ORIGINAL ARTICLE

Investigation of proteins' interaction network and the expression pattern of genes involved in the ABA biogenesis and antioxidant system under methanol spray in drought-stressed rapeseed

Mohammad Mohsenzadeh Golfazani[1] · Mohammad Mahdi Taghvaei[1] · Habibollah Samizadeh Lahiji[1] · Seddigheh Ashery[1] · Ali Raza[2]

Abstract

Drought is one of the most critical abiotic stresses, which significantly impair rapeseed (*Brassica napus* L.) productivity. Several factors can regulate the stress response, including changes in gene expression in biological pathways, extensive protein interaction networks, and post-translational regulatory factors like microRNAs. External factors can also affect the intensity of the stress response. Therefore, this study investigated protein–protein interactions of some essential genes involved in abscisic acid (ABA) production, antioxidant system, and Krebs cycle. The expression of phyton synthase (*PSY*), 9-cis-epoxycarotenoid dioxygenase (*NCED3*), aldehyde oxidase (*AAO3*), thioredoxin reductase (*NTRC*), and glutathione reductase (*GR*) genes in two rapeseed genotypes, i.e., Hyola308 (drought-sensitive) and SLM046 (drought-tolerant) were evaluated using qRT-PCR technique under 72 h of drought stress and methanol foliar application. In the SLM046 (tolerant) genotype, the expression levels of *PYS, NCED, AAO3,* and *GR* genes were increased after 8 h of foliar application. The expression level of the *NTR* gene was increased 8 and 24 h after stress and methanol treatment. In the Hyola308 genotype, *PYS, AAO3, NTR,* and *GR* genes' expression level was increased 8 h after methanol foliar application, and the *NCED* gene was increased 24 h after stress with methanol treatment. In general, methanol foliar application increased the expression levels of several genes. Particularly, the gene expression was considerably higher in the SLM046 genotype than in Hyola308. Bioinformatics prediction of microRNAs targeting *PSY, NCED, GR, NTRC,* and *AAO3* genes was performed, and 38, 38, 13, 11, and 11 microRNAs were predicted for these genes, respectively. The study of effective microRNAs showed that sometimes more than one type of microRNA could affect the desired gene, and in some cases, a conserved family of microRNAs caused the main effect on gene expression. Overall, our results lay the foundation for functional characterization of these genes or gene-miRNA modules in regulating drought stress tolerance in rapeseed.

Keywords Carotenoids · Gene expression · microRNA · Protein interaction · STRING

✉ Mohammad Mohsenzadeh Golfazani
mohsenzadeh.mohamad@guilan.ac.ir

Mohammad Mahdi Taghvaei
Mahdi.Taghvaei@gmail.com

Habibollah Samizadeh Lahiji
hsamizadeh@guilan.ac.ir

Seddigheh Ashery
ashery138@gmail.com

Ali Raza
alirazamughal143@gmail.com

1 Department of Agricultural Biotechnology, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

2 Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Center of Legume Crop Genetics and Systems Biology/College of Agriculture, Oil Crops Research Institute, Fujian Agriculture and Forestry University (FAFU), Fuzhou, China

Introduction

Rapeseed (*Brassica napus.* L) is one of the most valuable oilseeds crops belonging to Brassicaceae family. Studies have shown that water scarcity in the various stages of growth affects the quantity and the quality of oil production (Raza et al. 2020). Many genes are induced under drought stress in plants, and different organs are affected. Drought stress in plants leads to inhibition of photosynthesis and respiration in organs such as chloroplasts and mitochondria, accumulation of reactive oxygen species (ROS), and reprogramming of gene expression (Liu et al. 2021; Meng et al. 2020; Raza et al. 2020; Rezaie et al. 2020). Productions from these genes appear to be involved in stress tolerance, regulation of gene expression, and signal response to stress response (Ito et al. 2006).

Under dehydration conditions, abscisic acid (ABA) plays an essential role in expressing stress-responsive genes and stomatal closure. Plants use the carotenoid pathway as an indirect pathway for ABA synthesis. ABA biosynthesis begins with zeaxanthin, and zeaxanthin epoxidase (ZEP) converts zeaxanthin into violaxanthin (Ali et al. 2020). The phyton synthase gene (*PSY3*) also performs a specialized role in controlling ABA biosynthesis in response to abiotic stress (Li et al. 2007). The enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) is critical in ABA biosynthesis. In Arabidopsis, the enzyme aldehyde oxidase (AAO3) accelerates the finishing stage of ABA biosynthesis (Seo et al. 2004). *AAO3* was found to perform an essential role in ABA biosynthesis (Mittler et al. 2004). Thioredoxin (TRX) plays a key role in plant tolerance to oxidative stress (Dos Santos and Rey 2006). *TRX* uses Ferredoxin-dependent heterodimeric thioredoxin reductase (FTR) or NADPH-dependent thyrotoxin reductases (NTR) to reduce itself (Reichheld et al. 2005). NTRs are vital regulatory enzymes that modulate the redox state of the Trx system in plants. NTRs directly reduce ROS and lead to stress tolerance in plants. Arabidopsis has three NTR isoforms, *NTRA* and *NTRB*, found in the cytoplasm and mitochondria, and *NTRC* in chloroplasts. The important role of *NTRC* has been proven in plant growth and development and response to environmental conditions (Danaeipour and Haddad 2020; Pérez-Ruiz et al. 2006). The essential role of *NTRC* in response to abiotic stresses was also reported (Da et al. 2017). The study on Arabidopsis showed that the higher expression of the *NTR* gene increased tolerance to drought and photo-oxidative stresses. Increased *NTR* expression helped maintain the homeostasis of ROS under stress conditions (Kim et al. 2017).

Antioxidant defense mechanisms have been developed in plants to reduce the damaging effects of ROS on plant cells (Kalisz et al. 2016; Rezaie et al. 2020). Vital members of this defense system include superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR), catalase (CAT), etc. The study on cassava showed that the antioxidant response under drought stress might be associated with increased accumulation of ascorbate (AsA) and glutathione (GSH) and higher activities of SOD and CAT. However, in some of its genotypes, it mainly depended on total phenolic accumulation (TP) and increased GR activity (Chen et al. 2020). SOD catalyzes O_2 and H_2O_2 from superoxide (O_2^-) and consequently reduces its adverse effects. CAT is an antioxidant enzyme found in all aerobic organisms that catalyze H_2O_2 to water and oxygen in cells (Raza et al. 2021). Another enzyme, GR, plays a significant role in adapting to plants' oxidative stress (Salin 1991). This enzyme is responsible for converting oxidized glutathione to reduced glutathione and maintaining a high ratio of GSH to GSSG (Noctor and Foyer 1998). The alternative oxidase pathway is critical to reducing ROS production in chloroplast and mitochondrial organs (Mittler 2002). This process is carried out by the alternative oxidase enzyme (AOX). The lack of the *AOX1* gene in the plant reduces the ability to grow or tolerate stresses (Mohsenzadeh Golafazani et al. 2017). Alternative oxidase also plays a role in reducing cell damage under various environmental stresses. Therefore, many recent studies have focused on its role in response to salinity and drought stress (Li et al. 2013). One of the pathways affected by drought stress is the Krebs cycle (TCA), located in the mitochondrial matrix (Ramezanzadeh Bishegahi et al. 2021). TCA cycle enzymes are susceptible to oxidative stress (Fernie et al. 2004). Recent study showed that drought stress could affect the expression of pyruvate dehydrogenase 1 (*PDH1*), isocitrate dehydrogenase 1 (*IDH1*), and fumarase 1 (*FUM1*) genes involved in the Krebs cycle (Ramezanzadeh Bishegahi et al. 2021).

Foliar applications of aqueous methanol have been reported to increase yield, accelerate maturity, and reduce drought stress and irrigation requirements in crops grown in dry environments (Nonomura and Benson 1992; Ramezanzadeh Bishegahi et al. 2021). The increased growth and yield were attributed to the action of methanol as a C nutrient and as an inhibitor of photorespiration (Nonomura and Benson 1992). In another study, various crops such as tomato, bean, sugar beet, and oil seed rape treated with 20–30% methanol solutions showed an increase in yield (Zbieć et al. 2003). Methanol treatment on Arabidopsis and tobacco plants increased their fresh and dry weight (Ramírez et al. 2006). Plants can easily absorb methanol sprayed on leaves and utilize it as a carbon source (Hemming et al. 1995; Khalilzadeh et al. 2020). Rapid oxidation to CO_2 combined with methanol foliar application delays leaf aging by acting on stimulants

of ethylene production in the plant. Also, it increases leaf surface durability and increases the active photosynthetic period (Zbieć et al. 2003). Recent study on drought stress on two rapeseed genotypes, Hyola308 and SLM046, revealed that methanol treatment significantly affected the expression profiles of *IDH1, PDH, FUM, TRX,* and *AOX* genes and was able to modulate the effect of drought stress (Ramezanzadeh Bishegahi et al. 2021). Microarray-based gene expression study of 484 transcripts in leaves of Arabidopsis thaliana harvested at 1, 24, and 72 h after methanol treatment revealed that only two transcripts were significantly down-regulated, and the remainder were up-regulated. These included cytochrome P450's, glucosyl transferases, and the ABC transporter family members. Also, the analysis revealed an increase in the number of ABA- and auxin-related transcripts, aldehyde oxidase, and glutathione S-transferase. Therefore, methanol treatment led to the expression of hundreds of genes, and multiple detoxifications and signaling pathways were activated (Downie et al. 2004).

One of the most useful, standard, and widespread types of biomolecular networks is the protein–protein network. This network contains all the genes encoding proteins in a particular genome and highlights their functional relationship (Szklarczyk et al. 2021). Since proteins act primarily as interacting networks, protein–protein interaction networks can be considered as a valuable indicator for understanding cellular processes (Taghvaei et al. 2019). Examining these relationships and pathways of protein interactions provides researchers with very beneficial information. MicroRNAs are post-transcriptional gene regulatory elements influencing the expression of proteins. If these 21 to 22 nucleotide sequences bind to the gene sequence, they can regulate the activity of that gene. In other words, if the binding to the target region is complete, it leads to cleavage. On the other hand, if the binding is incomplete, it prevents the translation of the target gene (Bartel 2004).

Bioinformatics study of the genes network and microRNAs affecting the expression of specific genes cause removing extra data. Therefore, the desired data can be obtained more quickly and reduce the cost and time required in experimental tests. Accurately identifying the genes associated with drought stress provides the basis for improving drought tolerance in rapeseed. Expression data of mRNA transcripts under drought stress and non-stress conditions in sensitive and tolerant genotypes using qRT-PCR technique; data analysis of protein–protein interaction networks and microRNAs can provide a proper basis for improving drought stress tolerance in rapeseed as well as other similar plants. Therefore, in this study, both tolerant and sensitive rapeseed genotypes were studied to investigate their response to drought stress and treatment with methanol.

