INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
16–508/2017/19–2–273–281
DOI: 10.17957/IJAB/15.0274
http://www.fspublishers.org

Full Length Article

Identifying Differentially Expressed Genes Associated with Tolerance against Low Temperature Stress in *Brassica napus* through Transcriptome Analysis

Mengzhu Xian[1], Tao Luo[1], Mohammad Nauman Khan, Liyong Hu and Zhenghua Xu[*]
MOA Key Laboratory of Crop Ecophysiology and Farming System in the Middle Reaches of the Yangtze River/College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[*]For correspondence: xzh@mail.hzau.edu.cn
[1]These authors contributed equally to this work

Abstract

Under direct-seeding method of sowing, seedling survival is adversely affected due to low temperature. The purpose of this study was to understand the morphological, physiological and molecular response of rapeseed resistance to low temperature stress at seedling establishment. Two varieties Qinyou No.7 (resistant biotype) and Fengyou 730 (sensitive biotype) were used for morphological and physiological experiments under low temperature stress and the resistant variety was used for transcriptome analysis based on the Illumina HiSeq 2000 platform. Results showed resistant variety initiated osmotic regulation system firstly and then triggered the antioxidant enzyme system when exposed to low temperature stress for long duration. The MDA content firstly tended to increase and then decreased in resistant variety, but it continued to increase in sensitive variety. Through transcriptome analysis of Qinyou No. 7 under low temperature for 0 h, 24 h, 48 h and 96 h, 2254 differentially expressed unigenes clustering in 7 expression patterns were participated in low temperature stress. Protein-serine/threonine kinases, myo-inositol-1-phosphate synthases and calmodulins, as the members of ABA and IP3/Ca2+ signal transduction pathway, played an important role in the low temperature stress. Different expressed genes dataset provides useful candidate genes for functional analysis of rapeseed resistance to low temperature.

Keywords: Brassica; Low temperature stress; Transcriptome; Seedling growth
Abbreviations; CAT: Catalase; MDA: Malondialdehyde; NGS: Next-generation sequencing; POD: Peroxidase; RDW: Root dry weight; RFW: Root fresh weight; RL: Root length; ROS: Reactive oxygen species; SDW: Shoot dry weight; SFW: Shoot fresh weight; SL: Shoot length; SOD: Superoxide dismutase; SP: Soluble protein; TL: Total length; TFW: Total fresh weight; TDW: Total dry weight

Introduction

Rapeseed (*Brassica napus* L.) is one of the most important oilseed crops in the world because it is edible oil source for human and a potential future source for renewable biofuel (Tian *et al.,* 2016). The traditional method of seedling transplantation, which demands more labor force and cost, is nowadays replaced with direct-seeding method (Wang *et al.,* 2015). Healthy seedling establishment is a crucial developmental stage to determine the population density of rapeseed (*Brassica napus* L.) in field. However, reduction in seedling survival due to low temperature under direct-seeding is the limitation for the high yield acquisition and the promotion of planting area.

Previous researches revealed that the seasonal cold climate had become a direct obstacle for seedling establishment (Zheng *et al.,* 1998; Diepenbrock, 2000). An analysis of morphological traits under low temperature conditions indicated that seedling height, and shoot dry weights decreased as temperature decreased in sorghum (Yu *et al.,* 2004). Higher concentrations of soluble sugars, glucose and proline were positively associated with low temperature stress, which acted as osmotic substances to preserve protein structure and function (Patton *et al.,* 2007; Burbulis *et al.,* 2011; Trischuk *et al.,* 2014; Farooq *et al.,* 2016). Low temperature stress induced the accumulation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals (Suzuki and Mittler, 2006; Farooq *et al.,* 2009). These ROS may be signals inducing ROS scavengers and other protective mechanisms, as well as damaging agents contributing to stress injury in plants (Xiong *et al*., 2002; Airaki *et al*., 2012; Li *et al.,* 2013; ; Farooq *et al.,* 2016)). Diverse plant species tolerate cold stress to a varying degree, which depends on reprogramming gene expression to modify their physiology, metabolism, and growth (Chinnusamy *et al.,*

To cite this paper: Xian, M., T. Luo, M.N. Khan, L. Hu and Z. Xu, 2017. Identifying differentially expressed genes associated with tolerance against low temperature stress in *Brassica napus* through transcriptome analysis. *Int. J. Agric. Biol.*, 19: 273–281

2010). With the rapid advance of next-generation sequencing (NGS) technology, a large number of low temperature regulated genes had been identified to involve in a variety of biological processes in many crops (Xu *et al.*, 2011; Secco *et al.*, 2013; Zhang *et al.*, 2013; An *et al.*, 2015; Huang *et al.*, 2015). Complex gene networks, especially the unsaturated fatty acid and jasmonic acid biosynthesis pathways, were involved in the cold stress in *C. Japonica* (Li *et al.*, 2016). Many heat shock protein genes were triggered in plant cells when exposed to low temperature stress in Seabuckthorn (Sharma and Chaudhary, 2016). Information on the low-temperature transcriptome, proteome and metabolome is expected to continue to increase in the near future. This information is necessary for our understanding of the complex network of molecular changes that are important for low temperature tolerance (Zhu *et al.*, 2007). There are rare transcriptomic studies for seedling establishment of rapeseed to identify and characterize their cold-stress-induced gene expression.

To our knowledge, a comprehensive research from morphological physiological and molecular levels to study the effect of low temperature on the seedling establishment of rapeseed (*Brassica napus* L.) is still rare. The present study was to explore the low temperature resistance mechanisms in resistant and susceptible biotypes of rapeseed by morphological and physiological measurement and transcriptome analysis at the seedling establishment stage. The objectives of this study were to reveal the different morphological and physiological responses of resistant and sensitive varieties to low temperature stress, and to identify different expressed genes participated in low temperature stress, further to provide useful candidate genes for functional analysis and successful seedling establishment of rapeseed resistance to low temperature.

