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RESEARCH ARTICLE

Evaluation of protein's interaction and the regulatory network of some drought-responsive genes in Canola under drought and re-watering conditions

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Abstract

Drought stress is one of the most important environmental stresses that severely limits the growth and yield of Canola. The re-watering can compensate for the damage caused by drought stress. Investigation of protein's interaction of genes involved in important drought-responsive pathways and their regulatory network by microRNAs (miRNAs) under drought and re-watering conditions are helpful approaches to discovering drought-stress tolerance and recovery mechanisms. In this study, the protein's interaction and functional enrichment analyses of glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, heat shock factor main genes, and the regulatory network of key genes by miRNAs were investigated by in silico analysis. Then, the relative expression of key genes and their related miRNAs were investigated in tolerant and susceptible genotypes of Canola under drought and re-watering conditions by Real-time PCR technique. The bna-miR156b/c/g, bna-miR395d/e/f, bna-miR396a, and all the studied key genes except *HSFA1E* and *PK* showed changes in expression levels in one or both genotypes after re-watering. The *PPC1* and *HSFB2B* expression decreased, whereas the *MLS* and *CAC3* expression increased in both genotypes under re-watering treatment after drought stress. It could cause the regulation of oxaloacetate production, the increase of the glyoxylate cycle, lipid biosynthesis, and the reduction of the negative regulation of HSFs under re-watering conditions. It seems that *PPC1*, *G6PD2*, *MLS*, *CAC3*, and *HSFB2B* were involved in the recovery mechanisms after drought stress of Canola. They were regulated by drought-responsive miRNAs to respond appropriately to drought stress. Therefore, regulating these genes could be important in plant recovery mechanisms.

Keywords Fatty acid biosynthesis · Gene expression · Glyoxylate cycle · MiRNAs · STRING

Introduction

Canola (*Brassica napus*) is an essential oilseed crop cultivated for edible and industrial uses worldwide (Zhu et al. 2016; Batool et al. 2023; Secchi et al. 2023). Drought stress is one of the most important environmental stresses that severely limits the growth and yield of Canola and other crops in many regions of the world (Sevik and Cetin 2015; Poveda 2020; Varol et al. 2023).

Plants adapt to drought stress through morpho-physiological, biochemical, and molecular response defense mechanisms, which lead to adaptive changes in plants, such as changes in seed germination, growth rate, and regulation of stress response mechanisms (Yigit et al. 2016; Aghaie et al. 2020; Cicek et al. 2022; Muhammad Aslam et al. 2022). During drought stress, the accumulation of reactive oxygen species (ROS) can cause inhibition of the tricarboxylic acid cycle (TCA) in mitochondria, and it causes the induction of glycolysis and the oxidative pentose phosphate pathways (OPPP) (Gouda et al. 2020). Glycolysis is an essential pathway for energy generation in the cytosol of plant cells, and its regulation plays important roles during plant development and stress response (Zhang et al. 2017; Gouda et al. 2020). Many genes and enzymes are involved in glycolysis

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and pentose phosphate pathways. Phosphoglycerate kinase (PGK) is a regulatory glycolytic enzyme responsible for catalyzing the reversible conversion of 1,3-biphosphoglycerate to 3-phosphoglycerate (Chen et al. 2018). Pyruvate kinase (PK), one of the other regulatory enzymes, has a crucial role in plant defense signal transduction, and it provides energy for plant drought response (Si et al. 2009; Li et al. 2019). The produced oxaloacetate in glycolysis can be converted to malate and enter the TCA and glyoxylate cycle (Dai et al. 2013). The allosteric enzyme PPC1 is involved in many physiological and developmental processes, and it has a criteria role in tolerance to biotic and abiotic stresses (Bandyopadhyay et al. 2007; Hýsková et al. 2014; Liu et al. 2017).

The oxidative pentose phosphate pathway consists of oxidizing glucose-6-phosphate to pentose-phosphate by evolving the CO_2 molecule and reducing the $NADP^+$ to NADPH. The activation of the pentose phosphate pathway is necessary to balance of $NADP^+$ to NADPH (Landi et al. 2016). G6PD2 is the principal regulatory enzyme in the oxidative pentose phosphate pathway (Yang et al. 2019). G6PD2 is a source of NADPH in the cytoplasm of plant cells (Pan et al. 2016). The NADPH resulting from the pentose phosphate pathway is an essential reductive coenzyme in various biological processes (Corpas and Barroso 2014; Liu et al. 2019). But, excessive NADPH causes the production of ROS and damage to the cells (Sun et al. 2019).

The glyoxylate cycle is an anaplerotic pathway of the TCA cycle that allows the formation of 4-carbon compound succinate from two-carbon acetyl-CoA (Kunze and Hartig 2013). The involved enzymes of the glyoxylate cycle are affected by various stress conditions (He et al. 2019; Yuenyong et al. 2019; Brito et al. 2020). Isocitrate lyase (ICL) and malate synthase (MLS) are two unique vital enzymes that are involved in the glyoxylate cycle (Wu et al. 2020). MLS is an acyltransferase enzyme responsible for malate synthesis in the glyoxylate cycle. The malate could be transported to the cytosol, and it could convert to the oxaloacetate. Then, it enters in gluconeogenesis pathway (Pua et al. 2003; Brito et al. 2020). The fatty acid β-oxidation pathway is linked to the glyoxylate cycle in the peroxisome, which plays a particularly considerable role in germinating seeds and stress response (Olmedilla and Sandalio 2019; Sandalio et al. 2021). CAC3 is a subunit of acetyl-CoA carboxylase (ACC) enzyme in plants. It is involved in fatty acid biosynthesis and drought response mechanisms (Ke et al. 2000).

Among stress-related genes, the heat shock factor (HSF) family is an essential regulator of environmental stress responses (Priya et al. 2019). It is reported that A1 and A2 HSFs groups in Arabidopsis play crucial roles in heat shock response, and the B HSFs group negatively regulates heat-induced HSFs (Wang et al. 2020). HSPs are induced by different biotic and abiotic stress conditions (Balfagón et al. 2018). They participate in folding and unfolding to prevent protein accumulations and misfolded conformers (Ni et al. 2021). HSFA1s play a central role in the heat stress response in Arabidopsis. Glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor genes have been reported to be affected in response to stress conditions in different crops (Esposito 2016; Zhu et al. 2017; He et al. 2019; Yuenyong et al. 2019; Wulfert et al. 2020; Mi et al. 2022).

Re-watering or rehydrated treatment of plants restores the normal physiological functions of plants, and it could compensate for the damage caused by drought stress by precipitating growth and enhancing photosynthetic capacity (Qi et al. 2021). The response of plants to re-watering and regaining the original natural state after disturbance is very complex. It is defined as the complex interactions of various genes, microRNAs (miRNAs), transcription factors, and other drought-regulating mechanisms during drought stress (Guo and Ogle 2019; Qi et al. 2021). MiRNAs are small noncoding RNAs that regulate various aspects of biotic and abiotic stress responses in plants by regulating their target genes (Jian et al. 2016; Cao et al. 2018; Fourounjian et al. 2019; Sanz-Carbonell et al. 2020). Regulation of many miRNAs has been reported to be altered in response to drought and other stress conditions (Liu et al. 2008, 2016; Zhou et al. 2010; Hajdarpašić and Ruggenthaler 2012; Bhardwaj et al. 2014; Jian et al. 2016; Li et al. 2021; Pasandideh Arjmand et al. 2023).

Recently, some studies have focused on protein–protein interaction (PPI) network analysis by the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database to gain comprehensive information on the interaction network and KEGG pathway of the genes (Peng et al. 2020; Mohsenzadeh Golfazani et al. 2022; Taghvaei et al. 2022; Yan et al. 2022). The experimental data from PPI methods have been used in PPI network construction (Zainal-Abidin et al. 2022). Many studies have been conducted on the response of specific genes involved in important biological pathways such as glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor genes under drought stress in Canola and other crops (Muronetz et al. 2019; Brito et al. 2020; Gouda et al. 2020; Wang et al. 2020). However, there is little information about the protein's interaction of the main genes of these pathways and their regulatory network by miRNAs under drought stress and re-watering conditions in Canola. There is a high level of homology in the protein-coding sequences between Canola and Arabidopsis, which makes it possible to use Arabidopsis genome information for the functional interpretation of Canola (Shamloo-Dashtpagerdi et al. 2015).

It is hypothesized that some key drought-responsive genes could be regulated by important miRNAs and involved in the recovery mechanism after re-watering

conditions. Therefore, the PPI network analysis of important genes involved in the drought-responsive biological pathways based on the Arabidopsis model plant and the investigation of the expression pattern of mRNA transcripts and their related miRNA using quantitative Real-time PCR (qRT-PCR) technique could be essential and innovative for identifying the regulatory network of drought stress response and recovery mechanisms in Canola and other similar plants. The main aim of the present study is to identify genes and miRNAs involved in important biological pathways that play an influential role in drought stress response and recovery mechanisms. Therefore, the important glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor genes were investigated based on PPI network analysis. Then, the expression pattern of some key genes and the miRNAs affecting their expression were investigated in tolerant and susceptible genotypes of Canola under drought stress and re-watering conditions by Real-time PCR technique.