Materials and methods

Plant materials and treatment conditions

Drought-sensitive (Hayola308) and drought-tolerant (SLM046) genotypes' seeds were obtained from the Seed and Plant Improvement Institute (SPII), Iran (Mirzai et al. 2013). The seeds were placed 4 days in a petri dish using water and filter paper for germination in the biotechnology laboratory of Guilan University, Iran. Then at least three seedlings were planted in the soil in each 1 kg plastic pot (90 pots in total). The pots were placed in a growth chamber under a 16-h light (25 °C)/8-h dark (22 °C) photoperiod (photo intensity 6000 Lx) and were watered daily until sampling. Statistically, it was performed as a completely randomized factorial design in three replications with three factors, including methanol, genotype, and time. When the tolerant and sensitive plants reached the 3 to 4-leaf stage, they were divided into three groups, including: Control plants (CI) that were regularly irrigated, drought-stressed plants (DR) that were exposed to drought stress for 72 h (30% of field capacity) and methanol-sprayed (DM) plants under 72-h drought stress. The single-step spraying of methanol solution 20% v/v (Methanol, 99.9%, Thermo Scientific™) was performed after 72-h drought stress on the DM plants with a hand-held sprayer at a rate of 20 ml per pot, with the nozzle approximately 20 cm above the leaf surface (Armand et al. 2016; Ramírez et al. 2006). The CI, DR, and DM plants' leaves were harvested at 0 (72 h after water cut-off), 8, 24 h, and immediately frozen in liquid nitrogen, and stored in a freezer at −80° C for further analysis, RNA extraction and cDNA synthesize.

RNA extraction and cDNA synthesize

Total RNA was extracted using a total RNA isolation kit (DENAzist, IRAN) according to the manufacturer's instructions. The extracted RNA's quantity and quality were examined by Nanodrop® One (Thermo Fisher Scientific®) and 1% agarose gel electrophoresis. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used to synthesize cDNA following the manufacturer's instructions. cDNA was stored at – 80 C freezer.

Analysis of protein–protein interactions

The genes involved in the production of ABA, antioxidant system, and Krebs cycle, were selected to investigate their relationship and analyze their protein–protein interactions. Information and genetic identity of selected genes were taken using the UniProt database (Table 1). Afterward, it

Table 1 Selected important genes involved in the production of ABA, antioxidant system, Krebs cycle using *Arabidopsis thaliana* as a reference plant

Gene names	Identifier		UniProt
Glutathione reductase	AT3G54660	GR	https://www.uniprot.org/uniprot/P42770
NADPH-dependent thioredoxin reductase 3	AT2G41680	NTRC	https://www.uniprot.org/uniprot/O22229
Aldehyde oxidase 3	AT2G27150	AAO3	https://www.uniprot.org/uniprot/Q7G9P4
9-cis-epoxycarotenoid dioxygenase	AT3G14440	NCED3	https://www.uniprot.org/uniprot/Q9LRR7
Phytoene synthase	AT5G17230	PSY	https://www.uniprot.org/uniprot/P37271
Isocitrate dehydrogenase	AT4G35260	IDH1	https://www.uniprot.org/uniprot/Q8LFC0
Dihydrolipoyllysine-residue acetyltransferase component 1 of pyruvate dehydrogenase complex	AT3G52200	LTA3	https://www.uniprot.org/uniprot/Q0WQF7
Fumarate hydratase	AT2G47510	FUM1	https://www.uniprot.org/uniprot/P93033
Thioredoxin	AT5G42980	TRX3	https://www.uniprot.org/uniprot/Q42403
Alternative oxidase	AT3G22370	AOX1A	https://www.uniprot.org/uniprot/Q39219
Zeaxanthin epoxidase, chloroplastic	AT5G67030	ZEP	https://www.uniprot.org/uniprot/Q9FGC7
Catalase-2	AT4G35090	CAT	https://www.uniprot.org/uniprot/P25819
Superoxide dismutase [Cu–Zn] 1	AT1G08830	CSD1	https://www.uniprot.org/uniprot/P24704
Fumarate hydratase 2	AT5G50950	FUM2	https://www.uniprot.org/uniprot/Q9FI53
Succinate dehydrogenase [ubiquinone] flavoprotein subunit 2, mitochondrial	AT2G18450	SDH1-2	https://www.uniprot.org/uniprot/Q9ZPX5
Malate dehydrogenase 1, mitochondrial	AT1G53240	mMDH1	https://www.uniprot.org/uniprot/Q9ZP06
NADPH-dependent thioredoxin reductase 2	AT2G17420	NTRA	https://www.uniprot.org/uniprot/Q39242
NADPH-dependent thioredoxin reductase 1	AT4G35460	NTRB	https://www.uniprot.org/uniprot/Q39243
External alternative NAD(P)H-ubiquinone oxidoreductase B2, mitochondrial	AT4G05020	NDB2	https://www.uniprot.org/uniprot/Q94BV7
External alternative NAD(P)H-ubiquinone oxidoreductase B4, mitochondrial	AT2G20800	NDB4	https://www.uniprot.org/uniprot/Q9SKT7
Internal alternative NAD(P)H-ubiquinone oxidoreductase A2, mitochondrial	AT2G29990	NDA2	https://www.uniprot.org/uniprot/O80874

was utilized in the STRING database to identify protein–protein interactions (PPIs). This database contains information from various sources, including experimental data, computational forecasting methods, and it is updated continuously. In this database, each interaction is given a score from 0 to 1, and the minimum required interaction score was set to medium (0.4) (Szklarczyk et al. 2019; Taghvaei et al. 2022).

Design of specific primers

After examining the proteins' interaction and their functional relationships with each other, the candidate genes such as *PSY, NCED3, GR, NTRC,* and *AAO3* genes were selected to evaluate the expression using qRT-PCR. The actin gene was used as a reference gene to normalize the data (Wang et al. 2011). Sequences of the desired genes *PSY, NCED3, GR, NTRC,* and *AAO3* genes were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). The primers were designed using Tcoffee (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee) and primer3 (http://primer3.ut.ee) (Table 2). Finally, the primers were checked for specificity in NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

Investigation of gene expression by real-time PCR

Expression of selected genes was performed by qRT-PCR in cultivars SLM046 (tolerant) and Hyola308 (sensitive). The reactions were carried out in a LightCycler 96 real-time PCR machine (Roche, Basel, Switzerland) with the following amplification conditions: activation at 50 °C for 2 min; 95 °C for 2 min; followed by 40 cycles at 95 °C for 15 s and 58 °C for the 20 s; and 72 °C for 15 s and final holding at 4 °C. All reactions were performed in three biological replicates, each with three technical replicates. The Livak equation was used to compare the relative expression of the desired genes in real-time PCR (Livak and Schmittgen 2001). Graphs were constructed using Excel 2016. The standard error (SE) was used to compare the columns, and the relative expression rate was considered double (Mohsenzadeh Golfazani et al. 2019).

Table 2 Specifications of primers used in real-time PCR reaction

Gene	Primer sequence	Melt (°C)	PCR product	NCBI accession number
Actin-F	5-TCCCGAGTATTGTTGGTCGT-3′	54	157	AF111812
Actin-R	5-TCCATGTCATCCCAGTTGCT-3′			
AAO3-F	5-GAAGCAACCCACACGAGTTT-3′	54	175	XM_013862421.2
AAO3-R	5-AACCTCAGCCTCAAGACTCC-3′			
PSY-F	5-TCGCTGATACCGTTGCTAGA-3′	54	197	XM_013827623.2
PSY-R	5-TGTCGTTGCTTTGGACTTGG-3′			
NCED-F	5-AAGCAGTCCTCAACCTCTCC-3′	54	178	XM_013788802
NCED-R	5-TCTGAACACTAGGATCGGCG-3′			
GR -F	5′-GAGCAGATGTCTTTAAGAGGC-3′	53.3	159	XM_013855797.1
GR -R	5′-CTTTGTATTAGGCTTGCGAC-3′			
NTRC -F	5-GCTGTAACTGCTGCTGGATC-3′	53.8	178	XM_013892363.1
NTRC-R	5-ACTGTCCCCGATGCTTTGTA-3′			

Identification of microRNAs targeting the studied genes

All microRNA sequences belong to the Brassicaceae family plants, including *Arabidopsis thaliana, Arabidopsis lyrata, Brassica oleracea, Brassica rapa, Brassica napus,* and *Camelina sativa* were obtained from the miRbase database (https://www.mirbase.org) (Release 22.1, October 2018). To identify microRNAs targeting *PSY, NCED, GR, NTRC,* and *AAO3* genes, MicroRNA prediction was performed with psRNATarget database (expected value parameter 5) using CDS (https://www.zhaolab.org/psRNATarget/) (Dai et al. 2018). Cytoscape software version 3.8 was additionally used to map the relationship between the identified microRNAs and the studied genes (Shannon et al. 2003).

Results and discussion

Phenotypic changes of rapeseed after drought stress and methanol foliar spray

The Hyola308 genotype showed signs of gradual wilting after drought stress compared to the control. In contrast, the SLM046 genotype wilting symptoms were seen in the early hours under drought stress, and then in the following hours of 72-h drought stress, the wilting symptoms gradually disappeared, the wilting symptoms gradually disappeared. Examination of both genotypes after methanol foliar application under drought stress showed that the plants' freshness recovered again. It seems that methanol treatment increased plant tolerance to drought stress (Fig. 1A, B).

Protein–protein interaction network

In this study, the STRING platform was utilized to investigate protein interactions in which nodes represent proteins and lines represent known or predicted and direct or indirect
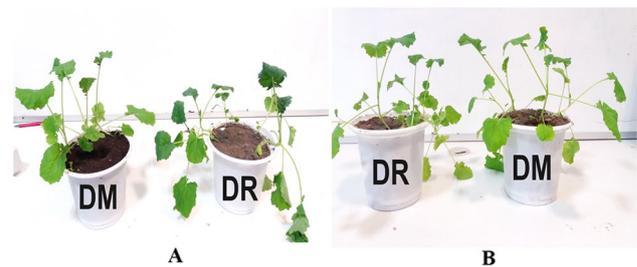
Fig. 1 The treated plants were separated into two groups: drought-stressed plants (DR) that were exposed to drought stress for 72 h (30% of field capacity) and methanol-sprayed (DM) plants which sprayed with methanol solution 20% v/v after 72-h drought stress. **A** Hyola308 (drought-sensitive) genotype: DM and DR plants. **B** SLM046 (drought-tolerant) genotype: DM and DR plants

interactions. The relationship between the nodes was based precisely on the present information extracted from relevant databases and articles. Accordingly, the results of the interactions between the genes (Table 1) indicated 21 nodes and 72 edges (Fig. 2), and all of them were located in the network.