Materials and Methods

Plant Materials and Growth Conditions

Two varieties of Qinyou No. 7 (resistant biotype) and Fengyou 730 (sensitive biotype) were used for morphological and physiological experiments (Xian *et al.*, 2015). While the resistant variety Qinyou No. 7 was used for transcriptome analysis. Newly-harvested seeds of Qinyou No. 7 and Fengyou 730 came from The Research Center of Rapeseed of Shanxi province and The Crops Research Institute of Hunan Province, China. For each variety, one hundred seeds (arranged in 10 × 10) were sown in each germination box (12 cm×12 cm×6 cm) and covered with 3-layer sterilized filter paper. The filter paper was moistened with 10 mL Hoagland's solution and 1 mL solution was added every day during the experimental period to provide adequate water for germination and seedling establishment. Seeds were germinated in incubator (day/night temperature at 25/20℃) with a photoperiod of 12 h light (150 μmol photons m^{-2} s^{-1}) and 12 h dark at 50% relative humidity. After 3 days, half of the germination boxes for each variety were placed in incubator (day/night temperature at 15/10℃) for low temperature treatment; the other germination boxes were placed in incubator (day/night temperature at 25/20℃) for normal temperature treatment. Roots and shoots for the low temperature treatment and the control were harvested separately at the time points of 0 h, 24 h, 48 h and 96 h after the low temperature treatment began.

Morphological and Physiological Parameters Measurement

Seedling morphological parameters such as shoot length (SL), root length (RL), total length (TL), shoot fresh weight (SFW), root fresh weight (RFW), total fresh weight (TFW), total dry weight (TDW), ratio of root to shoot length (RL/SL), ratio of root to shoot weight (RFW/SFW), ratio of shoot dry to fresh weight (SDW/SFW), ratio of root dry to fresh weight (RDW/RFW), and ratio of total dry to fresh weight (TDW/TFW) were recorded at each sampling time point. The harvested materials were dried to a constant weight at 80℃ to measure the shoot dry weight (SDW) and root dry weight (RDW).

Shoot material was collected for the determination of physiological parameters such as the content of proline, MDA and soluble protein, and the enzyme activity of SOD, POD and CAT. Proline was extracted with 5-sulfosalicylic acid, quantified by means of a colorimetric reaction with ninhydrin according to the method of Li Heseng (Li, 2000). MDA content was estimated by shoot homogenates reacted with thiobarbituric acid according to the method of Bhatugar-Mathur (Bhatnagar-Mathur *et al.*, 2009). Soluble protein content was determined by Coomassie brilliant blue (G-250) according to the Bradford method (Bradford, 1976). SOD activity was determined by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) as described by Donahue (Donahue *et al.*, 1997). POD activity was determined by measuring its ability to catalyze the colorimetric reaction of hydrogen peroxide (H_2O_2) and guaiacol as described by Raza *et al*. (2007). CAT activity was determined by measuring its ability to degrade the concentration of H_2O_2 as described by Raza *et al.*(2007).

RNA Extraction, Library Preparation and Sequencing

The four RNA samples, including 3 samples from the rapeseed shoots after 24 h, 48 h and 96 h chilling treatment and a mixed sample of shoots were harvested under control condition (25/20℃) at the same sampling time point，were collected from three replications, the replications for each treatment were pooled together to make one sample for transcriptome analysis.

Total RNA was extracted using TRK-1001 total RNA purification kit (LC Science, Houston, TX) following

the manufacturer's procedure. The total RNA quantity and purity were analysis of Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0.

Approximately 5 μg of total RNA representing a specific adipose type was subjected to isolate poly (A) mRNA with poly-T oligo attached magnetic beads (thermo-fisher). Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the d UTP method as described, the average insert size for the paired-end libraries was 300 (±50 bp). And then we performed the paired-end sequencing on an Illumina Hiseq 2000 (LC Sceiences, USA) platform using 100 bp paired-end reads.

Transcriptome Data Processing

All RNA-seq datas were aligned to *Brassica napus* L. reference genome (build 5) using Top Hat v2.0.9 with default parameters. The mapped reads were assembled using Cufflinks v2.11. All multiple assembled transcript files were then merged to produce a unique set of transcriptomes using the Cuffmerge utility provided by the Cufflinks package. Cuffdiff v2.11 was used for all differential expression analyses. Transcript abundances were estimated by Cufflinks in Fragments per Kilobase per Million mapped reads (FPKM) for paired-end reads. The differentially expressed genes (24 h compared with 0 h, 48 h compared with 0 h and 96 h compared with 0 h) were selected using a threshold of absolute value of $\log_2$ (fold change) >1 and FDR < 0.05 (Reiner *et al.*, 2003; Lu *et al.*, 2014; Wei *et al.*, 2015). Then, differentially expressed genes were clustered by STEM (v1.3.8) (Short Time-series Expression Miner) with the maximum number of model profiles was set to 20. The clustered profiles with P-value ≤ 0.01 were considered as significantly expressed.

qRT-PCR

For quantitative RT-PCR (qRT-PCR) analysis, total RNA was extracted from sample using Trizol Reagent (JingDao, China) with three replications. The cDNA was synthesized from 3 μg of total RNA using the TUREscript 1st Stand cDNA SYNTHESIS Kit (Aidlab, China), according to the manufacturer's instructions. The primers used for these experiments were listed in additional file 5. PCR reactions were set up in 96-well Hard-Shell PCR plates with 0.2 μM primers using SYBR® Green Supermix (BDI, German) in 5 μL. Reaction condition system was as follows: denatured at 95℃ for 3 min, 40 cycles of 95℃ for 10 s and 58℃ for 30 s, heating from 60℃ to 95℃ at a rate of 1℃ per 4 s for melt curve analysis. Finally the relative gene expression levels were normalized to the housekeeping gene *BnaA09g14410* (Zhang *et al.*, 2015a) and calculated by using the $2^{-\triangle\triangle Ct}$ method.

Experimental Design and Statistical Analysis

The experiment was conducted in Completely Randomized Design with factorial arrangement (2 varieties×2 temperatures×4 sampling times×3 replications). One-way analysis of variance (ANOVA) were performed to determine the effect of low temperature stress on morphological and physiological parameters using the statistical software package SAS 8.1, while Tukey's HSD (Honest Significant Difference) test at 5% level of probability was applied to distinguish significant treatments.

Results

Morphological Responses

Growth analysis showed that the shoot fresh weight (SFW), shoot length (SL) and root length (RL) were significantly affected by low temperature stress (Fig. 1; Fig. 2A, 2E and 2F). The SL and RL were about a fold lower under low temperature treatment than control conditions. There were just some time-points showed significant difference for shoot dry weight (SDW), root fresh weight (RFW) and root dry weight (RDW) (Fig. 2B, 2C and 2D). Total fresh weight (TFW) and total length (TL) were lower while SDW/SFW and TDW/TFW were higher when compared with that of control conditions (see additional file 1).

