in response to drought stress. *Plant Physiol.*, 154: 1232–1243

(Received 17 January 2019; Accepted 22 March 2019)