Based on STRING clustering (Fig. 2 and Table 3), the studied genes were classified into clusters including, superoxide metabolic process (CL: 10,321), Citrate cycle (TCA cycle) (CL: 9184), thioredoxin-disulfide reductase activity (CL: 10,322), thioredoxin reductase (CL: 10,344), alternative oxidase (CL: 38,330), carotenoid biosynthesis (CL: 14,843), respectively. These results indicated that there were direct and indirect associations and interactions between Krebs cycle enzymes (*FUM, SDHI,* and *IDH1*), effective enzymes in oxidation–reduction reactions and scavenging ROS (*CAT, CSD, NTR,* and *GR*), alternative oxidase pathway (AOX1A), and ABA production processes (*NCED, ABA1, AAO3,* and *PSY3*) (Fig. 2 and Table 3).

ABA is involved in various abiotic stresses like drought, cold, salinity, and growth and development processes such as seed development, dormancy, germination,

Fig. 2 Protein–protein interaction network (PPIs) using the STRING database. Colored nodes represent proteins, and each color represents a cluster. Colored lines represent their interactions with each other based on the references in the database
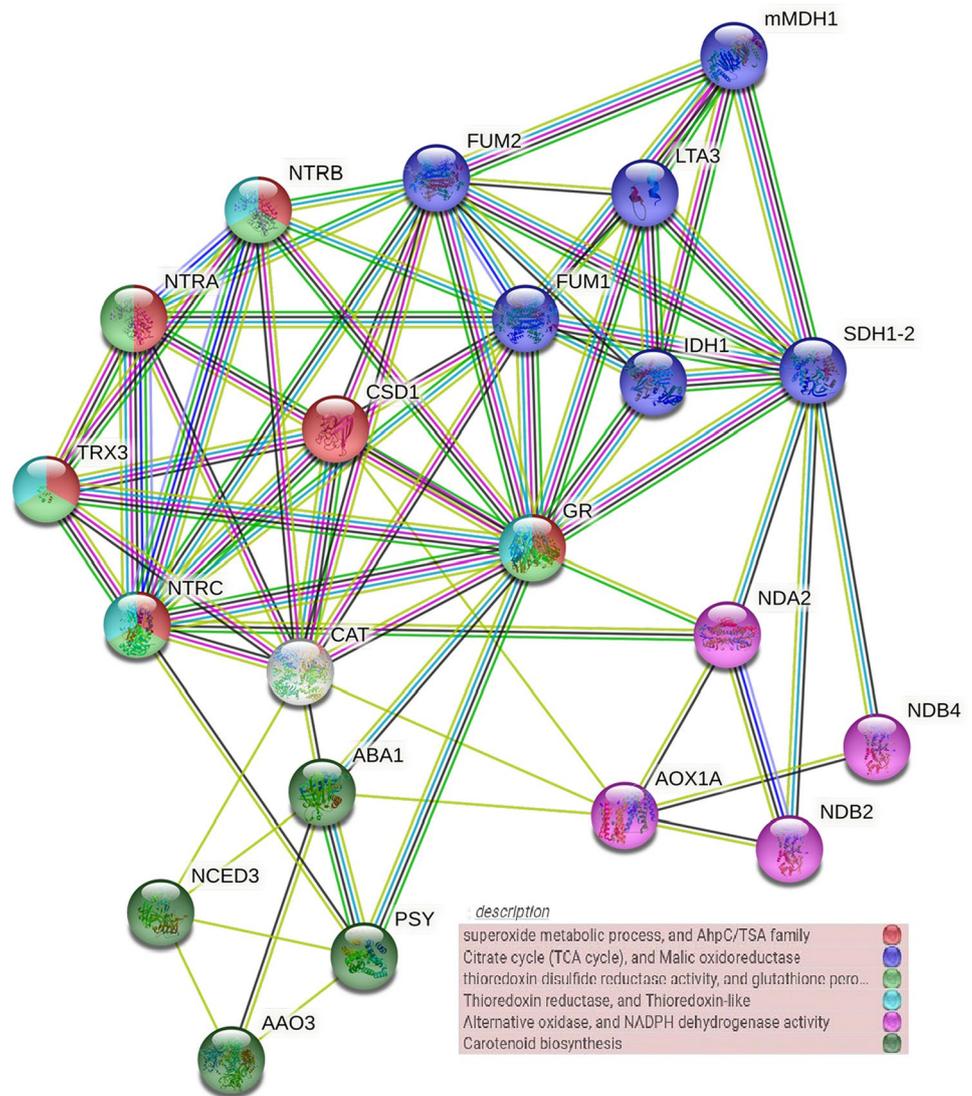
Table 3 Local network cluster based on STRING database

#term ID	Term description	Observed gene count	Background gene count	Strength	False discovery rate
CL:10,321	Superoxide metabolic process, and AhpC/TSA family	6	31	2.4	1.13E-11
CL:9184	Citrate cycle (TCA cycle) and malic oxidoreductase	6	85	1.96	7.17E-10
CL:10,322	Thioredoxin-disulfide reductase activity and glutathione peroxidase activity	5	20	2.51	1.49E-10
CL:10,344	Thioredoxin reductase and Thioredoxin-like	4	6	2.94	7.17E-10
CL:38,330	Alternative oxidase and NADPH dehydrogenase activity	4	11	2.68	2.07E-09
CL:14,843	Carotenoid biosynthesis	4	28	2.27	3.04E-08
CL:38,332	Alternative oxidase and extrinsic component of mitochondrial inner membrane	3	5	2.89	6.86E-08
CL:9306	Malate metabolic process	3	11	2.55	3.98E-07
CL:9186	Citrate cycle (TCA cycle)	3	57	1.84	2.57E-05
CL:14,881	Abscisic acid biosynthetic process	2	5	2.72	2.43E-05
CL:14,848	Carotenoid biosynthesis	2	13	2.3	0.0001
CL:9188	Citrate cycle (TCA cycle)	2	34	1.89	0.00058

and stomatal movement (Li et al. 2021). Therefore, observing the interaction between effective enzymes involved in ABA biosynthesis, ROS scavenging and oxidative stress-reducing was expected. According to Fig. 2, a direct relationship was seen between *ABA1* (ZEP), *NCED3, PSY3, GR, CAT*, and *NTRC* enzymes. Previous research reported that under drought stress conditions, the expression of *Arabidopsis NCED* gene (*AtNCED3*) was induced and controlled internal ABA levels (Bartel 2004; Iuchi et al. 2001). *PSY3* was also found to induce ABA production under drought and salinity stress in *Zea mays* (Li et al. 2007). GR is an NADPH-dependent antioxidant enzyme and effectively promotes GSH reduction and tolerance to oxidative stress (Gill et al. 2013). According to Fig. 2, we observed direct interactions between *GR* and *NTR* enzymes and Krebs cycle enzymes (*LTA3, FUM, IDH1,* and *SDH1*) and enzymes affecting ABA production (PSY, and ABA1). Overall, based on strong interactions and genes expression, our results provide strong evidence for future characterization of the genes.

Expression changes of the studied genes

The biosynthesis of carotenoid and ABA hormone

In plants, carotenoids serve as vital antioxidant pigments in stress tolerance. These pigments control ROS and free radicals and maintain redox balance. Plants, under stress, manage the accumulation and degradation of carotenoids by regulating genes involved in carotenoid metabolism. Accumulation and degradation of carotenoids affect a complex process of gene regulation (Wang et al. 2021). Based on STRING clustering, *PSY, NCED,* and *AAO3* genes were classified as carotenoid biosynthesis (CL: 14,843) (Table 3). The results of the gene network study (Fig. 2) revealed a direct association between the genes involved in ABA synthesis.

At the beginning of sampling (72 h without irrigation), an increase in the expression of *PSY* gene was observed in the drought-tolerant genotype (SLM046) (Fig. 3). Gene expression of DM samples showed that the highest gene expression was seen at 8 h after stress (6.7 times), and then it decreased at 24 h after stress. Nevertheless, *PSY* gene expression in DR samples had a decreasing trend at 8 and 24 h after
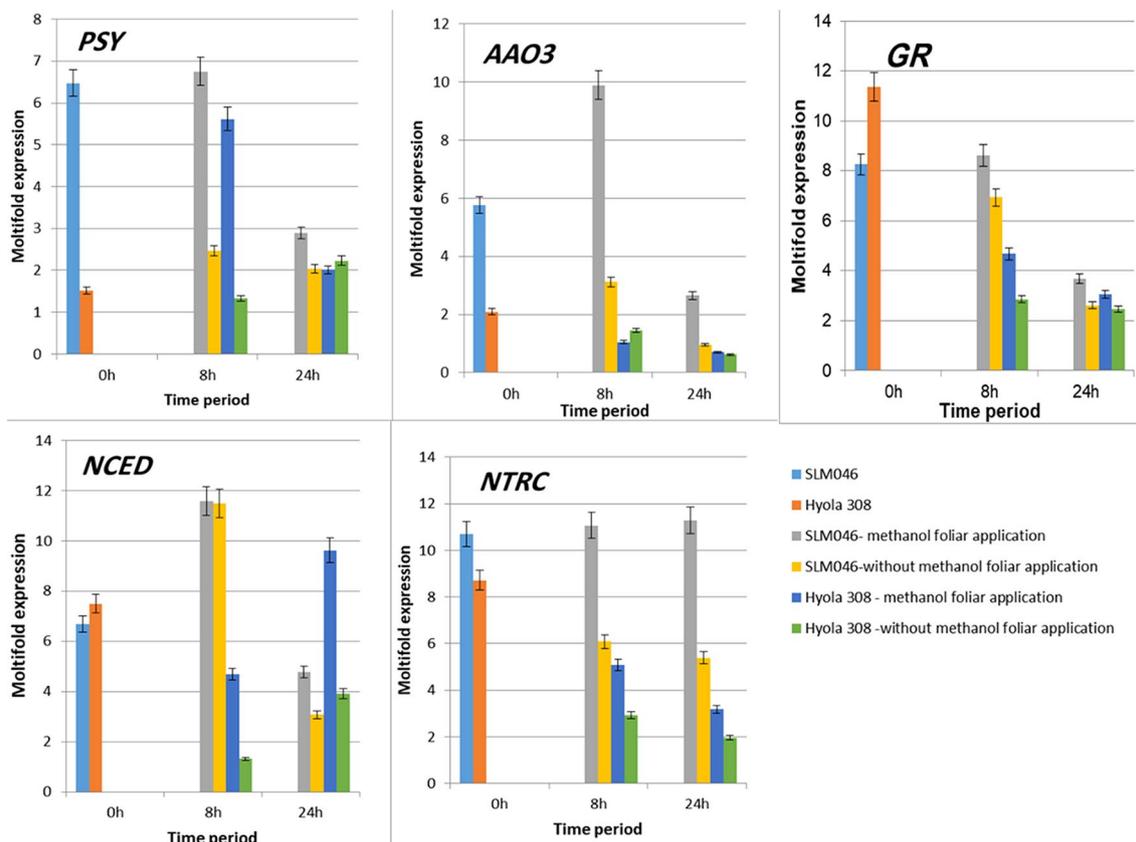
Fig. 3 Expression pattern of *PSY, NCED, GR, NTRC, AAO3* genes using real-time PCR under drought stress and methanol foliar application in seedling SLM046 and Hyola308