Dry weight of shoot after low temperature treatment was higher than untreated conditions, while the opposite situation was observed in dry weight of shoot (Fig. 2C and 2D). RL/SL ratio continued to increase with the seedling growth. After 96 h of low temperature treatment, RL/SL of Qinyou No. 7 was significantly lower than control plants, whereas RL/SL continued to increase with the seedling growth. After initiating temperature treatment for 24 h, RFW/SFW of varieties in low temperature was increased, and reached significantly higher level at 48 h. However, in the following 48 h (48–96 h period), RFW/SFW of Qinyou No. 7 was resume to control level, this parameter of Fengyou 730 increased sustainability and reached more than 1.4-fold difference after 96 h of low temperature treatment.

Physiological Responses

Low temperature stress raised the levels of reactive oxygen species (ROS) in cells, which resulted in membrane lipid peroxidation and accumulation of toxic chemicals, such as malondialdehyde (MDA). Protein and enzyme, such as peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD), were involved in resistance to low temperature by maintaining cell balance and scavenging the ROS. Physiological analysis revealed that both varieties actively initiated the osmotic regulation systems and reactive oxygen species (ROS) scavenging enzymes system to withstand low temperature stress (Fig. 3).

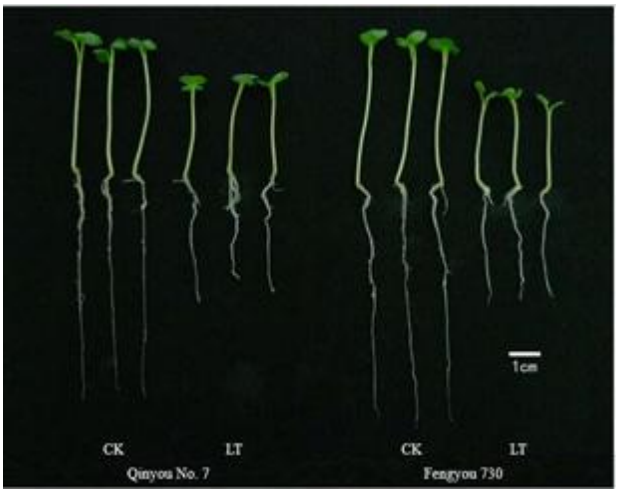

Fig. 1: Seedlings grown under different conditions after 96 h treatment

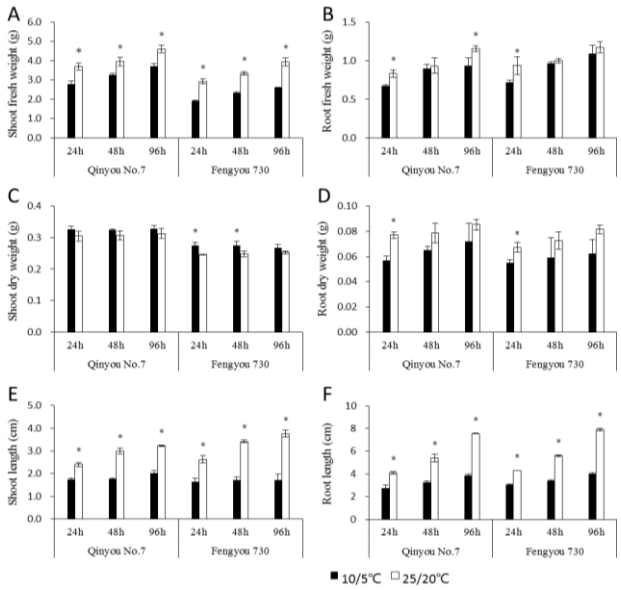

Fig. 2: Morphological behaviors of rapeseed seedlings to low temperature stress. (A) to (F) : Fresh weight (FW) ([A] and [B]), dry weight (DW) ([C] and [D]) and length ([E] and [F]) were assessed for shoots and roots. Errors bars are SD. "*" indicates statistically significant ($P < 0.05$) differences between samples grown under control and stress conditions

Low temperature resulted in significantly higher accumulation of proline content in Qinyou No. 7, but there was no obvious accumulation in Fengyou 730 (Fig. 3A). MDA content of plants under low temperature was higher than plants in control conditions. For Qinyou No.7, MDA content was more at 48 h, then decreased at 96 h under low temperature stress; For Fengyou 730, the MDA content continued to increase under low temperature stress (Fig.

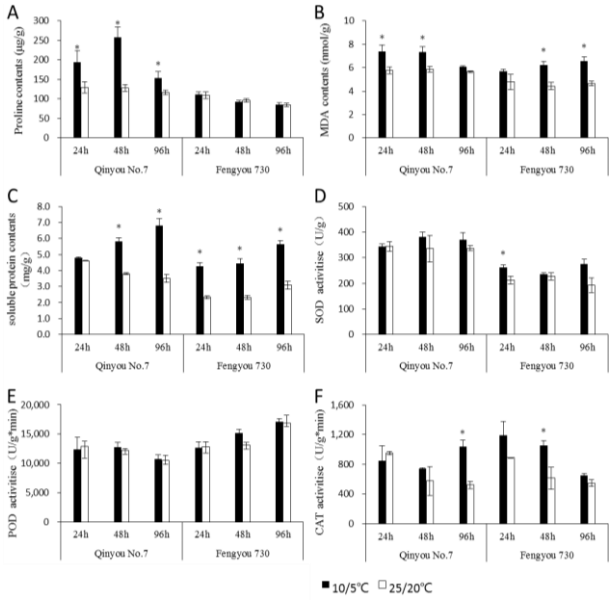

Fig. 3: Physiological responses of rapeseed seedlings to low temperature stress. (A) Proline concentration, (B) Malondialdehyde (MDA) content, (C) Soluble protein (SP) content, (D) Superoxide dismutase (SOD) activity, (E) Peroxidase (POD) activity and (F) Catalase (CAT) activity were assessed for shoots. Errors bars are SD. "*" indicates statistically significant differences between samples grown under control and stress conditions ($P < 0.05$)

3B). A gradual increase in soluble protein (SP) content was observed as the treating time was prolonged in both of the varieties (Fig. 3C). SOD activity was significantly increased in shoots of Fengyou 730 after 24 h treatment. The shoots of Qinyou No. 7 under low temperature showed a high SOD activity under low temperature stress (Fig. 3D). The similar situation was observed in the changes of POD activity for both varieties (Fig. 3E). The activity of CAT in Qinyou No. 7 was increased along with the extension of treatment period, while it was exactly opposite in Fengyou 730 (Fig. 3F).