Material and methods

Protein–protein interaction and functional enrichment analyses of genes

The main genes of some drought-responsive biological pathways, including glycolysis, oxidative pentose phosphate, glyoxylate cycle and fatty acid biosynthesis, and some heat shock factor genes, were selected based on UniProt database information (Table 1). They are the main genes of important biological pathways that respond to drought stress, as reported in previous studies (Ke et al. 2000; Bandyopadhyay et al. 2007; Si et al. 2009; Hýsková et al. 2014; Pan et al. 2016; Liu et al. 2017; Li et al. 2019; Wang et al. 2020). The protein–protein interaction network of these genes was carried out using the STRING database version 11.5 (https://string-db.org/) (Szklarczyk et al. 2019; Peng et al. 2020; Corpas et al. 2021; Mohsenzadeh Golfazani et al. 2022; Taghvaei et al. 2022). The STRING database information is collected from several online databases based on experiments (Szklarczyk et al. 2019). The network includes all the genes encoding proteins in the genome, and it highlights

Table 1 Selected glycolysis, oxidative pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor genes using *Arabidopsis thaliana* as a reference plant

Gene name	Identifier		UniProt ID
Malate synthase	AT5G03860	*MLS*	Q9LZC3
Isocitrate lyase	AT3G21720	*ICL*	P28297
Aconitase 3	AT2G05710	*ACO3*	Q9SIB9
Malate dehydrogenase 2, cytoplasmic	AT5G43330	*c-NAD-MDH2*	P57106
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic ·	AT3G04120	*GAPC1*	P25858
Phosphoglycerate kinase 3, cytosolic	AT1G79550	*PGK*	Q9SAJ4
Phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent)	AT1G70730	*PGM2*	F4I6W4
Cytosolic enolase 3	AT2G29560	*ENOC*	Q9ZW34
Pyruvate kinase	AT5G56350	*PK*	Q9FM97
Phosphoenolpyruvate carboxylase 1	AT1G53310	*PPC1*	Q9MAH0
Probable 6-phosphogluconolactonase 1	AT1G13700	*PGL1*	Q9LMX8
Probable 6-phosphogluconolactonase 5	AT5G24420	*PGL5*	Q8LEV7
Glucose-6-phosphate 1-dehydrogenase 2, chloroplastic	AT5G13110	*G6PD2*	Q9FY99
6-phosphogluconate dehydrogenase, decarboxylating 1, chloroplastic	AT1G64190	*6PGD1*	Q9SH69
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha, chloroplastic	AT2G38040	*CAC3*	Q9LD43
Biotin carboxyl carrier protein of acetyl-CoA carboxylase 2, chloroplastic	AT5G15530	*BCCP2*	Q9LLC1
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic	ATCG00500	*ACCD*	A0A1B1W4V3
Beta-ketoacyl-[acyl-carrier-protein] synthase III, chloroplastic	AT1G62640	*T3P18.20*	P49243
3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplastic	AT5G46290	*KASI*	P52410
Heat shock protein 90–4	AT5G56000	*Hsp81.4*	O03986
Heat shock protein 90–1	AT5G52640	*HSP90.1*	P27323
Heat stress transcription factor A-1e	AT3G02990	*HSFA1E*	Q9SCW5
Heat stress transcription factor B-2b	AT4G11660	*AT-HSFB2B*	Q9T0D3
Heat shock 70 kDa protein 4	AT3G12580	*HSP70*	Q9LHA8

their function and relationship. Therefore, the desired information is obtained faster, and the required time and cost are reduced (Jiang et al. 2021; Kumar and Bhalothia 2021; Bonilla et al. 2022; Mohsenzadeh Golfazani et al. 2022).

Then, the functional enrichment analysis of the genes was conducted by the SRPlot tool (https://www.bioinformatics.com.cn/en) based on the KEGG pathway analysis of STRING database version 11.5 (https://string-db.org/). It allows for outlining the potential functional protein–protein associations of plants (Szklarczyk et al. 2019; Corpas et al. 2021). After that, some genes, including *PK*, *PPC1*, *G6PD2*, *MLS*, *CAC3*, *HSFA1E*, and *HSFB2B*, were selected as candidate key genes for experimental analysis based on their function and protein–protein interaction with other genes.

Identification of potential miRNA of the key genes

The potential miRNAs of *PK*, *PPC1*, *G6PD2*, *MLS*, *CAC3*, *HSFA1E*, and *HSFB2B* genes were identified by the psRNATarget database (https://www.zhaolab.org/psRNATarget/) using the published miRNAs in *Brassica napus* (Dai et al. 2018). It is a free and user-friendly tool developed to identify complementary matching between plant miRNA and mRNA sequences (Dai et al. 2018). Cytoscape software version 3.9.1 was used to map the relationship between the identified miRNAs and the key genes based on the STRING database version 11.5 (https://string-db.org/) and psRNAtarget tool (https://www.zhaolab.org/psRNATarget/).

Plant materials and growth conditions

Seeds of the Canola genotypes (SLM046 and Hayola308) were obtained from the Seed and Plant Improvement Institute Karaj, Iran. The previous screening has identified that SLM046 is a tolerant genotype to drought stress, while Hayola308 is a drought-susceptible genotype (Mirzai et al. 2013; Pasandideh Arjmand et al. 2018). Seeds were surface sterilized with a 2% (v/v) sodium hypochlorite (NaOCl) solution for 10 min. Then, they were washed to remove NaOCl and germinated in Petri dishes on wet layers of filter paper at 25 °C in the growth chamber. After 4 days, suitable seedlings were planted in the soil. Plants were grown in a greenhouse at 23 ± 1 °C for a 12 h photoperiod, at 50–60% relative humidity, and controlled light of 100 µmol $m^{-2}s^{-1}$.

Drought and re-watering treatments

Plants were grown under well-watered conditions (non-stress) to 100% Field Capacity (FC). The soil samples were placed for 24 h in a pressure plate apparatus in three replicates for the FC calculation. Then the soil moisture content (%) was calculated by gravimetric method (Hansen et al. 1980). In addition, to the determination of the sampling time, the soil moisture was controlled by gypsum block (Soilmoisture Meter, 5910A) and tensiometer (Jet-Fill, 2725 ARL) devices during the experiment. Before starting the planting, these devices were randomly placed in three pots. The pots were completely saturated with water and placed at 23 °C. The numbers of the devices were read every day, and three soil samples were taken. Then, the soil moisture content at each point was obtained (Hansen et al. 1980). This information was used during the treatment and sampling of plants. 18 days after planting, drought stress was induced by withholding for 3 weeks to reduce soil moisture to 25% FC (39 days after planting at the five-leaf stage). 39 days after planting, one group of drought treatment samples of two genotypes was collected. At the same time, one group of samples was re-watered and allowed to recover (24 h) until the soil moisture was 100% FC (the five-leaf stage) (Hansen et al. 1980). Then, they were collected too. The well-watered samples were used as control samples. The leaf tissues were separately sampled from each treatment and quickly stored in liquid N_2.

RNA extraction, cDNA synthesis of mRNA, and design of specific primers

Total RNAs were extracted from the leaf tissues in 3 conditions (non-stress, drought stress, and re-watering conditions) using the super RNA extraction kit (AnaCell, Iran) according to the manufacturer's protocol. Then, total RNA was treated with RNase-free DNase I (CinnaGen, Iran). The RNA concentration and its quality were determined by a NanoDrop spectrophotometer (BioTek Epoch™ 2, USA) and 1% agarose gel electrophoresis, respectively. The treated RNA was used for cDNA synthesis of mRNA and miRNAs of each sample.

The first-strand cDNA synthesis kit (AnaCell, Iran) was used for the cDNA synthesis of mRNA according to the manufacturer's protocol. Primer pairs for qRT-PCR analysis of genes were designed using the Primer3 program (Kõressaar et al. 2018). The accuracy and appropriateness of primers were checked by the Oligo analyzer (https://www.idtdna.com/pages/tools/oligoanalyzer) and NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov) tools. The primers used for qRT-PCR analysis were listed in supplementary A.