stress. However, both cases of DM samples (SLM046 and Hyola308) showed higher gene expression than DR samples. In the SLM046 genotype, *PSY* gene expression in DM samples was about 6.7 times at 8 h after drought stress and almost three times at 24 h after stress. *PSY* gene expression in the drought-sensitive genotypes (Hyola308) increased slightly in the early hours of sampling compared to the tolerant genotype. In DM samples 8 h after stress, the highest gene expression (5.5 times) was observed and expression level was decreased at 24 h. *PSY* showed the lowest gene expression level (equivalent to 0 h) in DR samples at 8 h after drought stress and gradually increased over 24 h. Therefore, it seems that in sensitive plants, methanol foliar application has accelerated the response to drought stress. The *PSY* highest expression level was observed at 8 h in both DM genotypes under drought stress. *PSY* is the first reactive enzyme that limits the rate of carotenoid production, and the plant indirectly produces the ABA through this pathway (Li et al. 2020). This hormone plays a vital role as a regulator of development and in responding to drought stress (Ali et al. 2020). In both genotypes, *PSY* gene expression increased, leading to the production of the ABA. Also, *PSY* gene expression was increased by methanol application 8 h after stress. Methanol as an anti-stress agent (Hemming et al. 1995) increased the expression level of this gene, but it also decreased the expression level of *PSY* after 24 h. A recent study reported that salt stress and ABA could induce *DcPSY2* expression by binding the AREB transcription factor (possibly *DcAREB3*) to ABRE cis-element in the promoter. Therefore, the expression of *DcPSY2* in carrots increases carotenoid production, thereby increasing ABA levels and protecting the plant from abiotic stress (Simpson et al. 2018).

The *NCED* gene is a critical enzyme in ABA biosynthesis. *NCED* converts Neoxanthin into Xanthoxin, followed by the production of the phytohormone ABA (Wang et al. 2021). *NCED* gene expression increased in the tolerant genotype at 72 h without irrigation (Fig. 3). In both DM and DR samples in the drought-tolerant genotypes (SLM046), the *NCED* gene had the highest expression level at 8 h (11.5 and 11.4 times, respectively), and after 24 h, showed a decreasing trend. The expression pattern did not change in the DM samples, but the expression level in 24 h increased more than the DR samples. However, this expression pattern was similar to the *PSY* gene expression pattern in the tolerant plant. The tolerant plant reacts more rapidly with higher expression rates, and methanol spraying increases overall expression level. At 72 h without irrigation, the drought-sensitive genotypes (Hyola308) had higher expression of the *NCED* gene than DR samples (7.4 times). However, a similar expression pattern was observed in both DM and DR samples. In this way, initially, the expression decreased in the 8th hour

and then increased in the 24th hour. However, methanol foliar application in DM samples increased the expression level to a large extent (5 times at 8 h and 9 times at 24 h). In both genotypes, methanol increased the expression of this gene as an anti-stress factor. Increased expression of this gene would be directly related to increasing ABA as a stress-responsive plant hormone. The production of ABA would control drought stress and produce responsive genes and closed pores in response to stress. The effect of methanol was various on gene expression in two genotypes at different hours. The drought-sensitive genotype had the highest expression level 24 h after methanol foliar application, while the drought-tolerant genotype had the highest expression level 8 h after methanol treatment. These results also suggest *NCED* is involved in drought tolerance. Differences in the expression of this gene under drought stress conditions may be related to differences in cultivars, degree of drought, and other factors. In Arabidopsis, drought stress induced the expression of the *NCED* gene (*AtNCED3*), and this gene adjusted the level of endogenous ABA (Iuchi et al. 2001). On the other hand, overexpression of *AtNCED3* in transgenic plants increased the level of endogenous ABA. The results showed *AtNCED3* expression played a significant role in ABA biosynthesis in drought stress in Arabidopsis. In another study, the expression of *DcNCED1* and *DcNCED2* was significantly increased in three carrot cultivars under drought stress (Wang et al. 2021).

AAO3 gene expression increased in the tolerant genotype (SLM046) in the early hours of sampling (Fig. 3). However, *AAO3* gene expression decreased in DR samples at the 8th and 24th hours of drought stress. On the other hand, in DM samples, the highest expression was observed in the 8th hour (9.8 times), and then a decreasing trend was observed in the 24th hour. However, DM samples had higher expression levels than DR samples in the 8th and 24th hours. It seems that methanol changed the expression pattern in DM samples compared to DR samples. Also, the expression pattern of the *AAO3* gene observed in the tolerant plants in DM samples was similar to *PSY* and *NCED* genes. A study on peanuts showed that the expression of *AhZEP, AhNCED1, AhAAO2*, and *AhABA3* genes were significantly increased in response to drought stress (Long et al. 2019).

AAO3 gene expression was highest (2 times) in drought-sensitive genotype (Hyola308) in the first hours of sampling. In DR drought stress samples, gene expression decreased gradually at 8 and 24 h after drought stress. However, the expression level of the *AAO3* gene in DM samples did not change at 8 and 24 h. Therefore, it seems that methanol foliar application had little effect on gene expression in DM samples of sensitive plants and even reduced the expression to some extent in the 8th hour. In Arabidopsis, the enzyme aldehyde oxidase (AAO3) is an enzyme that accelerates

the finishing stage of ABA biosynthesis. According to the results, *AAO3* performs a significant role in ABA biosynthesis in leaves (Seo et al. 2004).

The results showed that the expression of the *AAO3* gene increased with methanol application. As a result, it will cause an increasing effect on ABA followed by controlling and coping with drought stress. However, this gene had the highest expression in the drought-tolerant genotype after 8 h in drought stress with methanol treatment. As mentioned, methanol changed the expression pattern in DM samples compared to DR samples, so it seems that methanol spraying was effective in stress-tolerant plants. In contrast, methanol did not significantly affect sensitive genotypes and even reduced gene expression. So the effectiveness of methanol was different in both sensitive and drought-tolerant genotypes. In Arabidopsis, *AtAAO3* is one of four abscisic aldehyde oxidases (*AtAAO1* to 4) that actively use abscisic aldehyde as a substrate. It is most likely the sole AAO involved in ABA biosynthesis (Long et al. 2019).

Drought is an essential factor in the biosynthesis and metabolism of carotenoids. Drought stress was reported affecting carotenoid content in many crops, including strawberries, corn, soybeans, etc. (Shafiq et al. 2015). The results showed that drought stress in the tolerant plant increased genes' expression, affecting the biosynthesis of carotenoids and ABA. In DR samples, the expression of *PSY, NCED, AAO3* genes increased at the beginning of stress and decreased after 24 h. However, the sensitive plant's expression increase was less than in the tolerant plant. It seems that the application of methanol on the aerial parts of the plant was effective in reducing the influence of drought stress and water requirement. Therefore, spraying methanol on the leaves led to easier absorption by the plant and reduced stress. Methanol foliar application in DM samples in the tolerant plants significantly increased the expression level of *PSY, NCED,* and *AAO3* genes. However, DM samples in the sensitive plants displayed an increasing effect of methanol only on *PSY* and *NCED* genes and had little impact on *AAO3* gene expression.

Expression changes in genes involved in the antioxidant defense system

The *GR* gene expression was elevated in the tolerant genotype (SLM046) at the initial hours of sampling (Fig. 3). However, gene expression decreased in DR drought stress samples at the 8th and 24th hours of drought stress. Also, it was less than the *GR* expression level at the beginning of stress. Methanol spraying appears to have altered the expression pattern in DM samples compared with DR samples. Since, in DM samples, the highest expression rate was observed in the 8th hour (8.6 times), which was the highest expression rate between both DM and DR groups. However,

a decreasing trend was observed again in the 24th hour. Nevertheless, at both 8th and 24th h, DM samples had higher expression levels than DR samples (8.6 times at 8 h and 3.6 times at 24 h).

The *GR* gene expression in the drought-sensitive genotype (Hyola308) had the highest expression (11.3 times) in the early hours of sampling, which was even higher than the expression in the tolerant plant. In DR drought stress samples, gene expression decreased significantly at 8 and 24 h after drought stress. It seems that the sensitive plant could not maintain the expression of the *GR* effective gene in regulating oxidative stress. However, *GR* gene expression decreased to a lesser extent in DM samples at the 8th and 24th hours. Therefore, it seems that methanol treatment had some compensatory effect on DM samples of sensitive plants.

GR is an NADP(H)-dependent antioxidant enzyme and effectively promotes GSH reduction and oxidative stress tolerance (Gill et al. 2013). The glutathione–ascorbate cycle is one of the ROS control cycles in most cell organs such as mitochondria, chloroplasts, and cytoplasm. *GR* is responsible for converting oxidized glutathione to reduced glutathione and maintaining a high ratio of GSH to GSSG (Noctor and Foyer 1998). In a study on *Nicotiana tabacum*, it was found that tolerance to photo-oxidative stress in plants is associated with the high activity of *GR* chloroplasts, so increasing the expression of the *GR* gene could prevent oxidative stress (Aono et al. 1993). Increased glutathione reductase gene expression was reported by drought stress in sensitive bean cultivars. They also stated the expression level of this gene remained constant in drought-resistant cultivars (Torres-Franklin et al. 2008). Other studies have reported increased glutathione reductase activity during drought stress compared to control plants (Chen et al. 2020; Ratnayaka et al. 2003; Yousuf et al. 2012).