Transcriptome Sequence Analysis and Assembly

Using the Illumina Hiseq2000 platform, 32327880, 29258470, 35524816 and 32984540 raw reads about 4G were generated in four samples of different low temperature durations (Table 1). After filtering and trimming the raw reads, 128089512 high-quality trimmed reads (Q20 > 99%) were used for further analysis. The raw sequence data have been submitted to the NCBI Short Read Archive with accession number SRP067875.

More than 77% of sample reads matched to the reference database (build 5), and more than 90% of these matching reads were positioned into exons region of the reference genome (see additional file 2 and 3).

Table 1: Throughput and quality of RNA-seq for the 4 libraries from low temperature treatment of 0 h, 24 h, 48 h and 96 h

Library	Raw Data		Valid Data		Valid Ratio (%)	Q20%
	Reads	Base (G)	Reads	Base (G)		
CK	32,327,880	4.04	31,731,236	3.97	98.15	99.55
LT 24h	29,258,470	3.66	28,806,142	3.6	98.45	99.38
LT 48h	35,524,816	4.44	35,033,202	4.38	98.62	99.38
LT 96h	32,984,540	4.12	32,448,932	4.06	98.38	99.25

All libraries were sequenced using HiSeq 2000. Q20 percentage indicates the percentage of sequences with sequencing error rate lower than 1%

There were 101040 genes assembled using the valid reads, in which 52086 genes showed significant annotated in GO database and 21218 genes were mapped to 269 KEGG pathways.

Differential Expression Gene Analyses

Compared with the control, a total number of 2001, 2001 and 1998 differentially expressed genes (DEGs) were discovered under low temperature treatment of 24 h, 48 h and 96 h, respectively. The number of up-regulated genes was increased by 40 when the low temperature treatment continued from 0 to 96 h. There were 428 up-regulated genes and 381 down-regulated genes among the LT-24 h vs. CK, LT-48h vs. CK and LT-96h vs. CK (Fig. 4).

The distribution of these genes is shown in Figure 5. Under biological processes, metabolic process was the most abundant GO term (13.6%), followed by cellular process (11.3%) and single-organism process (10.2%). In molecular function, genes-encoding proteins responsible for binding (18.2%) and catalytic activity (12.4%) were highly represented. For cellular component, the major categories included cell (4.9%), cell part (4.9%) and organelle (2.8%).

The KEGG pathway analysis showed that the most abundant pathways were related to metabolism (777 unigenes). Of the metabolic pathways that were identified, the most likely linked to low temperature stress is carbohydrate metabolism (206), followed by amino acid metabolism (158). There are 50 unigenes participated in low temperature-induced signal transduction (see additional file 4). Auxin responsive and transport protein, abscisic acid receptor, serine/threonine protein kinases and calmodulin were involved in signal transduction under low temperature stress.

Profiling Responses to the Low Temperature Duration

Using STEM clustering method to define a set of distinct and representative temporal gene expression mode under the four low temperature duration treatments, 2254 unigenes were clustered into 7 profiles (Fig. 7, including 3 up-regulated patterns (1248 unigenes) and 4 down-regulated patterns (1006 unigenes).

The continued up-regulated (profile 19) and down regulated (profile 0) DEGs were subjected to KEGG pathway analysis (Fig. 8). The KEGG enrichment analysis

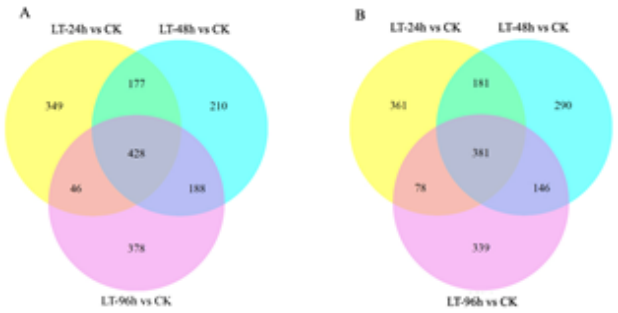

Fig. 4: Venn diagram of different expressed gene in LT-24 h vs. CK, LT-48 h vs. CK and LT-96 h vs. CK. (A) The up-regulated expressed gene. (B) The down-regulated expressed gene

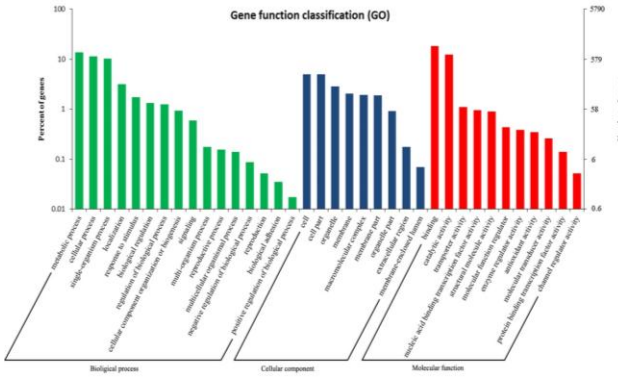

Fig. 5: GO annotations of all differentially expressed genes ($P < 0.05$)

showed that the unigenes mapped to protein processing in endoplasmic reticulum and protein export were up-regulated with the duration of low temperature ranging from 0–96 h, which demonstrated that the Qinyou No. 7 significantly increased protein synthesis and processing with positive transcriptional regulation to resist low temperature. Transcriptions related to the starch and sucrose metabolism were also increased.

Validation of the Different Expressed Genes by qRT-PCR

To confirm the accuracy and reproducibility of the transcriptome analysis results, 12 unigenes (8 from continue

to the up-regulated unigenes profile and 4 from continue to down-regulated unigenes profile) were selected for real-time quantitative reverse transcription PCR (qRT-PCR) validation. The expression profiles of the candidate unigenes revealed by qRT-PCR data were consistent with those derived from sequencing (Fig. 9).

Discussion

As the intuitionistic behavior of rapeseed seedling response to low temperature stress, morphological characteristics showed that seedling establishment was significantly inhibited under low temperature stress. Alteration in root-shoot length ratio was observed in this study and such kind of results were obtained for wheat crop (Equiza *et al.*, 2001). It was interesting that the dry weight of shoot was higher under low temperature than under normal temperature, which probably was associated with respiration inhibition under low temperature (Hurry *et al.*, 1995). These findings are supported by the previous study on rapeseed (Ping *et al.*, 2015), but are inconsistent with the results reported about sorghum (Yu *et al.*, 2004). Transcriptome analysis showed that the expression of many genes related to indole-3-acetic acid synthetase and auxin responsive and transport protein were down-regulated under low temperature stress. Indole-3-acetic acid (IAA), the main auxin in higher plants, has profound effects on plant growth and development (Zhao, 2010; Mashiguchia *et al.*, 2011). This implied that the auxin metabolism participated in controlling the length of root and shoot under low temperature stress at seedling establishment stage.