Investigation of gene expression by qRT-PCR

The relative expression pattern of genes was investigated by the quantitative Real-time PCR technique using a SYBR Green qPCR Master Mix 2X (AnaCell, Iran) based on manufacturer's instruction with the following amplification conditions: activation at 95 °C for 15 min; 95 °C for 30 s; followed by 40 cycles at 95 °C for 30 s and annealing temperature (°C) for the 30 s; and 72 °C for 30 s and final

holding at 4 °C. The relative expression of genes was performed in Roche LightCycler® 96 instrument (Germany) in three biological and technical replicates. The $2^{-\Delta\Delta Ct}$ method was used to obtain relative gene expression (Livak and Schmittgen 2001). The accuracy and sensitivity of qRT-PCR were obtained using the preparation of dilution series for cDNA in the qRT-PCR method (Schmittgen and Livak 2008; Ramzanzadeh et al. 2021).

cDNA synthesis and expression analysis of identified miRNAs by stem-loop method

Some identified miRNAs, such as bna-miR156b, bna-miR156c, bna-miR156g, bna-miR395d, bna-miR395e, bna-miR395f, and bna-miR396a were selected for expression analysis by Real-time PCR technique. The mature sequences of these miRNAs were obtained from the miRBase database (https://www.mirbase.org/), and it was found that the mature sequences of bna-miR156b, bna-miR156c, and bna-miR156g were similar. Also, the mature sequences of bna-miR395d, bna-miR395e, and bna-miR395f were similar. Specific stem-loop bna-miR156b/c/g, bna-miR395d/e/f, and bna-miR396a detection kits (Ana Cell, Iran) were used for miRNA-specific cDNA synthesis, and the qRT-PCR experiment based on manufacturer's instruction (Chen et al. 2005). The stem-loop method is an appropriate and cost-effective approach that employs a stem-loop reverse transcriptase primer for specific binding to a mature miRNA. Then, a miRNA-specific forward primer and a universal reverse primer are used for quantifying mature miRNA expression in qRT-PCR (Chen et al. 2005; Varkonyi-Gasic 2017). *U6* snRNA was used as an internal control of miRNAs (Supplementary A) (Jiang et al. 2021; Zhao et al. 2012). The reactions were carried out in a LightCycler® 96 instrument (Roche, Germany) with the following amplification conditions: activation at 95 °C for 15 min; 95 °C for 30 min, followed by 40 cycles at 95 °C for 30 s and 55 °C for the 60 s; and 72 °C for 30 s and final holding at 4 °C. The $2^{-\Delta\Delta Ct}$ method was used to obtain the relative gene expression (Livak and Schmittgen 2001).

Statistical analysis

The significance of qRT-PCR with three biological and technical replicates was calculated by SAS software (version 9.4) with analysis of variance (ANOVA). Tukey's test was used to distinguish differences between mean values, and a p-value < 0.01 was considered statistically significant. The experiment was conducted in a completely randomized design (CRD) in a factorial arrangement.

Results

Protein–protein interaction network and functional enrichment analyses

The interacting proteins in the network were involved in glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis pathways, etc. The main genes of these pathways were closely related to each other. Some genes were enrichment in carbon fixation in the photosynthetic organism pathway. There was no KEGG term for HSF genes in the STRING database. These genes are heat shock factors and could be effective in various biological pathways (Fig. 1, Table 2, respectively).

The result showed that the main genes were involved in other important pathways such as carbon metabolism, metabolic pathways, biosynthesis of secondary metabolites, pyruvate metabolism, fatty acid metabolism, biosynthesis of amino acids, propanoate metabolism, protein processing in the endoplasmic reticulum, citrate cycle (TCA cycle), purine metabolism, and glutathione metabolism (Fig. 2).

The potential miRNA of the key genes

After protein–protein interaction analysis and investigation of their functional relationships with each other, the candidate genes such as *PK* (AT4G26390, AT5G56350), *PPC1*, *G6PD2*, *MLS*, *CAC3*, *HSFA1E*, and *HSFB2B* genes were selected as glycolysis, oxidative pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor key genes. The results showed that these genes are regulated by 21 different miRNAs. Some genes are targeted by common miRNAs. *PPC1*, *G6PD2*, *HSFA1E*, and *HSFB2B* were target by bna-miR395d/e/f. *MLS* and *CAC3* genes were regulated by bna-miR396a (Table 3, Fig. 3, respectively).

Experimental investigation

Phenotypic changes of Canola after drought stress and re-watering treatments

The previous study on SLM046 and Hayola308 genotypes found that drought stress has specific morphological and physiological effects on genotypes. The SLM046 genotype, unlike the Hayola308 genotype, had traits that could assign tolerance to drought stress (Mirzai et al. 2013; Pasandideh Arjmand et al. 2018).

The Hyola308 genotype showed signs of drastically wilting after drought stress compared to the control (Fig. 4A). In contrast, the SLM046 genotype wilting symptoms were

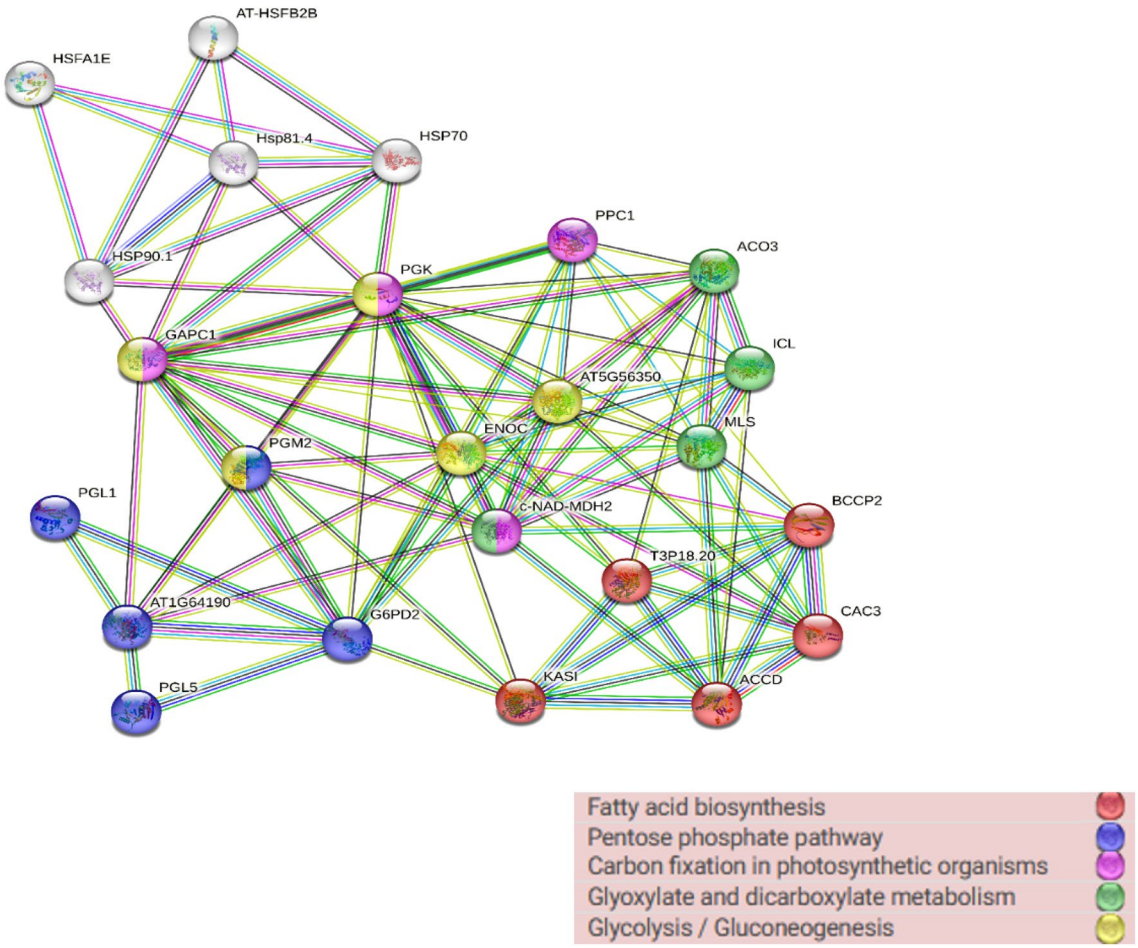

Fig. 1 Protein–protein interaction network of glycolysis, oxidative pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor main genes using the STRING database. Colored nodes denote proteins, and each color indicates a KEGG pathway. The edges represent their interactions with each other

Table 2 KEGG pathways of selected main genes based on STRING database. Gene count indicates the number of genes enriched in the KEGG pathways

KEGG ID	Term description	Observed gene count	Background gene count	Strength	False discovery rate
ath00061	Fatty acid biosynthesis	5	43	2.12	1.48E−08
ath00030	Pentose phosphate pathway	5	58	1.99	5.02E−08
ath00710	Carbon fixation in photosynthetic organisms	4	69	1.82	7.06E−06
ath00630	Glyoxylate and dicarboxylate metabolism	4	77	1.77	9.67E−06
ath00010	Glycolysis/Gluconeogenesis	5	116	1.69	1.03E−06

less (Fig. 4B). The shoot length of Hayola308 and SLM046 was similar in non-stress conditions (Fig. 4A, B). However, the shoot length of SLM046 was greater than Hayola308 under drought stress and re-watering conditions (Fig. 4C, D). The drought stress decreased leaf area in both genotypes (Fig. 4A, B). In addition, the leaves in SLM046 genotype looked bigger and greener than the leaves in Hyola308 genotype under drought stress and re-watering conditions (Fig. 4C, D).