Methanol application increased the expression of the GR gene in both tolerant and susceptible genotypes 8 h after stress, but this amount was higher in the tolerant plant. Increased expression of this gene has been reported in drought stress compared to controls (Ratnayaka et al. 2003; Yousuf et al. 2012). In the current study (72 h after final irrigation), GR gene expression was increased in both genotypes. The activity of the glutathione-ascorbate cycle increased by exposing the plant to drought stress and increasing ROS production. The amount of *GR* expression gradually decreases over time due to the activation of other antioxidant mechanisms. Unlike the susceptible plant, decreasing expression levels occurred to a lesser extent due to the tolerant plant's more robust stress tolerance mechanism. It also seems that methanol spraying was able to increase the intensity of the impact of this defensive mechanism in both genotypes. However, this increasing impact was higher in the tolerant plant.

At the beginning of sampling, *NTRC* gene expression increased in the tolerant genotypes (SLM046) (Fig. 3). The expression level of the *NTRC* gene decreased gradually after 8 h and reached its lowest level in DR samples in the 24th hour (5.3 times). Therefore, we observed an upward and then a downward pattern in DR samples. In contrast, methanol spray significantly increased the expression level of this gene at the 8th and 24th hour in DM samples compared to the same time in DR samples (11 times at 8 h and 11.2 times at 24 h).

NTRC gene expression in drought-sensitive genotypes (Hyola308) increased in the early hours of sampling but was less than the amount expressed in the tolerant plant. Similar to the tolerant plants, the expression level of the *NTRC* gene in DR samples of sensitive plants decreased at 8 h after stress and reached its lowest level after 24 h (1.9 times). Therefore, the expression pattern was first upward and then downward, similar to drought-tolerant plants. However, unlike the DM samples of the tolerant plant, methanol treatment did not alter the expression pattern. It merely increased the expression level at the 8th and 24th hours compared to the same time in the sensitive plant.

NTRC is located inside the chloroplast and is a significant player in the chloroplast redox detoxification system. Plants without *NTRC* showed susceptibility to bacterial pathogens, leading to a high accumulation of ROS (Mata-Pérez and Spoel 2019). As noticed, drought stress increased *GR* gene expression, and this factor could increase glutathione–ascorbate cycle activity. On the other hand, the activity of this cycle is dependent on NADPH, and one of its sources is *NTRC*. Therefore, the expression of the *NTRC* gene increases with increased antioxidant activity. As the activity of this system decreased, the expression level of the *NTRC* gene also gradually reduced. Prolonged stress causes ROS to accumulate in the plant and induces oxidative damage at the cellular level. Therefore, increasing antioxidant inhibitors is essential to lessen ROS accumulation. Plants cope with stress by increasing gene expression like *NTRC* (Cha et al. 2014). Based on the results (Fig. 2), there is a direct relationship between NTRs and *TRX*. *TRX* plays an essential role in the tolerance of plants to oxidative stress. *TRX* uses FTR or NTR to reduce itself (Reichheld et al. 2005). Drought stress increased the expression of the *NTRC* gene in both sensitive and tolerant genotypes; because of the more robust defense system of the tolerant plant against drought stress, the expression level of the *NTRC* gene was higher in the tolerant genotype. Moreover, methanol application effectively increased the expression of the *NTRC* gene. In both genotypes, increased expression was observed in 8 and 24 h after stress.

According to the results of the gene network, *GR* and *NTRC* genes were classified as superoxide metabolic processes (CL: 10,321) (Table 3). A direct interaction was observed between GR and NTR enzymes. It revealed the involvement of both of these enzymes in the antioxidant system. For example, *ntra* and *ntrb* mutants were able to survive but were highly sensitive to GSH reduction (Marty et al. 2009). The expression of GR and *NTRC* genes effective in regulating ROS activity increased during drought stress. A complex antioxidant defense system was activated in both sensitive and tolerant genotypes by exposing the plant to drought stress and increasing ROS production. Hence, the expression of *GR* and *NTRC* genes increased as part of this defense system. A downward trend in the expression level of *GR* and *NTRC* genes was observed at the 8th and 24th hours, caused by the activation of other elements of the antioxidant defense system. A severe effect of stress was observed because of the greater reduction in gene expression level in the sensitive plant and possibly its weaker antioxidant defense system. On the other hand, in both genotypes, methanol foliar application increased the expression level of both genes. The difference was that less expression was seen in sensitive genotypes at the 8th and 24th hours.

In comparison, an increasing trend was observed in the drought-tolerant genotype in the expression level of *GR* and *NTRC* genes. The treatment likely caused an enhancing effect on other genes involved in the antioxidant system, especially in the tolerant plant. This issue can be investigated in future research. Overall, drought stress increased ROS levels, followed by activation of the antioxidant system. Methanol was able to increase gene expression significantly. As a result, it could enhance anti-stress properties and support the plant cope with drought stress.

MicroRNAs targeting the studied genes

Examination of the Brassicaceae family microRNAs targeting *PSY, NCED, GR, NTRC*, and *AAO3* was performed. The detailed information of the identified miRNAs is presented in Table 4. As a result, 38 microRNAs were identified for the *PSY* gene. Five microRNAs used the translation inhibition mechanism, and the rest used the cleavage system (Table 4). Several families of microRNAs were identified for the *PSY* gene, most of which belonged to the miR397 family with six numbers. Also, bna-miR397a and bna-miR397b were specifically identified in rapeseed (Fig. 4). A recent study indicated that bna-miR397a/b also targeted *BnCSD10* in rapeseed and this genes' expression level under different abiotic stress like drought stress was changed (Su et al. 2021). This microRNA is recognized as one of the factors in response to abiotic stresses (Huang et al. 2020). Recently, it has been observed that *LAC2*, a negative regulator of lignin deposition, was regulated by an increase in miR397b in Arabidopsis roots under water-deficient conditions (Khandal et al. 2020). Another study showed that miR397 expression increased in alfalfa (*Medicago sativa*) under drought stress,

Table 4 Identified microRNAs affecting *PSY, NCED, GR, NTRC, AAO3 genes* using psRNATarget software with the expected value 5

miRNA_Acc	Target_Acc	Expectation	Target_start	Target_end	Inhibition	Target_Desc
aly-miR172a-5p	XM_013862421.2	3.5	1513	1533	Cleavage	AAO3
bra-miR5717	XM_013862421.2	3.5	593	613	Cleavage	AAO3
cas-miR172a-5p	XM_013862421.2	3.5	1513	1533	Cleavage	AAO3
bra-miR9569-5p	XM_013862421.2	4	686	706	Cleavage	AAO3
ath-miR4243	XM_013862421.2	4.5	1258	1278	Cleavage	AAO3
ath-miR8121	XM_013862421.2	4.5	1729	1752	Cleavage	AAO3
ath-miR830-5p	XM_013862421.2	4.5	1419	1440	Cleavage	AAO3
bra-miR5724	XM_013862421.2	4.5	854	874	Cleavage	AAO3
aly-miR156g-3p	XM_013862421.2	5	403	423	Cleavage	AAO3
aly-miR4249	XM_013862421.2	5	1153	1173	Cleavage	AAO3
ath-miR5018	XM_013862421.2	5	1389	1412	Cleavage	AAO3
ath-miR5014b	XM_022709985.1	3.5	1577	1597	Cleavage	GR
aly-miR838-3p	XM_022709985.1	4	1858	1878	Cleavage	GR
aly-miR834-3p	XM_022709985.1	4.5	284	304	Cleavage	GR
aly-miR856-3p	XM_022709985.1	4.5	772	792	Cleavage	GR
ath-miR5012	XM_022709985.1	4.5	1629	1649	Cleavage	GR
ath-miR834	XM_022709985.1	4.5	284	304	Cleavage	GR
aly-miR1887	XM_022709985.1	5	1567	1590	Translation	GR
ath-miR398b-5p	XM_022709985.1	5	1481	1501	Translation	GR
ath-miR398c-5p	XM_022709985.1	5	1481	1501	Translation	GR
ath-miR8181	XM_022709985.1	5	249	268	Cleavage	GR
ath-miR831-3p	XM_022709985.1	5	1461	1482	Cleavage	GR
ath-miR861-5p	XM_022709985.1	5	960	980	Cleavage	GR
bra-miR9555b-3p	XM_022709985.1	5	2025	2045	Translation	GR
ath-miR2111b-3p	XM_013892363.2	4	483	503	Cleavage	NTRC
aly-miR2111b-3p	XM_013892363.2	4.5	483	503	Cleavage	NTRC
ath-miR830-3p	XM_013892363.2	4.5	910	930	Cleavage	NTRC
bna-miR2111b-3p	XM_013892363.2	4.5	483	503	Cleavage	NTRC
aly-miR837-5p	XM_013892363.2	5	1223	1243	Cleavage	NTRC
aly-miR838-3p	XM_013892363.2	5	1546	1566	Cleavage	NTRC
ath-miR2934-5p	XM_013892363.2	5	408	428	Cleavage	NTRC
ath-miR870-3p	XM_013892363.2	5	1049	1069	Cleavage	NTRC
bra-miR2111b-3p	XM_013892363.2	5	483	503	Cleavage	NTRC
bra-miR9559-5p	XM_013892363.2	5	823	844	Cleavage	NTRC
cas-miR2111b	XM_013892363.2	5	483	503	Cleavage	NTRC
cas-miR159c-5	XM_013827623.2	2.5	416	436	Cleavage	PSY
ath-miR5029	XM_013827623.2	3	345	365	Cleavage	PSY
aly-miR838-3p	XM_013827623.2	3.5	1166	1186	Cleavage	PSY
ath-miR5021	XM_013827623.2	3.5	422	441	Cleavage	PSY
aly-miR169m-3p	XM_013827623.2	4	1450	1468	Cleavage	PSY
aly-miR834-3p	XM_013827623.2	4	1000	1020	Cleavage	PSY
aly-miR838-3p	XM_013827623.2	4	33	53	Cleavage	PSY
ath-miR834	XM_013827623.2	4	1000	1020	Cleavage	PSY
aly-miR319a-5p	XM_013827623.2	4.5	960	980	Cleavage	PSY
aly-miR4246	XM_013827623.2	4.5	949	969	Cleavage	PSY
aly-miR838-3p	XM_013827623.2	4.5	198	218	Cleavage	PSY
aly-miR862-3p	XM_013827623.2	4.5	240	260	Translation	PSY
ath-miR825	XM_013827623.2	4.5	154	174	Cleavage	PSY
bra-miR319-5p	XM_013827623.2	4.5	960	980	Cleavage	PSY
cas-miR159c-5	XM_013827623.2	4.5	278	298	Cleavage	PSY
cas-miR319a	XM_013827623.2	4.5	960	980	Cleavage	PSY