Changes in physiological characteristics reflected the process of seedling establishment responding to low temperature stress and the injury degree of stress. Plants under low temperature stress increased soluble matter concentration of cell to enhance the osmotic potential and drop the freezing point (Stitt and Hurry, 2002; Zhu *et al.*, 2007). In this study, the concentration of proline and soluble sugar were increased in shoots under low temperature, and the increase in resistant variety was significantly higher than sensitive variety. Similar results were also found in previous researches (Kushad and Yelenosky, 1987; Patton *et al.*, 2007; Burbulis *et al.*, 2011; Li *et al.*, 2013). Carbohydrate and protein metabolism is the foundation for the rapeseed seedling establishment (Patton *et al.*, 2007; Lutz, 2010). Transcriptome results revealed that beta-amylase and beta-glucosidase genes expression were up-regulated in low temperature stress to increase the sugar content. There are 8 heat shock protein expression, in which the heat shock 70kDa protein related gene *BnaA03g14210D* and *BnaA03g17100D* continued to up-regulate during the low temperature stress. The expression of molecular chaperone gene *BnaC05g35680D* was up-regulated firstly from 0–24 h and then down-regulated from 24–96 h in low temperature treatment. The up-regulated expression of molecular chaperone and heat shock protein helped to maintain the correct assembly of peptide and structure stability of protein, and then enhanced the resistance to low temperature (Hartl *et al.*, 2011; Aghdam *et al.*, 2013).

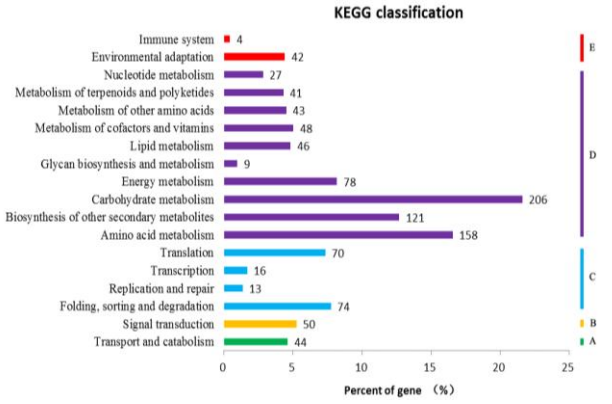

Fig. 6: The KEGG classification of all differentially expressed genes (P<0.05). (A) Cellular Processes. (B) Environmental Information Processing. (C) Genetic Information Processing. (D) Metabolism. (E) Organismal Systems

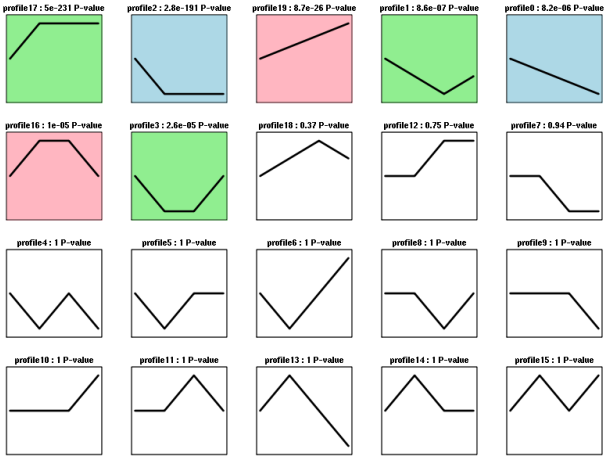

Fig. 7: Cluster analyses of the different expressed genes profiles. Developed by Short Time-series Expression Miner (v1.3.8) with the maximum number of model profiles was set to 20

Under low temperature stress, the reactive oxygen species (ROS) levels were raised in plant cells and the lipid peroxidation of the cell membrane was intense, which resulted in the accumulation of some toxic metabolites in plant (Wise and Naylor, 1987). As a product of membrane lipid peroxidation, MDA content in tissue reflected the degree of stress damage of biological membrane (An *et al.*, 2012). The MDA content firstly tended to increase and then decreased in resistant variety, but it continued to increase in sensitive variety. Research about physiological response of rapeseed to chilling stress showed that resistance materials

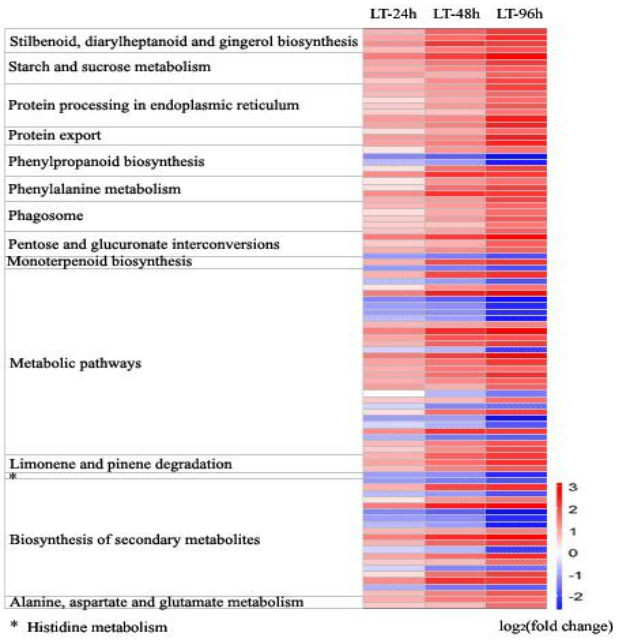

Fig. 8: The KEGG classification of continue to up and down-regulated genes

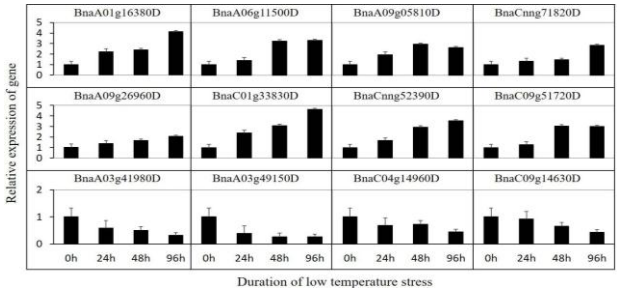

Fig. 9: Validation of DEGs using qRT-PCR (mean ± SE, n = 3)

