



Full Length Article

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

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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 second 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; Ji *et al.*, 2018). 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; Yang *et al.*, 2017). The miRNAs degrade or inhibit transcription by binding to target mRNAs, thereby regulating target genes expression (Zhu *et al.*, 2008; Zahoor *et al.*, 2018).

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 second generation high throughput sequencing technique. The miRNAs related to drought resistance were identified through bioinformatics analysis, target gene prediction and miRNA expression pattern analysis.

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% HgCl₂ 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 frozen 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^{-ΔΔCt} 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



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

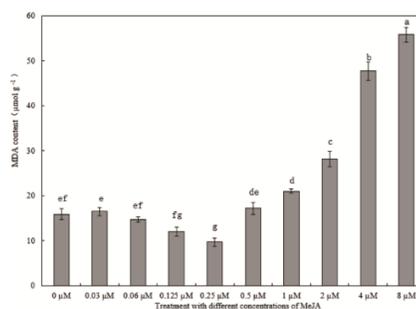


Fig. 2: Effects of different concentrations of MeJA on MDA content in roots of wheat seedling



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

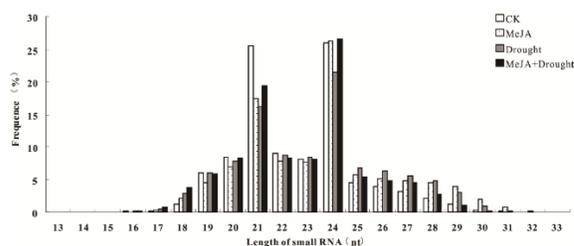


Fig. 4: Length of small RNA in different treatments

nt was the highest among the lengths. The sequences with a length of 21 nt represented the sec category. However, the percentages of these two lengths under drought treatment were the lowest compared to other treatments.

Comparison of Differentially Expressed miRNAs among Treatments

The differentially expressed miRNAs of control, 0.25 μM

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

MeJA, drought, and MeJA + D treatments were compared, 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).

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

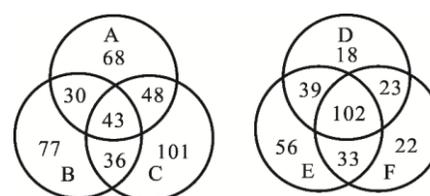


Fig. 5: Differentially expressed miRNA among different treatments. The up-regulated miRNAs in CK and MeJA(A), CK and Drought(B), CK and MeJA + D (C); the downregulated miRNAs in CK and MeJA (D), CK and Drought (E), CK and MeJA + D (F)

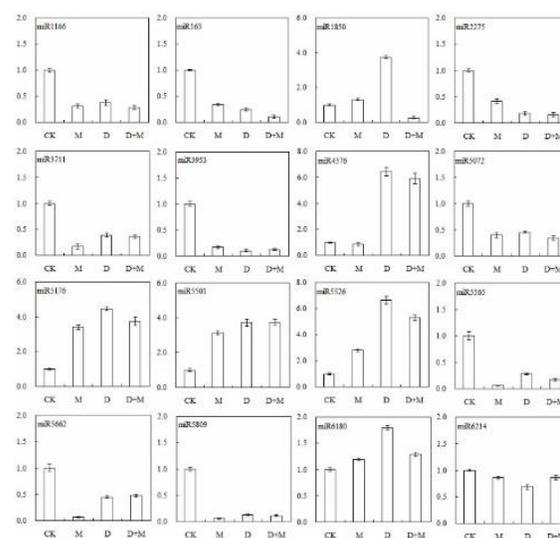


Fig. 6: Validation of the expression patterns of 16 miRNAs in different treatments

gene, including miR163, miR5809, miR3711, miR5565, miR2275, 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 in different treatments

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	58535 (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%)	

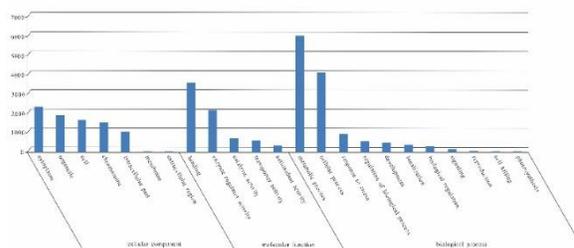


Fig. 7: GO analysis of the predicted targets for sequenced miRNAs in three ontologies: biological process, molecular function and cellular component

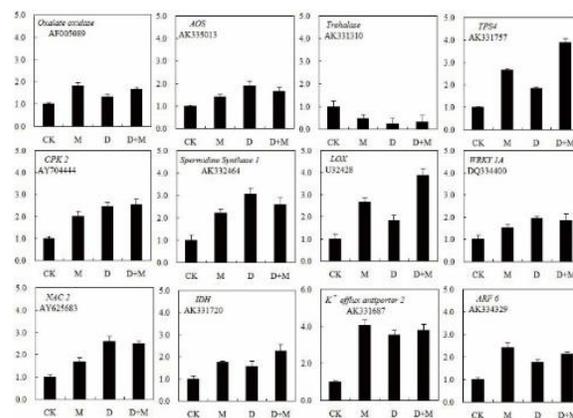


Fig. 8: Expression patterns of screened target genes in different treatments

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



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 addition, another predicted target gene of miR5809 also encodes the PAL, which are involved in secondary metabolism

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
miR2594	0.09	1.25	0.19	WRKY transcription factor auxin transporter-like protein protein WAX2-like nucleobase-ascorbate transporter S-type anion channel SLAH3-like BURP domain-containing protein chlorophyllase-1-like
miR3440	2.46	3.45	3.16	ethylene constitutive triple response protein 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 (Nambesee 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 enhance 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 *AMI* from rice, highly expressed in the leaves. The expression of *AMI* was up-regulated under salt stress. Moreover, the wild type was more sensitive to KCl than the *ami1* mutant, suggesting that *AMI* plays a critical role in regulating cation transport and maintaining plant cell ion homeostasis. Furthermore, *ami1* 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://plantfdb.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 differentiation and 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 *TPS*s 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 secondary 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 μ M 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 second 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.

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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. Plantarum*, 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. Plantarum*, 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. MicroRNAs: 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. Hamamci, 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. *Proc. 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 Crops 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
- Ji, B., Z. Li, W. Gu, J. Li, T. Xie and S. Wei, 2018. Methyl jasmonate pretreatment promotes the growth and photosynthesis of maize seedlings under saline conditions by enhancing the antioxidant defense system. *Intl. J. Agric. Biol.*, 20: 1454–1462
- 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. Plantarum*, 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. Proteomics*, 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.L., 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
- Mailoux, 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 Crops 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, Patricia Ringler 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 salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plantarum*, 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. Lipoxigenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. *Physiol. Plantarum*, 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. *Proc. 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. Genomics*, 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. *Proc. 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
- Yang, Y., M. Li and Y. Yi, 2017. The miRNAomes in *Rehmannia glutinosa* roots exposed to different levels of replanting disease pressure. *Intl. J. Agric. Biol.*, 19: 77–84
- 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. Plantarum*, 129: 296–306
- Zahoor, S., H.M.N. Cheema, A.A. Khan, M.S. Nawaz ur Rehman and A.K. Mattoo, 2018. Co-Expression analysis of miRNAs and target NBS-LRR genes in *Cucumis sativus*. *Intl. J. Agric. Biol.* 20: 100–108
- 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 H₂O₂-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 Ca²⁺-mediated stomatal regulation in response to drought stress. *Plant Physiol.*, 154: 1232–1243

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