Table 4 (continued)

miRNA_Acc	Target_Acc	Expectation	Target_start	Target_end	Inhibition	Target_Desc
aly-miR172a-5p	XM_013827623.2	5	584	603	Cleavage	PSY
aly-miR3445-3p.2	XM_013827623.2	5	351	371	Cleavage	PSY
aly-miR3449-5p	XM_013827623.2	5	729	749	Cleavage	PSY
aly-miR397a-5p	XM_013827623.2	5	880	900	Cleavage	PSY
aly-miR397b-5p	XM_013827623.2	5	880	900	Cleavage	PSY
aly-miR4243	XM_013827623.2	5	98	118	Cleavage	PSY
aly-miR825-3p	XM_013827623.2	5	154	174	Cleavage	PSY
ath-miR397a	XM_013827623.2	5	880	900	Cleavage	PSY
ath-miR5645c	XM_013827623.2	5	814	834	Cleavage	PSY
ath-miR829-3p.2	XM_013827623.2	5	623	643	Cleavage	PSY
ath-miR838	XM_013827623.2	5	1166	1186	Translation	PSY
ath-miR838	XM_013827623.2	5	39	58	Translation	PSY
ath-miR838	XM_013827623.2	5	198	217	Translation	PSY
ath-miR862-3p	XM_013827623.2	5	240	260	Translation	PSY
ath-miR865-3p	XM_013827623.2	5	958	978	Cleavage	PSY
bna-miR397a	XM_013827623.2	5	879	900	Cleavage	PSY
bna-miR397b	XM_013827623.2	5	879	900	Cleavage	PSY
bra-miR5726	XM_013827623.2	5	537	557	Cleavage	PSY
bra-miR9561-3p	XM_013827623.2	5	338	360	Cleavage	PSY
cas-miR156k-3p	XM_013827623.2	5	820	841	Cleavage	PSY
cas-miR172a-5p	XM_013827623.2	5	584	603	Cleavage	PSY
cas-miR397	XM_013827623.2	5	880	900	Cleavage	PSY
aly-miR838-3p	XM_013788802.2	3	2031	2051	Cleavage	NCED3
aly-miR157d-5p	XM_013788802.2	3.5	2107	2126	Cleavage	NCED3
ath-miR157d	XM_013788802.2	3.5	2107	2126	Cleavage	NCED3
cas-miR156i	XM_013788802.2	3.5	2104	2123	Cleavage	NCED3
aly-miR3445-3p.1	XM_013788802.2	4	149	169	Cleavage	NCED3
ath-miR847	XM_013788802.2	4	2032	2052	Cleavage	NCED3
cas-miR157d	XM_013788802.2	4	2104	2123	Cleavage	NCED3
aly-miR161-5p.1	XM_013788802.2	4.5	946	966	Cleavage	NCED3
aly-miR169b-3p	XM_013788802.2	4.5	981	1002	Cleavage	NCED3
ath-miR169b-3p	XM_013788802.2	4.5	981	1002	Cleavage	NCED3
ath-miR5021	XM_013788802.2	4.5	2114	2133	Translation	NCED3
ath-miR838	XM_013788802.2	4.5	2031	2051	Translation	NCED3
ath-miR867	XM_013788802.2	4.5	2311	2331	Cleavage	NCED3
aly-miR156h-3p	XM_013788802.2	5	2034	2054	Translation	NCED3
aly-miR156h-5p	XM_013788802.2	5	2107	2126	Translation	NCED3
aly-miR157a-5p	XM_013788802.2	5	2107	2127	Cleavage	NCED3
aly-miR157b-5p	XM_013788802.2	5	2107	2127	Cleavage	NCED3
aly-miR157c-5p	XM_013788802.2	5	2107	2127	Cleavage	NCED3
aly-miR3439-5p	XM_013788802.2	5	1139	1159	Cleavage	NCED3
aly-miR3441-3p.2	XM_013788802.2	5	448	468	Cleavage	NCED3
aly-miR395i	XM_013788802.2	5	1153	1173	Cleavage	NCED3
aly-miR847-3p	XM_013788802.2	5	2032	2052	Cleavage	NCED3
ath-miR156h	XM_013788802.2	5	2107	2126	Translation	NCED3
ath-miR156i	XM_013788802.2	5	2107	2126	Translation	NCED3
ath-miR156j	XM_013788802.2	5	2107	2126	Translation	NCED3
ath-miR157a-5p	XM_013788802.2	5	2107	2127	Cleavage	NCED3
ath-miR157b-5p	XM_013788802.2	5	2107	2127	Cleavage	NCED3
ath-miR157c-5p	XM_013788802.2	5	2107	2127	Cleavage	NCED3
ath-miR416	XM_013788802.2	5	650	670	Cleavage	NCED3

Table 4 (continued)

miRNA_Acc	Target_Acc	Expectation	Target_start	Target_end	Inhibition	Target_Desc
ath-miR858b	XM_013788802.2	5	1213	1233	Cleavage	NCED3
ath-miR867	XM_013788802.2	5	1276	1296	Cleavage	NCED3
bna-miR156b	XM_013788802.2	5	2107	2127	Cleavage	NCED3
bna-miR156c	XM_013788802.2	5	2107	2127	Cleavage	NCED3
bna-miR156g	XM_013788802.2	5	2107	2127	Cleavage	NCED3
bol-miR157a	XM_013788802.2	5	2107	2127	Cleavage	NCED3
bra-miR157a	XM_013788802.2	5	2107	2127	Cleavage	NCED3
cas-miR156h	XM_013788802.2	5	2107	2126	Translation	NCED3
cas-miR156j	XM_013788802.2	5	2107	2126	Translation	NCED3

and by overexpression of alfalfa's miR397 in the tobacco plant, drought stress resistance occurred (Chen et al. 2020). According to the expectation index, the lowest value of the index belonged to miR159 (Table 4). The miR159 family is highly protected among monocotyledonous and dicotyledonous plants. However, the miR159's relative abundance behaves differently in plants under drought stress because of the tissue and plant species (López-Galiano et al. 2019). For example, miR159 was reported to increase in Arabidopsis and maize under drought stress (Liu et al. 2008; Wei et al. 2009) but decreased in cotton and potato (Xie et al. 2015; Yang et al. 2014). While in barley and alfalfa, miR159 decreased in roots and increased in leaves in response to drought stress (Da et al. 2017; Hackenberg et al. 2015).

However, some microRNAs were mutual among various genes, including miR838 among *PSY, NCED, GR, NTRC*, and miR834 genes between *PSY* and *GR* genes, miR172 between *PSY* and *AAO3* genes, and miR5021 between *PSY* and *NCED* genes (Fig. 4). 38 microRNAs were identified for the *NCED* gene, of which nine microRNAs used the translation inhibition mechanism, and the rest used the cleavage system (Table 4). Several microRNAs were identified for the *NCED* gene; most of them belonged to miR156, miR157 families, with 11 numbers. Also, bna-miR156b, bna-miR156c, bna-miR156g were specifically identified in rapeseed (Fig. 4). The miR156 family is highly conserved in plants and plays a significant role in responding to abiotic stresses (Feyissa et al. 2021). Studies on alfalfa (*Medicago sativa*) have shown that miR156 has a regulatory role in drought stress (Feyissa et al. 2019). The overexpression of miR156's *Oryza sativa* affected the expression of genes associated with abiotic stress resistance and growth in alfalfa (Wang et al. 2021). The lowest expectations were for miR838 and miR157 (Table 4). It was observed that miR838 was effective in drought stress in wheat (Akdogan et al. 2016). The miR157, which belonged to the same miRNA family as that of miR156, showed the same expression trend.

Studies have shown that miR157 is a factor involved in response to drought stress and high temperatures (Chen et al. 2020; Li et al. 2013). 13 microRNAs were identified for the

GR gene, of which four microRNAs used the translation inhibition mechanism, and the rest used the cleavage system. No microRNA was found in rapeseed for this gene. However, the lowest Expectations belonged to ath-miR5014b, aly-miR838-3p, and aly-miR834-3p, respectively (Table 4). Specifically, ath-miR5014b has been identified in Arabidopsis (Borges et al. 2011), and no evidence has been found for its involvement in stress regulation. aly-miR838-3p was in common with *PSY* and *NTRC*, and aly-miR834-3p was also in common with *PSY*. The miR838 and miR834 families are conserved in the Brassicaceae family, and their regulatory role in regulating abiotic stress has been identified (Çelik and Akdaş 2019; Srivastava et al. 2013). Examination of the *Zanthoxylum bungeanum* revealed that miR834 had a regulatory role in drought stress (Fei et al. 2020). miR838 was also influential in drought stress in *Triticum aestivum* (Akdogan et al. 2016).

Eleven microRNAs were identified for the *NTRC* gene, and all microRNAs used the cleavage mechanism (Table 4). Several families of microRNAs were identified for the NTRC gene, most of which belonged to the miR2111 family with five numbers. Bna-miR2111b-3p was also detected in rapeseed (Fig. 4). Studies have also indicated the role of this microRNA in drought stress (Ghorecha et al. 2017; Wang et al. 2011). MiR2111 has also been involved in phosphate homeostasis and plant survival (Li et al. 2013). Studies have shown that simulated drought stress increases miR2111 expression (Zheng et al. 2016). In soybean gma-miR2111b/c/e/f had increased in leaves, but its expression decreased in roots (Huang et al. 2020).

Eleven microRNAs were identified for the *AAO3* gene, and all microRNAs used the cleavage mechanism (Table 4). Several families of microRNAs were identified for the *AAO3* gene, most of which belonged to the miR172 family with two numbers (Fig. 4). The lowest expectation value belonged to aly-miR172a-5p, bra-miR5717 (Table 4). miR172 is involved in the development of anther in many species like Arabidopsis, cotton, and alfalfa miR172. It regulates flower organ recognition genes, such as the *AP2* and *AP2* genes. Its expression was significantly inhibited

Fig. 4 Relationships between microRNAs affecting *PSY*, *NCED3*, *GR*, *NTRC*, *AAO3* genes were mapped using Cytoscape software. The hexagon shapes represent rapeseed microRNAs, and the cyan color indicates individually located microRNAs, other groups with the same color represent the microRNA family

under high-temperature stress, while its target gene (TOE) expression was increased (Chen et al. 2020). A recent study demonstrated that bna-miR172a/b/c/d targeted *BnCSD2* in rapeseed (Su et al. 2021). The role of this microRNA under drought stress has been clarified; mir172 expression was affected in *Brassica juncea*, *Helianthus annuus*, and *Malus domestica* (Ali et al. 2020; Bhardwaj et al. 2014; Liang et al. 2020).