After re-watering of plants, the wilting symptoms gradually disappeared in both genotypes (Fig. 4D), and it could cause the plants' freshness to recover again. However, examining the response at the molecular level could reveal more differences.

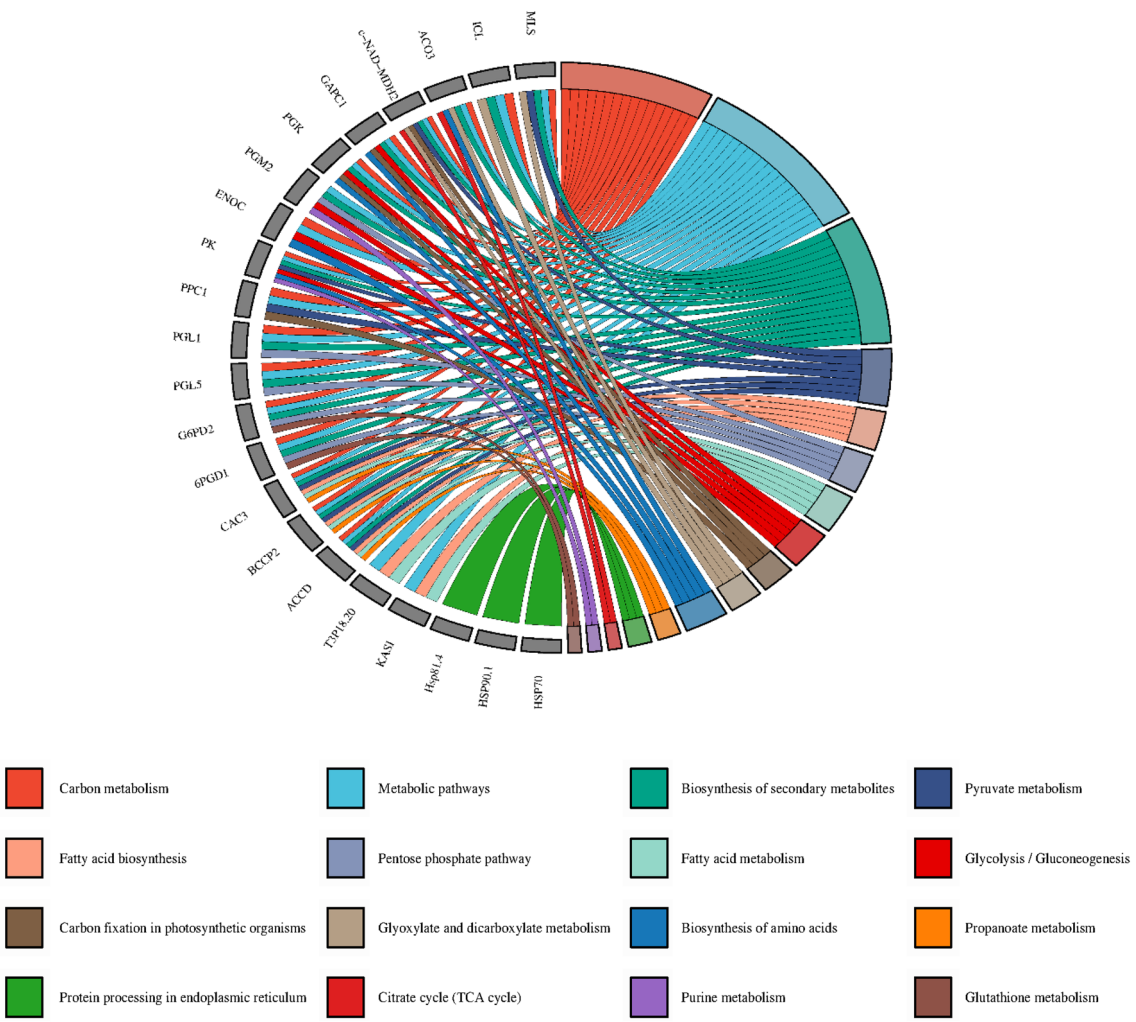

Fig. 2 Functional enrichment analysis of the main genes based on the STRING database. Colors represent biological pathways. The same color in the genes indicates the same biological pathway for the genes

Expression analysis of the key genes and their identified miRNAs

The level of *PK* expression was higher in the SLM046 genotype than in Hayola308 under drought stress conditions. Re-watering did not significantly change the expression of this gene in genotypes (Fig. 5A).

The results illustrated that the *PK* gene was regulated by bna-miR156b/c/g (Table 3, Fig. 3, respectively). As shown in Fig. 5B, the expression level of bna-miR156b/c/g was higher in the SLM046 genotype than in the Hayola308 genotype under drought stress conditions. Re-watering treatment decreased bna-miR156b/c/g expression in the SLM046 genotype. However, the re-watering treatment caused the expression of bna-miR156b/c/g to increase 12 times in the Hayola308 genotype.

There was no significant difference in the expression of *G6PD2* in Hayola308 and SLM046 genotypes under drought stress conditions. Re-watering did not cause significant changes in *G6PD2* expression in the SLM046 genotype. However, the expression of *G6PD2* in the Hayola308 genotype increased by re-watering treatment. The expression level of this gene in the Hayola308 genotype was almost two times higher than in the SLM046 genotype (Fig. 5C).

The expression level of *PPC1* was higher in Hayola308 genotype than the SLM046 genotype under drought stress and re-watering conditions. Re-watering decreased the expression of *PPC1* in both genotypes (Fig. 5D).

The result showed that the expression of *HSFA1E* in Hayola308 and SLM046 genotypes was not significantly different under drought stress conditions. Re-watering did not cause a significant change in the expression level of *HSFA1E* in the genotypes. The expression level of *HSFA1E* in the Hayola308 genotype was higher than SLM046 in the re-watering treatment (Fig. 5E). The expression of *HSFB2B* in SLM046 was almost twice as high as in Hayola308 under

Table 3 Identified microRNAs affecting *PK*, *PPC1*, *G6PD2*, *MLS*, *CAC3*, *HSFA1E*, and *HSFB2B* genes using the psRNATarget tool with corresponding expected values 5. A higher expectation value indicates less similarity between miRNA and the target genes

miRNA_Acc	Target_Acc	Expectation	Target_start	Target_end	Inhibition	Target_Desc
bna-miR156b	XM_013878587.2	5	708	728	Cleavage	*PK*
bna-miR156c	XM_013878587.2	5	708	728	Cleavage	*PK*
bna-miR156g	XM_013878587.2	5	708	728	Cleavage	*PK*
bna-miR164a	XM_013800011.2	3.5	3199	3219	Cleavage	*PPC1*
bna-miR164b	XM_013800011.2	3.5	3199	3219	Cleavage	*PPC1*
bna-miR164c	XM_013800011.2	3.5	3199	3219	Cleavage	*PPC1*
bna-miR164d	XM_013800011.2	3.5	3199	3219	Cleavage	*PPC1*
bna-miR395a	XM_013800011.2	4.5	2182	2202	Cleavage	*PPC1*
bna-miR395b	XM_013800011.2	4.5	2182	2202	Cleavage	*PPC1*
bna-miR395c	XM_013800011.2	4.5	2182	2202	Cleavage	*PPC1*
bna-miR395d	XM_013800011.2	4.5	2182	2202	Cleavage	*PPC1*
bna-miR395e	XM_013800011.2	4.5	2182	2202	Cleavage	*PPC1*
bna-miR395f	XM_013800011.2	4.5	2182	2202	Cleavage	*PPC1*
bna-miR6032	XM_013800011.2	4.5	602	621	Cleavage	*PPC1*
bna-miR395d	XM_013896871.2	4.5	572	592	Cleavage	*G6PD2*
bna-miR395e	XM_013896871.2	4.5	572	592	Cleavage	*G6PD2*
bna-miR395f	XM_013896871.2	4.5	572	592	Cleavage	*G6PD2*
bna-miR396a	XM_013864819.2	2.5	1527	1547	Cleavage	*MLS*
bna-miR159	XM_013794785.2	5	2617	2637	Cleavage	*CAC3*
bna-miR2111a-5p	XM_013794785.2	5	1604	1624	Cleavage	*CAC3*
bna-miR2111b-5p	XM_013794785.2	5	1604	1624	Cleavage	*CAC3*
bna-miR2111d	XM_013794785.2	5	1604	1624	Cleavage	*CAC3*
bna-miR396a	XM_013794785.2	2.5	1990	2010	Cleavage	*CAC3*
bna-miR395a	XM_013876884.2	3.5	907	927	Cleavage	*HSFA1E*
bna-miR395b	XM_013876884.2	3.5	907	927	Cleavage	*HSFA1E*
bna-miR395c	XM_013876884.2	3.5	907	927	Cleavage	*HSFA1E*
bna-miR395d	XM_013876884.2	4	907	927	Cleavage	*HSFA1E*
bna-miR395e	XM_013876884.2	4	907	927	Cleavage	*HSFA1E*
bna-miR395f	XM_013876884.2	4	907	927	Cleavage	*HSFA1E*
bna-miR395a	XM_013859043.2	5	707	727	Cleavage	*HSFB2B*
bna-miR395b	XM_013859043.2	5	707	727	Cleavage	*HSFB2B*
bna-miR395c	XM_013859043.2	5	707	727	Cleavage	*HSFB2B*
bna-miR395d	XM_013859043.2	4	707	727	Cleavage	*HSFB2B*
bna-miR395e	XM_013859043.2	4	707	727	Cleavage	*HSFB2B*
bna-miR395f	XM_013859043.2	4	707	727	Cleavage	*HSFB2B*
bna-miR403	XM_013859043.2	5	339	359	Cleavage	*HSFB2B*
bna-miR403	XM_013859043.2	5	424	444	Cleavage	*HSFB2B*
bna-miR6028	XM_013859043.2	5	1804	1824	Translation	*HSFB2B*