persisted less MDA content under chilling stress (Pu and Sun, 2010; Zhang *et al.*, 2015b). Peroxidases are a kind of antioxidant enzymes, which can remove ROS in plant cell (Breusegem *et al.*, 1999). Previous studies mainly focused on SOD, POD and CAT activities, however, due to the material properties and the difference of stress duration and intensity, changes in antioxidant enzymes under low temperature stress were not so regular (Gechev *et al.*, 2003; Zhang *et al.*, 2011; Li *et al.*, 2013). In our experiment, the resistant variety initiated osmotic regulation system firstly and then triggered the antioxidant enzyme system when exposed to low temperature stress for long duration. The enzyme activity of sensitive material increased slightly earlier than resistant variety, but along with extension of low temperature stress, CAT activity of sensitive variety gradually reduced. Expression of 5 peroxidases genes increased in resistant variety, among which the *BnaA06g16150D* expression was up-regulated to 5 times of the control as the low temperature duration extended from 0–96 h in resistant variety. There were 12 glutathione S-transferase (GST) genes up-regulated. Overexpression of GST can stimulate seedling growth under chilling and salt stress, and this effect could be caused by oxidation of the glutathione pool (Roxas *et al.*, 1997). Overexpression of a tobacco glutathione S-transferase with glutathione peroxidase activity (GST/GPX) in transgenic tobacco (*Nicotiana tabacum* L.) enhanced seedling growth under a variety of stressful conditions (Roxas *et al.*, 2000). Secondary metabolism and its products also play an important role in rapeseed response to low temperature stress response. There were 8 flavonol synthase genes up-regulated, in which *BnaC07g29940D* expression continued to rise. The flavonoids from phenylpropanoid pathway could also protect membrane lipid in cold stress (Crifo *et al.*, 2011; Gao *et al.*, 2013).

The mechanism of low temperature response in rapeseed seedling at establishment stage is very complex, including the chilling signal transduction induced related genes expression and its metabolites involved in this process. A generic signal transduction pathway starts with the low temperature stress, followed by the generation of second messengers (Shinozaki *et al.*, 2003; Chinnusamy *et al.*, 2007). Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes (Xiong *et al.*, 2002; Kudla *et al.*, 2010; Batistic and Kudla, 2012). IP3 levels increased in plants under stress, and the time frame for the increase correlated with changes in cytosolic Ca^{2+} levels (Dewald *et al.*, 2001). In our experiment, 4 myo-inositol-1-phosphate synthase genes and 3 calmodulin genes participated in the resistance to low temperature stress, and the expression of calmodulin genes continued to increase during low temperature treatment. It demonstrates that IP3/Ca^{2+} signal transduction pathway plays an important role in the low temperature stress resistance. Many transcription factors have been reported in response to cold stress and alter the expression levels of some cold stress-responsive genes, eventually affect several physiological traits to overcome the adverse conditions (Gilmour *et al.*, 1998; Kasuga *et al.*, 1999; Sakuma *et al.*, 2002). Fourteen transcription factors from zinc finger (2), MYB (9), MADS (3) were considered to be associated with low temperature stress in seedling establishment. Among these, the expression of the zinc finger, 7 of the MYB and 1 of the MADS were up-regulated. Protein-serine/threonine kinases are one of the key kinases in ABA signal transduction pathway, regulating the activity of some protein by the phosphorylation (Mizoguchi *et al.*, 1995; Brock *et al.*, 2010; Roskoski, 2010). The up-regulated expression of the protein-serine/threonine kinases in low temperature stress reveals that ABA signal transduction pathway participates in the low temperature stress at seedling establishment stage.

Conclusion

Complex mechanisms including morphological, physiological and transcriptional level were involved in seedling establishment of rapeseed responding to low temperature stress. Shoot and root elongation was inhibited under low temperature stress, but the dry weight was increased. The resistant variety firstly initiated osmotic regulation system, and then triggered the antioxidant enzyme system under low temperature stress. Through transcriptome analysis, 2254 differentially expressed unigenes clustering in 7 expression patterns were participated in resistance to low temperature stress. The differentially expressed genes dataset will also be served as a very useful genetic resource to analysis the resistance of rapeseed to low temperature. Further studies are needed to verify the functions of candidate genes for improving low temperature tolerance ability through genetic engineering.

Acknowledgments

This research was funded by National Natural Science Foundation of China (31301268), Project 2013QC043 supported by the Fundamental Research Funds for the Central Universities and National Modern Rapeseed Industry Technology System (NYCYTC-00510).

References

Aghdam, M.S., L. Sevillano, F.B. Flores and S. Bodbodak, 2013. Heat shock proteins as biochemical markers for postharvest chilling stress in fruits and vegetables. *Sci. Hortic.*, 160: 54–64

Airaki, M., M. Leterrier, R.M. Mateos, R. Valderrama, M. Chaki, J.B. Barroso, L.A. Del Rio, J.M. Palma and F.J. Corpas, 2012. Metabolism of reactive oxygen species and reactive nitrogen species in pepper (*Capsicum annuum* L.) plants under low temperature stress. *Plant Cell Environ*., 35: 281–295

An, D., J. Yang and P. Zhang, 2012. Transcriptome profiling of low temperature-treated cassava apical shoots showed dynamic responses of tropical plant to cold stress. *BMC Genomics*, 13: 64

An, X., J. Chen, J. Zhang, Y. Liao, L. Dai, B. Wang, L. Liu and D. Peng, 2015. Transcriptome Profiling and Identification of Transcription Factors in Ramie (*Boehmeria nivea* L. Gaud) in Response to PEG Treatment, Using Illumina Paired-End Sequencing Technology. *Int. J. Mol. Sci.*, 16: 3493–3511

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

Bhatnagar-Mathur, P., M.J. Devi, V. Vadez and K.K. Sharma, 2009. Differential antioxidative responses in transgenic peanut bear no relationship to their superior transpiration efficiency under drought stress. *J. Plant Physiol*., 166: 1207–1217

Bradford, M.M., 1976. A rapid and sensitive method for thequantitation of microgram quantities of protein utilizing the principle ofprotein–dye binding. *Analytical Biochem.*, 72: 248–254