Conclusion

Morphological observation showed that rapeseed plants, both sensitive and tolerant genotypes, regained their freshness after methanol foliar application under irrigation cutoff stress. This study showed that the expression of *PSY, NCED, GR, NTRC, AAO3* genes in rapeseed drought-tolerant genotype (SLM046) increased more than the sensitive genotype (Hyola308). Drought stress reduces plant growth by changing photosynthesis and water status. The cell also tries to prevent oxidative stress and ROS accumulation by implementing special measures (including altering gene expression). The protein–protein interaction results showed extensive correlations between enzymes in the Krebs cycle and the mechanisms involved in oxidation–reduction reactions effective in controlling ROS and the pathway of carotenoid synthesis and ABA synthesis. This study observed that the expression of *GR* and *NTRC* genes increased during drought stress. The plant tries to prevent the accumulation and damage of ROS and activates the antioxidant system. Also, the expression of *PSY, NCED, AAO3* genes effective in the pathway of carotenoid synthesis and ABA synthesis during drought stress was increased. Therefore, this pathway was activated under drought stress, and the production of the ABA increased as one of the factors controlling stress. This study showed that after methanol treatment, the expression of *PSY, NCED, GR, NTRC, AAO3* genes in rapeseed drought-tolerant genotype (SLM046) increased significantly compared to sensitive genotype (Hyola308). Apparently, methanol increased tolerance, especially in the SLM046 genotype. In the early hours of stress in the sensitive genotypes, the expression of these genes increased after methanol treatment compared to plants without foliar application; however, in the next hour, the amount decreased. Considering the effect of methanol on the expression of genes under drought stress, it seems that methanol causes a positive impact under drought stress. Carbon dioxide fixation occurred, and hydrogen peroxide production decreased by the application of methanol. The study of effective microRNAs showed that sometimes more than one type of microRNA could affect the desired gene, and in some cases, a conserved family of microRNAs caused the main effect on gene expression. Therefore, by examining and manipulating these main groups of microRNAs, the genes expression level can be affected. This issue can be further investigated in future experiments. Further research on rapeseed and the genes involved in stress resistance may reveal relevant molecular patterns for identifying drought resistance.

Declarations

References

Akdogan G, Tufekci ED, Uranbey S, Unver T (2016) miRNA-based drought regulation in wheat. Funct Inte Genom 16:221–233. https://doi.org/10.1007/s10142-015-0452-1

Ali S, Hayat K, Iqbal A, Xie L (2020) Implications of abscisic acid in the drought stress tolerance of plants. Agronomy 10:1323. https://doi.org/10.3390/agronomy10091323

Aono M, Kubo A, Saji H, Tanaka K, Kondo N (1993) Enhanced tolerance to photooxidative stress of transgenic Nicotiana tabacum with high chloroplastic glutathione reductase activity. Plant Cell Physiol 34:129–135. https://doi.org/10.1093/oxfordjournals.pcp.a078386

Armand N, Amiri H, Ismaili A (2016) The effect of methanol on photosynthetic parameters of bean (*Phaseolus vulgaris* L.) under water deficit. Photosynthetica 54:288–294. https://doi.org/10.1007/s11099-015-0178-2

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297. https://doi.org/10.1016/S0092-8674(04)00045-5

Bhardwaj AR, Joshi G, Pandey R, Kukreja B, Goel S, Jagannath A, Kumar A, Katiyar-Agarwal S, Agarwal M (2014) A genome-wide perspective of miRNAome in response to high temperature, salinity and drought stresses in *Brassica juncea* (Czern) L. PLoS ONE. https://doi.org/10.1371/journal.pone.0092456

Borges F, Pereira PA, Slotkin RK, Martienssen RA, Becker JD (2011) MicroRNA activity in the *Arabidopsis* male germline. J Exp Bot 62:1611–1620. https://doi.org/10.1093/jxb/erq452

Çelik Ö, Akdaş EY (2019) Tissue-specific transcriptional regulation of seven heavy metal stress-responsive miRNAs and their putative targets in nickel indicator castor bean (*R. communis* L.) plants. Ecotoxicology Env Safety 170:682–690. https://doi.org/10.1016/j.ecoenv.2018.12.006

Cha J-Y, Kim JY, Jung IJ, Kim MR, Melencion A, Alam SS, Yun D-J, Lee SY, Kim MG, Kim W-Y (2014) NADPH-dependent thioredoxin reductase A (NTRA) confers elevated tolerance to oxidative stress and drought. Plant Physiol Biochem 80:184–191. https://doi.org/10.1016/j.plaphy.2014.04.008

Chen J, Pan A, He S, Su P, Yuan X, Zhu S, Liu Z (2020) Different microRNA families involved in regulating high temperature stress response during cotton (*Gossypium hirsutum* L.) anther development. Int J Mol Sci. https://doi.org/10.3390/ijms21041280

Da Q, Wang P, Wang M, Sun T, Jin H, Liu B, Wang J, Grimm B, Wang H-B (2017) Thioredoxin and NADPH-dependent

thioredoxin reductase C regulation of tetrapyrrole biosynthesis. Plant Physiol 175:652–666. https://doi.org/10.1104/pp.16.01500

Dai X, Zhuang Z, Zhao PX (2018) psRNATarget: a plant small RNA target analysis server (2017 release). Nucleic Acids Res 46:W49–W54. https://doi.org/10.1093/nar/gky316

Danaeipour Z, Haddad R (2020) Influence of drought stress on photosynthetic characteristics and protective enzymes in plants. Iranian J Gene Plant. 9:114–129

Dos Santos CV, Rey P (2006) Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci 11:329–334. https://doi.org/10.1016/j.tplants.2006.05.005

Downie A, Miyazaki S, Bohnert H, John P, Coleman J, Parry M, Haslam R (2004) Expression profiling of the response of *Arabidopsis thaliana* to methanol stimulation. Phytochem 65:2305–2316. https://doi.org/10.1016/j.phytochem.2004.07.006

Fei X, Li J, Kong L, Hu H, Tian J, Liu Y, Wei A, Biochemistry, (2020) miRNAs and their target genes regulate the antioxidant system of *Zanthoxylum bungeanum* under drought stress. Plant Physiol 150:196–203. https://doi.org/10.1016/j.plaphy.2020.01.040

Fernie AR, Carrari F, Sweetlove LJ (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Curr Opin Plant Biol 7:254–261. https://doi.org/10.1016/j.pbi.2004.03.007

Feyissa BA, Arshad M, Gruber MY, Kohalmi SE, Hannoufa A (2019) The interplay between miR156/SPL13 and DFR/WD40–1 regulate drought tolerance in alfalfa. BMC Plant Biol 19:1–19. https://doi.org/10.1186/s12870-019-2059-5

Feyissa BA, Amyot L, Nasrollahi V, Papadopoulos Y, Kohalmi SE, Hannoufa A (2021) Involvement of the miR156/SPL module in flooding response in *Medicago sativa*. Sci Rep 11:1–16. https://doi.org/10.1038/s41598-021-82450-7

Ghorecha V, Zheng Y, Liu L, Sunkar R, Krishnayya N (2017) MicroRNA dynamics in a wild and cultivated species of Convolvulaceae exposed to drought stress. Physiol Mol Biol Plants 23:291. https://doi.org/10.1007/s12298-017-0426-y

Gill SS, Anjum NA, Hasanuzzaman M, Gill R, Trivedi DK, Ahmad I, Pereira E, Tuteja N (2013) Glutathione and glutathione reductase: a boon in disguise for plant abiotic stress defense operations. Plant Physiol Biochem 70:204–212. https://doi.org/10.1016/j.plaphy.2013.05.032

Hackenberg M, Gustafson P, Langridge P, Shi BJ (2015) Differential expression of micro RNA s and other small RNA s in barley between water and drought conditions. Plant Biotechnol J 13:2–13. https://doi.org/10.1111/pbi.12220

Hemming DJ, Criddle RS, Hansen LD (1995) Effects of methanol on plant respiration. J Plant Physiol 146:193–198. https://doi.org/10.1016/S0176-1617(11)82040-7

Huang S, Zhou J, Gao L, Tang Y (2020) Plant miR397 and its functions. Funct Plant Biol 48:361–370. https://doi.org/10.1071/fp20342

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. Plant J 27:325–333. https://doi.org/10.1046/j.1365-313x.2001.01096.x

Kalisz A, Pokluda R, Jezdinský A, Sȩkara A, Grabowska A, Gil J, Neugebauerová J (2016) Chilling-induced changes in the antioxidant status of basil plants. Acta Physiol Plant 38:196. https://doi.org/10.1007/s11738-016-2214-7

Khalilzadeh R, Seid Sharifi R, Pirzad A (2020) Mitigation of drought stress in pot marigold (*Calendula officinalis*) plant by foliar application of methanol. J Plant Physiol Breed. 10:71–84

Khandal H, Singh AP, Chattopadhyay D (2020) The MicroRNA397b-LACCASE2 module regulates root lignification under water and phosphate deficiency. Plant Physiol 182:1387–1403. https://doi.org/10.1104/pp.19.00921

Kim MR, Khaleda L, Jung IJ, Kim JY, Lee SY, Cha J-Y, Kim W-Y (2017) Overexpression of chloroplast-localized NADPH-dependent thioredoxin reductase C (NTRC) enhances tolerance to photo-oxidative and drought stresses in *Arabidopsis thaliana*. J Plant Bio 60:175–180. https://doi.org/10.1007/s12374-016-0464-y

Li F, Murillo C, Wurtzel ET (2007) Maize Y9 encodes a product essential for 15-cis-zeta-carotene isomerization. Plant Physiol 144:1181–1189. https://doi.org/10.1104/pp.107.098996

Li C, Liang D, Xu R, Li H, Zhang Y, Qin R, Li L, Wei P, Yang J (2013) Overexpression of an alternative oxidase gene, *OsAOX1a*, improves cold tolerance in *Oryza sativa* L. Genet Mol 12:5424–5432. https://doi.org/10.4238/2013.November.11.4

Li X, Chen P, Xie Y, Yan Y, Wang L, Dang H, Zhang J, Xu L, Ma F, Guan Q (2020) Apple SERRATE negatively mediates drought resistance by regulating MdMYB88 and MdMYB124 and microRNA biogenesis. Hortic Res 7:1–11. https://doi.org/10.1038/s41438-020-0320-6

Li M, Yang Y, Raza A, Yin S, Wang H, Zhang Y, Dong J, Wang G, Zhong C, Zhang H et al (2021) Heterologous expression of *Arabidopsis thaliana* rty gene in strawberry (Fragaria × ananassa Duch.) improves drought tolerance. BMC Plant Biol. https://doi.org/10.1186/s12870-021-02839-4

Liang C, Wang W, Ma J, Wang J, Zhou F, Li W, Yu Y, Zhang L, Huang W, Huang X (2020) Identification of differentially expressed microRNAs of sunflower seedlings under drought stress. Agronomy 112:2472–2484. https://doi.org/10.1002/agj2.20254