drought-stress conditions. Re-watering decreased the expression of *HSFB2B* in both genotypes. The expression level of *HSFB2B* was similar in both genotypes under re-watering conditions (Fig. 5F). The result demonstrated that *G6PD2*, *PPC1*, *HSFA1E*, and *HSFB2B* could be regulated by bna-miR395d/e/f in Canola. The result showed that these genes, except *G6PD2* were regulated by miR395a/b/c (Table 3, Fig. 3, respectively).

There was no significant difference in the expression level of bna-miR395d/e/f in SLM046 and Hayola308 genotypes under drought stress conditions. After re-watering, there was no significant change in the expression of bna-miR395d/e/f in the SLM046 genotype. But re-watering caused a significant increase in bna-miR395d/e/f expression in the Hayola308 genotype (Fig. 5G).

There was no significant difference in the expression of *MLS* in both genotypes under drought stress conditions. Re-watering increased the expression of *MLS* in both genotypes. *MLS* expression in the Hayola308 genotype was almost 2.5 times higher than SLM046 in re-watering conditions (Fig. 5H).

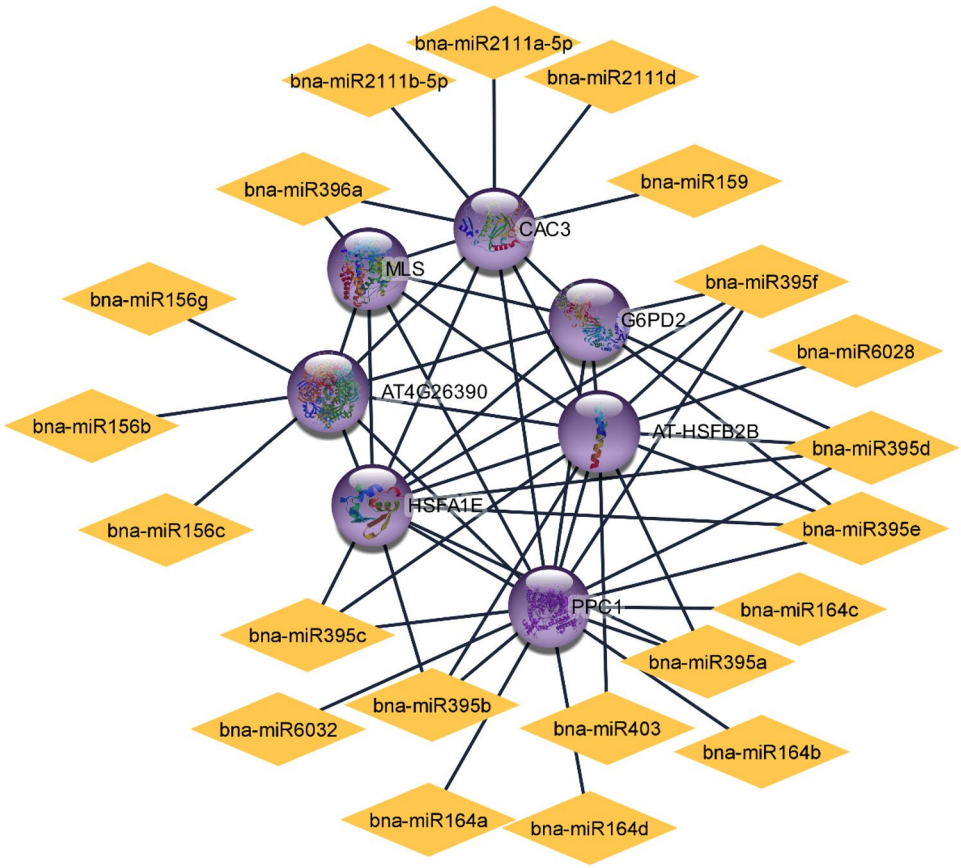

Fig. 3 Relationships between microRNAs affecting *PK*, *PPC1*, *G6PD2*, *MLS*, *CAC3*, *HSFA1E*, and *HSFB2B* genes were mapped using Cytoscape software. The rhombus shapes represent microRNAs, and the circles indicate the key genes. The edges represent the interaction between miRNAs and key genes based on the STRING database and psRNAtarget tool

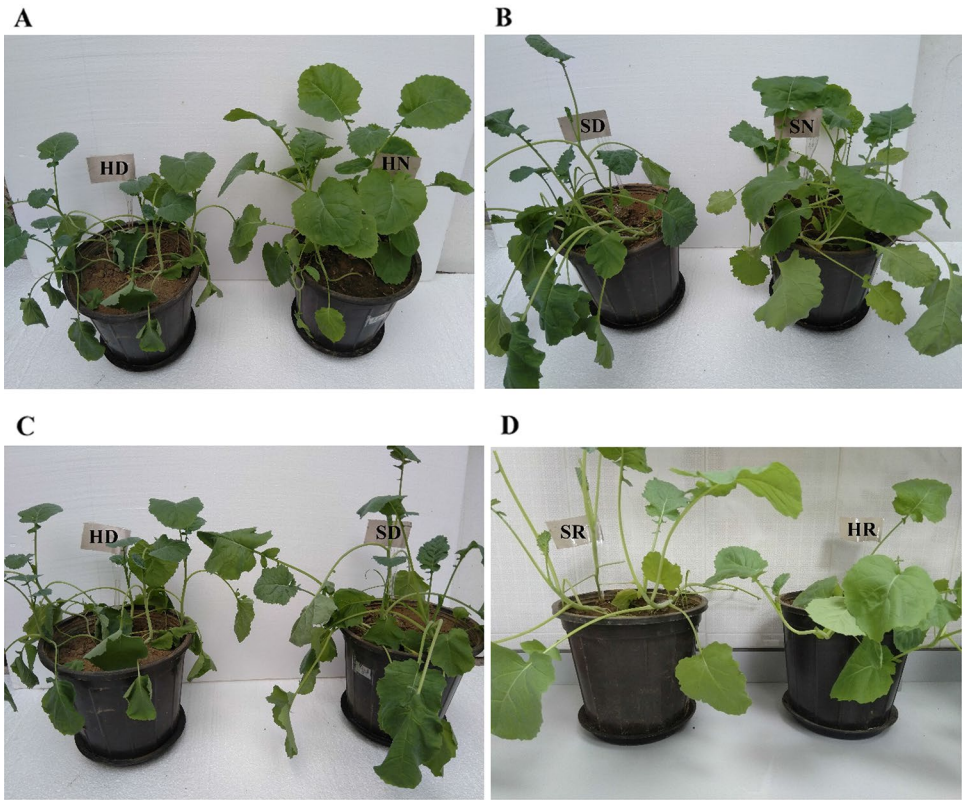

Fig. 4 Phenotypic differences between the SLM046 and Hayola308 genotypes under non-stress (control), drought stress (withholding for 3 weeks), and re-watering treatment (24 h after re-watering of drought treatment samples) conditions at sampling time (five-leaf stage). **A** The Hayola308 genotype under drought stress (HD) and non-stress (HN) conditions. **B** The SLM046 genotypes under drought stress (SD) and non-stress (SN) conditions. **C** The drought stress treatment of SLM046 (SD) and Hayola308 (HD) genotypes. **D** The re-watering treatment of SLM046 (SR) and Hayola308 (HR) genotypes