Breusegem, F.V., L. Slooten, J.M. Stassart, J. Botterman, T. Moens, M.V. Montagu and D. Inze´, 1999. Effects of overproduction of tobacco MnSOD in maize chloroplasts on foliar tolerance to cold and oxidative stress. *J. Experim. Bot.*, 50: 71–78

Brock, A.K., R. Willmann, D. Kolb, L. Grefen, H.M. Lajunen, G. Bethke, J. Lee, T. Nurnberger and A.A. Gust, 2010. The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. *Plant Physiol*., 153: 1098–1111

Burbulis, N., V. Jonytienė, R. Kuprienė and A. Blinstrubienė, 2011. Changes in proline and soluble sugars content during cold acclimation of winter rapeseed shoots in vitro. *J. Food Agric. Environ.,* 9: 371–374

Chinnusamy, V., J. Zhu and J.K. Zhu, 2007. Cold stress regulation of gene expression in plants. *Trends Plant Sci*., 12: 444–451

Chinnusamy, V., J.K. Zhu and R. Sunkar, 2010. Gene regulation during cold stress acclimation in plants. *Methods Mol. Biol.*, 639: 39–55

Crifo, T., I. Puglisi, G. Petrone, G.R. Recupero and A.R. Lo Piero, 2011. Expression analysis in response to low temperature stress in blood oranges: implication of the flavonoid biosynthetic pathway. *Gene,* 476: 1–9

Dewald, D.B., J. Torabinejad, C.A. Jones, J.C. Shope, A.R. Cangelosi, J.E. Thompson, G.D. Prestwich and H. Hama, 2001. Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed arabidopsis. *Plant Physiol.,* 126: 759–769

Diepenbrock, W., 2000. Yield analysis of winter oilseed rape (*Brassica napus* L): a review. *Field Crops Res.,* 67: 35–49

Donahue, J.L., C.M. Okpodu, C.L. Cramer, E.A. Grabau and R.G. Alscher, 1997. Responses of Antioxidants to Paraquat in Pea Leaves. *Plant Physiol*., 113: 249–257

Equiza, M.A., J.P. Mirave and J.A. Tognetti, 2001. Morphological, anatomical and physiological responses related to differential shoot vs. root growth inhibition at low temper. *Ann. Bot.*, 87: 67–76

Farooq, M., M. Hussain, A. Nawaz, D.-J. Lee, S.S. Alghamdi and K.H.M. Siddique, 2016. Seed priming improves chilling tolerance in chickpea by modulating germination metabolism, trehalose accumulation and carbon assimilation. *Plant Physiol. Biochem*., doi: 10.1016/j.plaphy.2016.12.012

Farooq, M., T. Aziz, A. Wahid, D.J. Lee and K.H.M. Siddique, 2009. Chilling tolerance in maize: agronomic and physiological applications. *Crop Pasture Sci*., 60: 501–516.

Gao, J., N. Jiang, X. Qin, X. Zhu, T. Ai, T. Peng, T. Peng, J. Wu, Y. Xu and F. Chen, 2013. Physiological and Metabolic Responses of Jatropha to Chilling Stress. *Int. J. Agric. Biol.*, 15: 871–877

Gechev, T., H. Willekens, M. Van Montagu, D. Inzé, Van Camp, Wim, V. Toneva and I. Minkov, 2003. Different responses of tobacco antioxidant enzymes to light and chilling stress. *J. Plant Physiol.*, 160: 509–515

Gilmour, S.J., D.G. Zarka, E.J. Stockinger, M.P. Salazar, J.M. Houghton and M.F. Thomashow, 1998. Low temperature regulation of theArabidopsis CBF family of AP2 transcriptional activators as anearly step in cold-induced COR gene expression. *The Plant J.*, 16: 433–442

Hartl, F.U., A. Bracher and M. Hayer-Hartl, 2011. Molecular chaperones in protein folding and proteostasis. *Nature,* 475: 324–332

Huang, L.K., H.D. Yan, X.X. Zhao, X.Q. Zhang, J. Wang, T. Frazier, G. Yin, X. Huang, D.F. Yan, W.J. Zang, X. Ma, Y. Peng, Y.H. Yan and W. Liu, 2015. Identifying differentially expressed genes under heat stress and developing molecular markers in orchardgrass (*Dactylis glomerata* L.) through transcriptome analysis. *Mol. Ecol. Resour.*, 15: 1497–509

Hurry, V.M., A. Strand, M. Tobiaeson, P. Gardestrom and G. Oquist, 1995. Cold Hardening of Spring and Winter Wheat and Rape Results in Differential Effects on Growth, Carbon Metabolism, and Carbohydrate Content. *Plant Physiol*, 109: 697–706

Kasuga, M, Q. Liu, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki, 1999. Improving plant drought, salt, andfreezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.,* 17: 287–291

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

Kushad, M.M. and G. Yelenosky, 1987. Evaluation of Polyamine and Proline Levels during LowTemperature Acclimation of Citrus. *Plant Physiol*., 84: 692–695

Li, H.S., 2000. *Plant Physiology Experiment Technology,* 2th edition. Higher Education Press of Beijin, China

Li, Q., S. Lei, K. Du, L. Li, X. Pang, Z. Wang, M. Wei, S. Fu, L. Hu and L. Xu, 2016. RNA-seq based transcriptomic analysis uncovers alpha-linolenic acid and jasmonic acid biosynthesis pathways respond to cold acclimation in Camellia japonica. *Sci. Rep.*, 6: 36463

Li, Z.G., L.X. Yuan, Q.L. Wang, Z.L. Ding and C.Y. Dong, 2013. Combined action of antioxidant defense system and osmolytes in chilling shock-induced chilling tolerance in Jatropha curcas seedlings. *Acta Physiol. Plant.*, 35: 2127–2136

Lu, X., H. Kim, S. Zhong, H. Chen, Z. Hu and B. Zhou, 2014. De novo transcriptome assembly for rudimentary leaves in Litchi chinesis Sonn. and identification of differentially expressed genes in response to reactive oxygen species. *BMC Genom.*, 18: 805

Lutz, C., 2010. Cell physiology of plants growing in cold environments. *Protoplasma,* 244: 53–73

Mashiguchia, K., K. Tanaka, T. Sakai, S. Sugawara, H. Kawaide, M. Natsume, A. Hanada, T. Yaeno, K. Shirasu, H. Yao, P. Y. Zhao, K.I. Hayashi, Y. Kamiya and H. Kasahara, 2011. The main auxin biosynthesis pathway in Arabidopsis. *PNAS*, 108: 18512–18517