Liu H-H, Tian X, Li Y-J, Wu C-A, Zheng C-C (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. RNA 14:836–843. https://doi.org/10.1261/rna.895308

Liu D, Liu H, Du H, Liu H, Kurtenbach R (2021) Relationship between polyamines conjugated to mitochondrion membrane and mitochondrion conformation from developing wheat embryos under drought stress. J Biosci 46:1–11. https://doi.org/10.1007/s12038-021-00155-5

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Long H, Zheng Z, Zhang Y, Xing P, Wan X, Zheng Y, Li L (2019) An abscisic acid (ABA) homeostasis regulated by its production, catabolism and transport in peanut leaves in response to drought stress. PLoS ONE 14:e0213963. https://doi.org/10.1371/journal.pone.0213963

López-Galiano MJ, García-Robles I, González-Hernández AI, Camañes G, Vicedo B, Real MD, Rausell C (2019) Expression of miR159 is altered in tomato plants undergoing drought stress. Plants 8:201. https://doi.org/10.3390/plants8070201

Marty L, Siala W, Schwarzländer M, Fricker MD, Wirtz M, Sweetlove LJ, Meyer Y, Meyer AJ, Reichheld J-P, Hell R (2009) The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in *Arabidopsis*. Proc Natl Acad Sci 106:9109–9114. https://doi.org/10.1073/pnas.0900206106

Mata-Pérez C, Spoel SH (2019) Thioredoxin-mediated redox signalling in plant immunity. Plant Sci 279:27–33. https://doi.org/10.1016/j.plantsci.2018.05.001

Meng C, Yang M, Wang Y, Chen C, Sui N, Meng Q, Zhuang K, Lv W (2020) SlWHY2 interacts with SlRECA2 to maintain mitochondrial function under drought stress in tomato. Plant Sci 301:110674. https://doi.org/10.1016/j.plantsci.2020.110674

Mirzai M, Moeini A, Ghanati F (2013) Effects of drought stress on the lipid peroxidation and antioxidant enzyme activities in two canola (*Brassica napus* L.) cultivars. J Agric 15:593–602

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410. https://doi.org/10.1016/s1360-1385(02)02312-9

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490–498. https://doi.org/10.1016/j.tplants.2004.08.009

Mohsenzadeh Golafazani M, Samizadeh Lahiji H, Hassani Kumleh H (2017) Patterns of mitochondrial gene expression in rapeseed leaves (*Brassica napus* L) at early growth stage in response to drought stress. Iranian J Plant Sci. 48:67–77

Mohsenzadeh Golfazani M, Pasandideh Arjmand M, Hassani Kumleh H, Samizadeh Lahiji H, Vahedi R, Ramezanzadeh Bishegahi S (2019) The effect of iron toxicity on some of morphological traits, relative gene expression of G6PDH and peroxidase enzyme activity in resistant and susceptible genotypes of Rice (*Oryza sativa*). Cereal Res 9:207–220

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Biol 49:249–279. https://doi.org/10.1146/annurev.arplant.49.1.249

Nonomura AM, Benson AA (1992) The path of carbon in photosynthesis: improved crop yields with methanol. Proc Natl Acad Sci U S A 89:9794–9798. https://doi.org/10.1073/pnas.89.20.9794

Pérez-Ruiz JM, Spínola MC, Kirchsteiger K, Moreno J, Sahrawy M, Cejudo FJ (2006) Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage. Plant Cell 18:2356–2368. https://doi.org/10.1105/tpc.106.041541

Ramezanzadeh Bishegahi S, Mohsenzadeh M, Samizadeh H (2021) Effect of methanol foliar application on expression changes of some mitochondrial genes in canola under drought stress. Iranian J Plant Sci. 52:113–128

Ramírez I, Dorta F, Espinoza V, Jiménez E, Mercado A, Peña-Cortés H (2006) Effects of foliar and root applications of methanol on the growth of *Arabidopsis*, tobacco, and tomato plants. J Plant Growth Regul 25:30–44. https://doi.org/10.1007/s00344-005-0027-9

Ratnayaka HH, Molin WT, Sterling TM (2003) Physiological and antioxidant responses of cotton and spurred anoda under interference and mild drought. J Exp Bot 54:2293–2305. https://doi.org/10.1093/jxb/erg251

Raza A, Charagh S, Razzaq A, Javed R, Khan RSA, Hasanuzzaman M (2020) Brassicaceae plants response and tolerance to drought stress: physiological and molecular interventions. In: Hasanuzzaman M (ed) The plant family Brassicaceae: biology and physiological responses to environmental stresses. Singapore, Springer Singapore, pp 229–261

Raza A, Su W, Gao A, Mehmood SS, Hussain MA, Nie W, Lv Y, Zou X, Zhang X (2021) Catalase (CAT) gene family in rapeseed (*Brassica napus* L.): genome-wide analysis, identification, and expression pattern in response to multiple hormones and abiotic stress conditions. Int J Mol Sci. https://doi.org/10.3390/ijms22084281

Reichheld J-P, Meyer E, Khafif M, Bonnard G, Meyer Y (2005) AtNTRB is the major mitochondrial thioredoxin reductase in *Arabidopsis thaliana*. FEBS Lett 579:337–342. https://doi.org/10.1016/j.febslet.2004.11.094

Rezaie R, Mandoulakani BA, Fattahi M (2020) Cold stress changes antioxidant defense system, phenylpropanoid contents and expression of genes involved in their biosynthesis in *Ocimum basilicum* L. Sci Rep 10:1–10. https://doi.org/10.1038/s41598-020-62090-z

Salin ML (1991) Chloroplast and mitochondrial mechanisms for protection against oxygen toxicity. Free Radic Res Commun 13:851–858. https://doi.org/10.3109/10715769109145867

Seo M, Aoki H, Koiwai H, Kamiya Y, Nambara E, Koshiba T (2004) Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant Cell Physiol 45:1694–1703. https://doi.org/10.1093/pcp/pch198

Shafiq S, Akram NA, Ashraf M (2015) Does exogenously-applied trehalose alter oxidative defense system in the edible part of radish (*Raphanus sativus* L.) under water-deficit conditions? Sci Hortic 185:68–75. https://doi.org/10.1016/j.scienta.2015.01.010

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13:2498–2504. https://doi.org/10.1101/gr.1239303

Simpson K, Fuentes P, Quiroz-Iturra LF, Flores-Ortiz C, Contreras R, Handford M, Stange C (2018) Unraveling the induction of phytoene synthase 2 expression by salt stress and abscisic acid in *Daucus carota*. J Exp Bot 69:4113–4126. https://doi.org/10.1093/jxb/ery207

Srivastava S, Srivastava AK, Suprasanna P, D'souza S (2013) Identification and profiling of arsenic stress-induced microRNAs in *Brassica juncea*. J Exp Bot 64:303–315. https://doi.org/10.1093/jxb/ers333

Su W, Raza A, Gao A, Jia Z, Zhang Y, Hussain MA, Mehmood SS, Cheng Y, Lv Y, Zou X (2021) Genome-wide analysis and expression profile of superoxide dismutase (SOD) gene family in rapeseed (*Brassica napus* L) under different hormones and abiotic stress conditions. Antioxidants (basel) 10:1182. https://doi.org/10.3390/antiox10081182

Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork, P.J.N.a.r. (2019) STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47:D607–D613. https://doi.org/10.1093/nar/gky1131

Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, Doncheva NT, Legeay M, Fang T, Bork P (2021) The STRING database in 2021: customizable protein–protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Res 49:D605–D612. https://doi.org/10.1093/nar/gkaa1074

Taghvaei MM, Samizadeh Lahiji H, Bakhtiarizadeh MR, Mohsenzadeh Golafazani M (2019) Bioinformatics analysis of microRNAs related to cold Stress and their effects on proteins associated with fatty acids metabolism in rapeseed (*Brassica napus* L). J Crop Biotech. 9:41

Taghvaei MM, Lahiji HS, Golfazani MM (2022) Evaluation of expression changes, proteins interaction network, and microRNAs targeting catalase and superoxide dismutase genes under cold stress in rapeseed (*Brassica napus* L.). OCL. https://doi.org/10.1051/ocl/2021051

Torres-Franklin ML, Contour-Ansel D, Zuily-Fodil Y, Pham-Thi A-T (2008) Molecular cloning of glutathione reductase cDNAs and analysis of GR gene expression in cowpea and common bean leaves during recovery from moderate drought stress. J Plant Physiol 165:514–521. https://doi.org/10.1016/j.jplph.2007.03.011

Wang T, Chen L, Zhao M, Tian Q, Zhang WH (2011) Identification of drought-responsive microRNAs in *Medicago truncatula* by genome-wide high-throughput sequencing. BMC Genomics 12:367. https://doi.org/10.1186/1471-2164-12-367

Wang K, Liu Y, Teng F, Cen H, Yan J, Lin S, Li D, Zhang W (2021) Heterogeneous expression of Osa-MIR156bc increases abiotic stress resistance and forage quality of alfalfa. Crop J 1:949–963. https://doi.org/10.1016/j.cj.2020.11.009

Wei L, Zhang D, Xiang F, Zhang Z (2009) Differentially expressed miRNAs potentially involved in the regulation of defense mechanism to drought stress in maize seedlings. Int J Plant Sci 170:979–989. https://doi.org/10.1086/605122

Xie F, Wang Q, Sun R, Zhang B (2015) Deep sequencing reveals important roles of microRNAs in response to drought and salinity

stress in cotton. J Exp Bot 66:789–804. https://doi.org/10.1093/jxb/eru437

Yang J, Zhang N, Mi X, Wu L, Ma R, Zhu X, Yao L, Jin X, Si H, Wang D (2014) Identification of miR159s and their target genes and expression analysis under drought stress in potato. Comput Biol Chem 53:204–213. https://doi.org/10.1016/j.compbiolchem.2014.09.009

Yousuf, P.Y., Hakeem, K.U.R., Chandna, R., and Ahmad, P. (2012). Role of glutathione reductase in plant abiotic stress. In Abiotic Stress Responses in Plants (Springer), pp. 149–158

Zbieć I, Karczmarczyk S, Podsiadło C (2003) Response of some cultivated plants to methanol as compared to supplemental irrigation. Electron J Pol Agric Univ 6:1–7

Zheng Y, Hivrale V, Zhang X, Valliyodan B, Lelandais-Brière C, Farmer AD, May GD, Crespi M, Nguyen HT, Sunkar R (2016) Small RNA profiles in soybean primary root tips under water deficit. BMC Syst Biol 10:1–10. https://doi.org/10.1186/s12918-016-0374-0