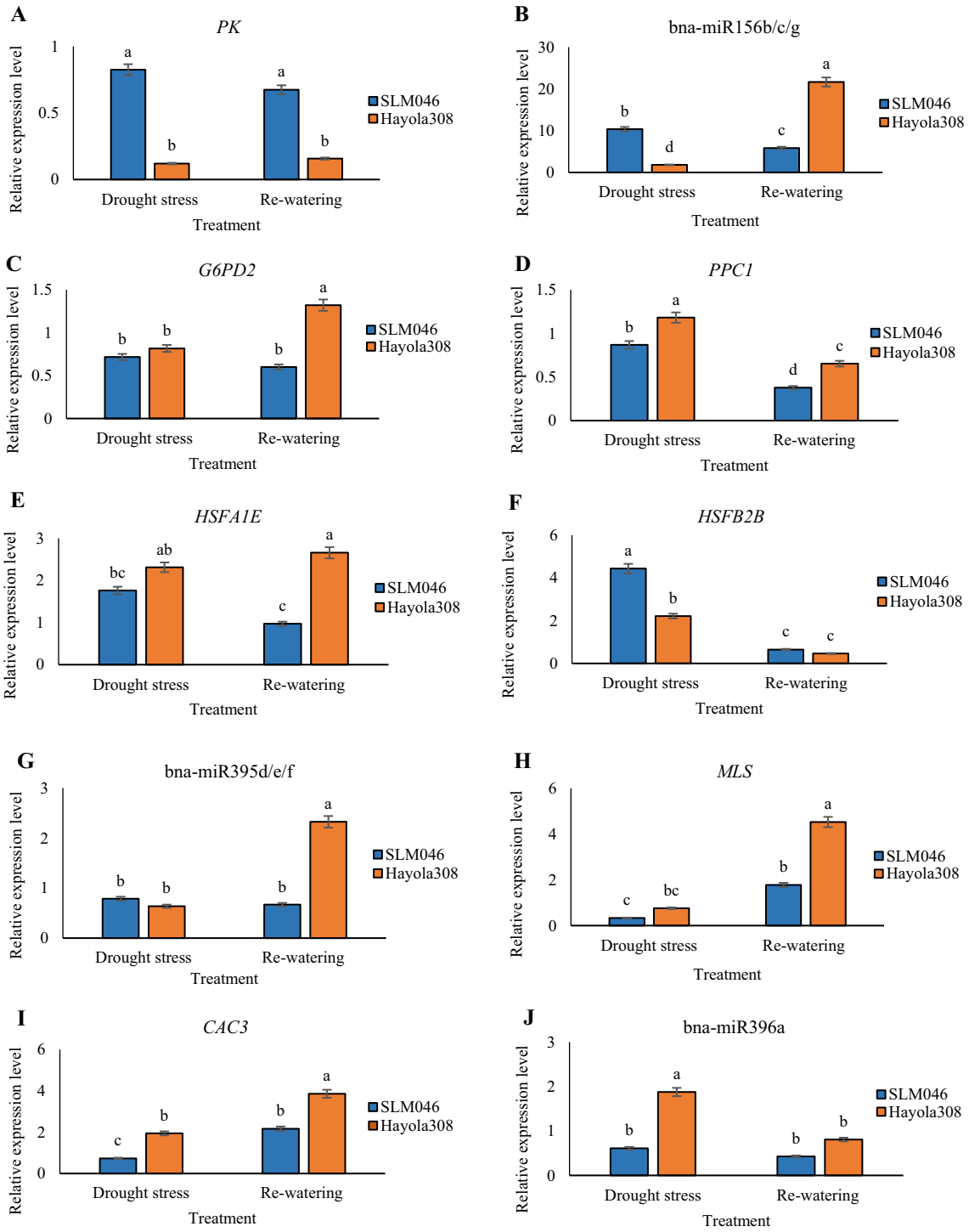

Fig. 5 The expression pattern of *PK* (**A**), bna-miR156b/c/g (**B**), *G6PD2* (**C**), *PPC1* (**D**), *HSFA1E* (**E**), *HSFB2B* (**F**), bna-miR395d/e/f (**G**), *MLS* (**H**), *CAC3* (**I**), and bna-miR396a (**J**) using qRT- PCR in tolerant (SLM046) and susceptible (Hyola308) genotypes under drought stress and re-watering conditions. The 'a', 'b' and 'c' indicate variables that are statistically different from each other. The same letter indicates that the variable is not statistically different from another variable

CAC3 gene expression was higher in Hayola308 genotype than in the SLM046 genotype under drought stress conditions. Re-watering increased the expression of this gene in both genotypes. The results showed that the expression of *CAC3* in both genotypes under drought stress conditions was lower than in re-watering conditions. The expression

level of *CAC3* in the Hayola308 genotype was higher than the SLM046 genotype under drought stress and re-watering conditions (Fig. 5I).

The result showed that the *MLS* and *CAC3* were regulated by bna-miR396a in Canola (Table 3, Fig. 3, respectively). The expression level of bna-miR396a in the Hayola308 genotype was higher than SLM046 under drought stress. Re-watering did not significantly change the expression of bna-miR396a in the SLM046 genotype. But re-watering decreased the expression of bna-miR396a in the Hayola308 genotype (Fig. 5J).

Drought stress-related traits, such as greener leaves and more shoot length and leaf area, were observed in the tolerant genotype, which showed that this genotype was more tolerance to drought stress (Fig. 4). The examination of phenotypic changes showed that the drought stress caused a decrease in leaf area in both genotypes (Fig. 4A, B). However, the leaves of SLM046 were greener and more extensive than Hayola308 under drought stress and re-watering conditions (Fig. 4C, D). It might cause an increase in leaf chlorophyll content and photosynthesis capacity in the tolerant genotype (Pasandideh Arjmand et al. 2018). It seems that the phenotypic changes observed in genotypes under drought stress and re-watering conditions were due to the difference in the expression of drought-responsive genes and their regulation by miRNAs in tolerant and susceptible genotypes. It causes the morphological difference in the genotypes and the difference in tolerance to drought stress.

The result showed that the glycolysis, oxidative pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor key genes had close protein–protein interactions (Fig. 1). In addition, these genes were involved in other important biological pathways, such as carbon metabolism, metabolic pathways, biosynthesis of secondary metabolites, pyruvate metabolism, biosynthesis of amino acids, TCA cycle, etc., which can indicate the importance of these genes in regulating biological processes under drought stress conditions (Fig. 2).

The candidate key genes are regulated by different miRNAs via cleavage and translation inhibition (Table 3, Fig. 3, respectively). It could cause the regulation and coordination of the expression of these key genes in response to drought stress.

Glycolysis and oxidative pentose phosphate pathways

The relative expression level of *PK* in the tolerant genotype was higher than in the susceptible genotype under drought and re-watering treatments (Fig. 5A). PK catalyzes the final stage of glycolysis to produce pyruvic acid and ATP. Pyruvate could enter the TCA cycle or be converted into oxaloacetate by PEPC and enter the gluconeogenesis pathway to produce carbohydrates by consuming energy (Nakagawa et al. 2018). The *PK* was induced significantly in drought stress, and it is reported that the increased *PK* expression may produce more energy through the glycolysis pathway. Therefore, the plant can tolerate drought stress (Zeng et al. 2019). It could be assumed that the higher expression of the *PK* gene in the tolerant genotype causes an increase in the amount of ATP in the cell. The expression of the *PK* gene in different plants has different responses to drought stress. It has been reported that drought stress increased *PK* gene expression in maize genotypes (Zeng et al. 2019). Another study revealed that drought stress reduces the *PK* gene and increases the expression of the *MLS* gene in the soybean (Nakagawa et al. 2018). The *PK* limits the rate of glycolysis, and reducing its expression can reduce energy (Si et al. 2009; Li et al. 2019).

Although the expression of *PK* in the tolerant genotype was higher than in the susceptible genotype, the expression of *PK* did not show a significant change in both genotypes after re-watering. However, *PK* is an important gene in response to drought stress, it probably does not play a role in re-watering conditions and recovery mechanisms.

The result showed that the *PK* gene was regulated by bna-miR156b/c/g in Canola (Table 3, Fig. 3, respectively). It is demonstrated that the target genes of miR156 are important in Maize (Kong et al. 2010), Alfalfa (Feyissa et al. 2019), and Cotton (Wang et al. 2013) under drought and salinity stress conditions. The result illustrated that the expression level of bna-miR156b/c/g in the tolerant genotype was five times higher than in the susceptible genotype (Fig. 5B). It appears that the higher expression of bna-miR156b/c/g in the tolerant genotype could be effective in tolerance to drought stress. The miR156 is induced by various abiotic stresses such as drought, heat, cold, and salinity (Arshad et al. 2017; Yang et al. 2017; Feyissa et al. 2019; Visentin et al. 2020; Ma et al. 2021). The results of studies demonstrated that miR156 is one of the most important stress-responsive miRNAs in Canola and other plants (Jian et al. 2016; Ahmed et al. 2020). The results showed that the expression level of bna-miR156b/c/g in the tolerant genotype decreased with reducing the effects of drought stress under re-watering treatment. The decrease in the expression of bna-miR156b/c/g, which is a drought-responsive miRNA, could regulate *PK* gene expression even after plant recovery. The results illustrated that the expression level of bna-miR156b/c/g increases in the susceptible genotype under re-watering treatment. It could regulate the *PK* expression in recovery conditions after stress and, as a result, less ATP production in the susceptible genotype. The increase in the expression of bna-miR156b/c/g under re-watering treatment might be one of the limiting factors of ATP production from glycolysis. The family of miR156 is involved in regulating various processes, such as biotic and abiotic stress tolerance and

recovery mechanism in plants (Cui et al. 2014; Wang et al. 2016; Visentin et al. 2020).