Mizoguchi, T., N. Hayashida, K. Yamaguchi-Shinozakiax, H. Kamada and K. Shinozaki, 1995. Two genes that encode ribosomal-protein S6 kinase homologs are induced by cold or salinity stress in Arabidopsis thaliana. *FEBS Lett.*, 358: 199–204

Patton, A.J., S.M. Cunningham, J.J. Volenec and Z.J. Reicher, 2007. Differences in Freeze Tolerance of Zoysiagrasses: II. Carbohydrate and Proline Accumulation. *Crop Sci.*, 47: 2170–2180

Ping, Y., X. Meng-Zhu, Z. Zhe, Z. Xiao-Hong, H.U. Li-Yong and X.U. Zheng-Hua, 2015. Cluster analysis for different rapeseed varieties on seed germination and seedling growth under low temperature stress. *Res. on Crops*, 16: 68–77

Pu, Y. and W. Sun, 2010. The Relationship between Cold Resistance of Winter Turnip Rape Varieties and its Physiological Characteristics. *Mol. Plant Breed.*, 8: 335–339

Raza, S.H., H.R. Athar, M. Ashraf and A. Hameed, 2007. Glycinebetaine-induced modulation of antioxidant enzymes activities and ion accumulation in two wheat cultivars differing in salt tolerance. *Environ. Experim. Bot.*, 60: 368–376

Reiner, A., D. Yekutieli and Y. Benjamini, 2003. Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics*, 19: 365–378

Roskoski, R. Jr., 2010. RAF protein-serine/threonine kinases: structure and regulation. *Biochem. Biophys. Res. Commun.*, 399: 313–317

Roxas, V.P., S.A. Lodhi, Daniel K. Garrett, J.R. Mahan and R.D. Allen, 2000. Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. *Plant Cell Physiol*., 41: 1229–1234

Roxas, V.P., R.K. Smith Jr., E.R. Allen and R.D. Allen, 1997. Overexpression of glutathione S-transferase/ glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotechnol.*, 15: 988–991

Sakuma, Y., Q. Liu, J.G. Dubouzet, H. Abe, K. Shinozaki and K. Yamaguchi-Shinozaki, 2002. DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem. Biophys. Res. Commun.,* 290: 998–1009

Secco, D., M. Jabnoune, H. Walker, H. Shou, P. Wu, Y. Poirier and J. Whelan, 2013. Spatio-temporal transcript profiling of rice roots and shoots in response to phosphate starvation and recovery. *Plant Cell,* 25: 4285–4304

Sharma, P.C. and S. Chaudhary, 2016. NGS-Based Expression Profiling of HSP Genes During Cold and Freeze Stress in Seabuckthorn (*Hippophae rhamnoides* L.). *Heat Shock Proteins Plants*, 10: 309–327

Shinozaki, K., K. Yamaguchi-Shinozaki and M. Seki, 2003. Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol.*, 6: 410–417

Stitt, M. and V. Hurry, 2002. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. *Curr. Opin. Plant Biol.*, 5: 199–206

Suzuki, N. and R. Mittler, 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. *Physiol. Plant.,* 126: 45–51

Tian, H.Y., S.A. Channa and S.W. Hu, 2016. Relationships between genetic distance, combining ability and heterosis in rapeseed (*Brassica napus* L.). *Euphytica*, 213: 1

Trischuk, R.G., B.S. Schilling, N.H. Low, G.R. Gray and L.V. Gusta, 2014. Cold acclimation, de-acclimation and re-acclimation of spring canola, winter canola and winter wheat: The role of carbohydrates, cold-induced stress proteins and vernalization. *Environ. Experim. Bot.*, 106: 156–163

Wang, R., T. Cheng and L. Hu, 2015. Effect of wide–narrow row arrangement and plant density on yield and radiation use efficiency of mechanized direct-seeded canola in Central China. *Field Crops Res.*, 172: 42–52

Wise, R.R. and A.W. Naylor, 1987. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. *Plant Physiol.,* 83: 272–277

Xian, M., P. Yang, L. Hu and Z. Xu, 2015. Comprehensive evaluation of low temperature tolerance in rapeseed during germination and emergence periods. *Crops*, 5: 116–122

Xiong, L., K.S. Schumaker and J.K. Zhu, 2002. Cell Signaling during Cold, Drought, and Salt Stress. *Plant Cell*, pp: 165–183

Xu, L., L. Zhu, L. Tu, L. Liu, D. Yuan, L. Jin, L. Long and X. Zhang, 2011. Lignin metabolism has a central role in the resistance of cotton to the wilt fungus Verticillium dahliae as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. *J. Exp. Bot.*, 62: 5607–5621

Yu, J., M.R. Tuinstra, M.M. Claassen, W.B. Gordon and M.D. Witt, 2004. Analysis of cold tolerance in sorghum under controlled environment conditions. *Field Crops Res.,* 85: 21–30

Zhang, J, A.S. Mason, J. Wu, S. Liu, X. Zhang, T. Luo, R. Redden, J. Batley, L. Hu and G. Yan, 2015a. Identification of Putative Candidate Genes for Water Stress Tolerance in Canola (*Brassica napus*). *Front Plant Sci.*, 6: 1058

Zhang, J, K. Wu, S. Zeng, J.A.T.d. Silva, X. Zhao, C.E. Tian, H. Xia and J. Duan, 2013. Transcriptome analysis of Cymbidium sinense and its application to the identification of genes associated with floral development. *BMC Genomics,* 14: 279

Zhang, S., H. Jiang, S. Peng, H. Korpelainen and C. Li, 2011. Sex-related differences in morphological, physiological, and ultrastructural responses of Populus cathayana to chilling. *J. Exp. Bot.,* 62: 675–686

Zhang, X., L.J. Feng, T. Yang, Z. Xu and L. Hu, 2015b. Effects of chilling stress on physiological characteristics of rapeseed seedlings in winter. *Plant Physiol. J.*, 51: 737–746

Zhao, Y., 2010. Auxin biosynthesis and its role in plant development. *Annu. Rev. Plant Biol.*, 61: 49–64

Zheng, G.H., Y.P. Gao, R.W. Wilen and L.V. Gusta, 1998. Canola seed germination and seedling emergence from pre-hydrated and re-dried seeds subjected to salt and water stresses at low temperatures. *Ann. Appl. Biol.*, 132: 339–348

Zhu, J., C.H. Dong and J.K. Zhu, 2007. Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.*, 10: 290–295

(Received 08 September 2016; Accepted 29 December 2016)