The *G6PD2* and *PPC1* genes are involved in the oxidative pentose phosphate and glycolysis pathways, respectively (Dai et al. 2013). The result showed that the relative expression of *G6PD2* in tolerant and susceptible genotypes was similar under drought stress. Re-watering of the tolerant genotype did not change the expression of *G6PD2*. The relative expression level of *G6PD2* increased in the susceptible genotype by re-watering treatment (Fig. 5C). It seems that re-watering of the susceptible genotype increases the conversion of glucose 6 phosphate to 6-phosphogluconolactone and it enters the pentose phosphate pathway. In the oxidative pentose phosphate, glucose 6-phosphate is finally converted to ribulose 5-phosphate and CO_2, reducing two molecules of $NADP^+$ to NADPH. Probably, re-watering of the susceptible genotype increases the pentose phosphate pathway activity, and it produces more NADPH in the cell. However, excessive NADPH has the potential to cause oxidative damage to cells during drought stress, it is demonstrated that NADPH plays a key role in mediating responses to stress (Sagi and Fluhr 2006; Miller et al. 2010; Sun et al. 2019; Corpas et al. 2021). Although photosynthesis and growth could be higher in the tolerant genotype, the expression of *G6PD2* was the same in both genotypes under drought stress conditions. Re-watering could not change the expression of this gene in the tolerant genotype. It proposed that the NADPH regeneration through the pentose phosphate pathway is different in tolerant and susceptible genotypes under re-watering conditions.

The result of KEGG enrichment showed that *PPC1* is involved in carbon fixation in photosynthetic organisms (Fig. 1, Table 2, respectively). The expression level of *PPC1* in genotypes was higher in drought stress than in re-watering conditions (Fig. 5D). It could indicate the importance of the *PPC1* gene in genotypes under drought stress. It might compensate for the reduction of photosynthesis and growth in genotypes. The expression of *PPC1* under drought could have an important role in compensating for the decrease in photosynthesis caused by drought stress (Qin et al. 2016). Expression of the *PPC1* gene can increase plant biomass and partially compensate for stress damage (Waseem and Ahmad 2019). The result demonstrated that the general pattern of *PPC1* showed a similar trend in tolerant and susceptible genotypes. The expression of *PPC1* decreased in re-watering conditions in both genotypes, indicating that the conversion of phosphoenolpyruvate to oxaloacetate reduces in recovery conditions. Probably, re-watering treatment reduces the gluconeogenesis pathway to prevent more energy consumption.

The result illustrated that *G6PD2* and *PPC1* could be regulated by bna-miR395d/e/f in Canola. The *PPC1* was regulated by bna-miR395a/b/c (Table 3, Fig. 3, respectively). MiR395 is an important stress-responsive miRNA that is involved in drought stress (Jian et al. 2016), cadmium stress tolerance (Zhang et al. 2013), sulfate assimilation regulatory (Matthewman et al. 2012), and regulating of transcription factors (Zhang et al. 2017). The result illustrated that the expression of bna-miR395d/e/f in the tolerant genotype did not change under drought stress and re-watering conditions. Re-watering of the susceptible genotype after drought increased the expression of bna-miR395d/e/f (Fig. 5G). It is reported that the expression of bna-miR395d/e/f in Canola was down-regulated under drought stress (Jian et al. 2016). The result shows that bna-miR395d/e/f could respond to re-watering treatment in the susceptible genotype (Fig. 5G). The significant increase in the expression of bna-miR395d/e/f in the susceptible genotype can be due to the regulation of target genes under re-watering conditions. The result showed that *PPC1* also was regulated by bna-miR164a/b/c/d and bna-miR6032 (Table 3, Fig. 3, respectively). It is reported that bna-miR164 is related to drought-responsive genes in Canola (Sarwar et al. 2021). A study reported that bna-miR164 is involved in oxidative stress in Canola (Jian et al. 2018). The bna-miR6032 is involved in fatty acid and lipid metabolism in Canola (Wang et al. 2017).

The investigation of the key genes involved in the glycolysis pathway could show that the more production of ATP and pyruvate and then production of oxaloacetate and entry into the gluconeogenesis pathway accurse in the tolerant genotype than in susceptible genotype under drought stress. This could be related to higher photosynthesis and growth in this genotype. It seems that the re-watering of the tolerant genotype causes the produced pyruvate to enter the TCA and other pathways to reduce the consumption of more ATP in the gluconeogenesis pathway.

Heat shock factor genes

As shown in Fig. 5E, the relative expression level of the *HSFA1E* gene was similar in both genotypes under drought stress. The expression level of this gene in the susceptible genotype was higher than the tolerant genotype in the re-watering treatment. It is reported that *HSFA1* directly interacts with a brassinosteroid signaling transcription factor, and it could activate several heat shock protein genes under stress conditions. This interaction increases the tolerance to stress, but it suppresses the growth of plants (Haider et al. 2022). Therefore, increasing the expression of this gene can cause a decrease in plant growth. The examination of phenotypic changes in genotypes showed that the shoot length and the leaf area in the susceptible genotype were lower than the tolerant genotype under drought stress and re-watering conditions (Fig. 4C, D). The not changing of the *HSFA1E* expression could prevent more growth reduction of genotypes under drought stress and re-watering conditions.

The relative expression level of the *HSFB2B* gene in the tolerant genotype was higher than in the susceptible

genotype under drought stress (Fig. 5F). It is documented that *HSFB2B* represses the HSFs activation in normal conditions. It negatively regulates heat shock-responsive gene expression but contributes positively to acquired thermotolerance under stress conditions. The thermo-tolerance of plants could be effective on the growth and yield under drought stress (Ikeda et al. 2011; Fu et al. 2022). Therefore, the higher expression of *HSFB2B* in the tolerant genotype could be one of the influential factors in the proper growth of this genotype under drought stress.

Re-watering treatment did not cause a significant change in the expression of the *HSFA1E* gene in tolerant and susceptible genotypes. It suggested that *HSFA1E* probably does not play a role in re-watering conditions and recovery mechanisms. But re-watering caused the relative expression of *HSFB2B* to be reduced in both genotypes. It seems that re-watering may reduce the repression of downstream genes by *HSFB2B*. The result illustrated that *HSFA1E* and *HSFB2B* could be regulated by bna-miR395a/b/c/d/e/f in Canola (Table 3, Fig. 3, respectively).

A study demonstrated that the bna-miR395d/e/f were drought-responsive in Canola (Jian et al. 2016). PPI network analysis revealed that HSEs genes are related to glycolysis, pentose phosphate, glyoxylate cycle, and fatty acid biosynthesis (Fig. 1, Table 2, respectively). The bna-miRNA395d/e/f might relate the central carbon metabolism to key heat shock factor genes by the coordinated regulation of *PPC1*, *G6PD2*, and HSEs genes. It could be effective in the expression of genes that are involved in these pathways. Therefore, changes in the expression of glycolysis and pentose phosphate key genes, which could be an important factor for phenotypic changes in genotypes, could be related to HSF genes and their regulation by stress-responsive miRNAs under drought stress and re-watering conditions.

The result demonstrated that *HSFB2B* was regulated by bna-miR6028 and bna-miR403 (Table 3, Fig. 3, respectively). It is reported that both miRNAs were stress-responsive. The expression level of bna-miR6028 decreased under cadmium stress conditions and pathogen infection in Canola (Zhou et al. 2017; Jian et al. 2018). The relative expression level of miR403 was changed under pathogen infection in Canola (Cao et al. 2016). The result demonstrated that different types of HSF genes show different expression patterns under drought stress and re-watering conditions, which might indicate the importance of regulating the expression of other HSFs by these genes.

Glyoxylate cycle and Fatty acid biosynthesis

As shown in Fig. 5H, the general pattern of *MLS* showed a similar trend in genotypes under drought and re-watering conditions. The relative expression of the *MLS* gene in genotypes was increased under re-watering treatment. It might indicate an increase in the conversion of fatty acid to carbohydrates under re-watering conditions. The result suggested that the activity of the glyoxylate cycle decreases under drought stress and increases in re-watering conditions. The glyoxylate cycle produces succinate, which is needed to regenerate malate to continue the cycle to produce sugars in glycolysis. As a result, cells do not lose carbon dioxide molecules and can survive with less carbon (Su et al. 2019).

The result showed that the relative expression of the *CAC3* gene of the susceptible genotype was more than the tolerant genotype under drought stress conditions. However, the relative expression of *CAC3* in both genotypes increased in re-watering conditions (Fig. 5I). The *CAC3* is involved in fatty acid biosynthesis and stress tolerance (Deeba et al. 2012). Lipid biosynthesis in genotypes might increase with re-watering treatment and decreasing stress pressure to increase membrane tolerance.

The result demonstrated that the *MLS* and *CAC3* genes were regulated by bna-miR396a (Table 3, Fig. 3, respectively). The miR396 is another important stress-responsive miRNA that is involved in drought stress in *Brassica napus* (Shamloo Dashtpagerdi et al. 2015; Jian et al. 2016), *Arabidopsis thaliana* (Liu et al. 2008), *Oryza sativa* (Zhou et al. 2010), *Sorghum bicolor* (Hamza et al. 2016), *Nicotiana tabacum* (Frazier et al. 2011), and *Triticum dicoccoides* (Kantar et al. 2011). The result showed that the expression level of bna-miR396a in the susceptible genotype was higher than the tolerant genotype under drought stress. Re-watering did not significantly change the expression of bna-miR396a in the tolerant genotype. However, re-watering decreased the expression of this miRNA in the susceptible genotype (Fig. 5J). It is reported that the expression of miR396 was significantly induced under drought stress conditions in tobacco (Yang and Yu 2009). Another study reported that the expression of miR396 was downregulated in Rice under drought stress conditions (Zhou et al. 2010). The general trend of *MLS* and *CAC3* expression was similar in tolerant and susceptible genotypes after re-watering. The expression of these genes increased after re-watering compared to drought conditions (Fig. 5H, I). Therefore, the higher expression of bna-miR396a in the susceptible genotype under drought stress, in addition to regulation of *MLS* and *CAC3*, could be due to the regulation of other target genes in response to drought stress.

The connection between glyoxylate cycle activity and fatty acid biosynthesis might be made through the regulation of *CAC3* and *MLS* genes by bna-miR396a. Also, the *CAC3* was regulated by bna-miR159, bna-miR2111a-5p, bna-miR2111b-5p, and bna-miR2111d (Table 3, Fig. 3, respectively). It is reported that these miRNAs are involved in fatty acid biosynthesis in Canola. Therefore, the regulation of *CAC3* by miRNAs could regulate fatty acid biosynthesis. The expression of *KASII* and *KASIII*

is regulated by the bna-miR159 (Wang et al. 2016). The bna-miR2111 has a vital role in fatty acid biosynthesis. In addition, zinc finger protein (ZFP) is co-regulated by the bna-miR2111 family in Canola and is related to drought stress response (Wang et al. 2016).

The investigated genes could play a key role in drought stress through the regulation of important pathways, which causes phenotypic changes in genotypes. It occurs through changes in the expression of genes involved in important drought-responsive pathways and their regulation by miRNAs in genotypes. The bna-miR156b/c/g, bna-miR395d/e/f, and bna-miR396a could cause the coordinated regulation of glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor genes in response to drought stress and re-watering conditions. The expression patterns of drought-responsive miRNAs might be different in tolerant and susceptible genotypes. As shown in Fig. 6, the expression of these miRNAs was different in susceptible and tolerant genotypes in re-watering conditions compared to drought stress, and it probably caused the expression of target genes to be regulated in a different way. All the studied genes except *HSFA1E* and *PK* showed changes in expression levels after re-watering. The *PPC1* and *HSFB2B* expression decreased, whereas the *MLS* and *CAC3* expression increased in both genotypes under re-watering treatment after drought stress. It seems that *PPC1*, *G6PD2*, *MLS*, *CAC3*, and *HSFB2B* were involved in the recovery mechanisms in Canola. They were regulated by drought-responsive miRNAs to respond appropriately and coordinately to drought stress.

Conclusions

The results showed that glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor genes had close protein–protein interactions. The key genes of these pathways in Canola were regulated by drought-responsive miRNAs, such as bna-miR156b/c/g, bna-miR395d/e/f, and bna-miR396a to the drought stress response. The expression of these miRNAs were different in susceptible and tolerant genotypes in re-watering conditions compared to drought stress, and it probably caused the expression of target genes to be regulated differently. It causes phenotypic changes in the genotypes under drought stress and re-watering conditions. The higher expression of the *PK* in the tolerant genotype could cause an increase in the amount of ATP in the glycolysis pathway under drought stress and re-watering conditions, and it could be regulated by drought-responsive bna-miR156b/c/g in Canola. Re-watering treatment of the susceptible genotype increased the NADPH production in the cell through the oxidative pentose phosphate pathway. However, excessive NADPH has the potential to cause oxidative damage to cells during drought stress, the regeneration of NADPH plays a key role in mediating responses to stress in susceptible genotype. The result illustrated that the conversion of phosphoenolpyruvate to oxaloacetate reduced in recovery conditions. Therefore, re-watering might reduce the gluconeogenesis pathway to prevent more energy consumption. In addition, it could balance by increasing the expression of the glyoxylate cycle and fatty acid biosynthesis key drought-responsive genes. The result showed that one of the regulation factors of *PPC1* and *G6PD2* genes in Canola was drought-responsive

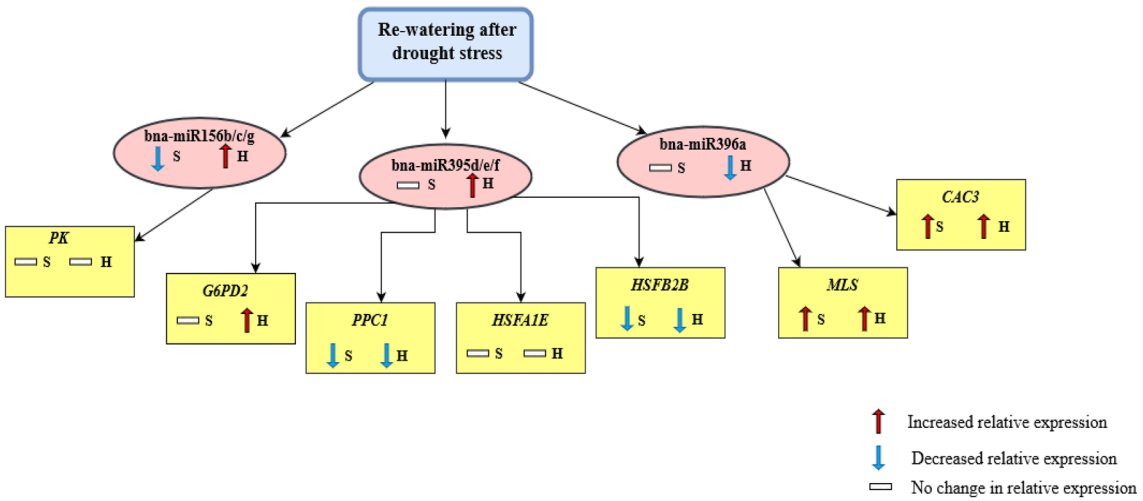

Fig. 6 The flow chart of relative expression changes of genes and their regulatory miRNAs in tolerant (S) and susceptible (H) genotypes of Canola under re-watering post to drought stress

bna-miR395d/e/f. The bna-miR395d/e/f might relate these genes to the key heat shock factor genes, including *HSFA1E* and *HSFB2B*. It is possible that not changing the expression of *HSFA1E* in the genotypes could prevent growth reduction under drought stress and re-watering conditions. Re-watering could reduce the repression of downstream genes by *HSFB2B*. Therefore, it could be effective in the expression of genes that are involved in these pathways. The result demonstrated that the glyoxylate cycle activity and fatty acid biosynthesis could be increased under the re-watering condition, and the key genes of these pathways could be regulated by drought-responsive bna-miR396a in Canola. The results illustrated that some glycolysis, pentose phosphate, glyoxylate cycle, fatty acid biosynthesis, and heat shock factor key drought-responsive genes, including *PPC1*, *G6PD2*, *MLS*, *CAC3*, and *HSFB2B*, could be involved in recovery mechanism after drought stress. They were regulated by drought-responsive miRNAs to respond appropriately and coordinately to drought stress. Therefore, regulating these genes in tolerant and susceptible genotypes could be important in plant recovery mechanisms.

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Author contributions MPA: Investigation, Formal analysis, Resources, Writing-Review & Editing. HSL: Investigation, Methodology, Conceptualization, Resources, Writing-Review & Editing. MMG: Investigation, Formal analysis, Writing-Review & Editing. MHB: Investigation, Writing- Review & Editing.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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