Evaluation of expression changes, proteins interaction network, and microRNAs targeting catalase and superoxide dismutase genes under cold stress in rapeseed (*Brassica napus* L.)

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Abstract – Rapeseed is the third-largest source of plant oil and one of the essential oil plants worldwide. Cold stress is one of the critical factors that affect plant yield. Therefore, improving cold stress tolerance is necessary for yield increase. The present study investigated *BnCAT1* and *BnCSD1* genes’ expression behavior in a tolerant and sensitive cultivar under cold stress (4°C). Besides, protein-protein interaction networks of CATs and CSDs enzymes, and their association with other antioxidant enzymes were analyzed. Moreover, the microRNAs targeting *BnCAT1* and *BnCSD1* genes were predicted. This study indicated many direct and indirect interactions and the association between the components of the plant antioxidant system. However, not only did the CATs and CSDs enzymes have a relationship with each other, but they also interacted directly with ascorbate peroxidase and glutathione reductase enzymes. Also, 23 and 35 effective microRNAs were predicted for *BnCAT1* and *BnCSD1* genes, respectively. The gene expression results indicated an elevated expression of *BnCAT1* and *BnCSD1* in both tolerant and sensitive cultivars. However, this increase was more noticeable in the tolerant cultivar. Thus, the *BnCSD1* gene had the highest expression in the early hour of cold stress, especially in the 12th h, and the *BnCAT1* gene showed the highest expression in the 48th h. This result may indicate a functional relationship between these enzymes.

Keywords: free radicals / gene expression / microRNA / protein interactions / string

**Résumé** – Évaluation des changements d’expression, du réseau d’interaction des protéines et des microARN ciblant les gènes de la catalase et de la superoxyde dismutase en cas de stress lié au froid chez le colza (*Brassica napus* L.). Le colza représente la troisième source d’huile végétale et un oléoprotagineux essentiel dans le monde. Le stress lié au froid est l’un des facteurs critiques qui affectent le rendement des plantes. Par conséquent, l’amélioration de la tolérance au stress froid s’avère nécessaire pour augmenter le rendement. La présente étude a examiné la variation de l’expression des gènes *BnCAT1* et *BnCSD1* chez un cultivar tolérant et un autre sensible au stress lié au froid (4°C). En outre, les réseaux d’interaction protéine-protéine d’enzymes (catalase CAT et superoxide dismutase CSD), et leur association avec d’autres enzymes antioxydantes ont été analysés. De plus, les microARNs ciblant les gènes *BnCAT1* et *BnCSD1* ont été identifiés. Cette étude a souligné de nombreuses interactions directes et indirectes et l’association entre les composants du système antioxydant des plantes. Non seulement les enzymes CATs et CDSs étaient liées entre elles, mais elles interagissaient également directement avec l’ascorbate peroxidase et la glutathion réductase. De plus, respectivement 23 et 35 microARN étaient associés à une modification de l’expression des gènes *BnCAT1* et *BnCSD1*. Les résultats de l’expression génétique ont indiqué une expression élevée de *BnCAT1* et *BnCSD1* chez les cultivars tolérants et sensibles. Cependant, cette augmentation était plus marquée chez le cultivar tolérant. Ainsi, le gène *BnCSD1* avait l’expression la plus élevée dans les premières heures d’exposition au froid, en particulier à la 12e h, tandis que le gène *BnCAT1* a montré l’expression la plus élevée à la 48e h. Ce résultat peut indiquer une relation fonctionnelle entre ces enzymes.

**Mots clés** : radicaux libres / expression génétique / microARN / interactions protéiques / colza

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1 Introduction

Rapeseed has a significant place among annual oilseeds and is considered the most important annual oil plant in temperate, cold, and humid regions. Rapeseed is the third-largest source of plant oil in the world after soybeans and palm oil (Wanasundara et al., 2017). Plant growth and development are adversely altered by environmental stresses, including abiotic stresses (Raza, 2020). Abiotic stresses (drought, flood, salinity, oxidative, cold, heat, heavy metals) are the leading cause of global crop decline. They are responsible for the reduced yields of more than 50% of primary agricultural products (Raza et al., 2019, 2020a).

Cold stress is one of the main factors limiting the growth and production of crops; therefore, increasing crop cold tolerance is crucial to increase crop yield. Cold causes adverse effects on plant cells exposed to environmental stress. Catalase exists in all cellular compartments of plants, transforming the membrane’s physical state from the liquid crystalline phase to the gel-like solid phase. These changes occur in the cell membrane causing other changes in the cell or plant. The cold temperature causes intracellular ice crystals to form, disrupting the sensitive plant organs (Raza, 2020). Moreover, the cold affects the accumulation of reactive oxygen species (ROS). As the temperature decreases, enzymes and ROS inhibitory systems’ activities will typically decrease. Therefore, the plant can’t cope adequately with ROS production. Investigating influential factors in cell damage has shown that ROS are the principal causes of damage during abiotic stresses, leading to changes in the factors involved in preserving membrane compounds, antifreeze compounds, antioxidants, and many other processes (Hasanuzzaman et al., 2020; He et al., 2021). Excessive production of reactive oxygen species under cold stress can cause severe oxidative damage to plants. Antioxidant defense mechanisms have been developed in plants to reduce ROS’s adverse effects on plant cells (Kalisz et al., 2016; Rezaie et al., 2020). Accumulation of ROS will have detrimental impacts, primarily on membranes, and can lead to ion leakage. Besides, low temperatures cause the formation of secondary structures in RNA, therefore affecting gene expression. Moreover, the freezing temperature will have far more destructive effects (Gusta et al., 2004). Plants have developed their antioxidant defense mechanism to maintain ROS’s balance because an excessive increase in ROS leads to the loss of key intracellular signaling molecules (Xie et al., 2019).

ROS, such as hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), and hydroxyl radicals (HO$_2$), are oxygen (O$_2$) derivatives that are more reactive and are considered general responses to biotic and abiotic stresses. ROS are naturally produced in chloroplasts, mitochondria, plasma membranes, apoplasts, endoplasmic reticulum, peroxisome, and cell wall. Therefore, the plant should maintain its scavenging systems to deal with these destructive factors. Several studies have confirmed the critical importance of the intracellular antioxidant defense system against various stresses (Cavallini et al., 2016; Hasanuzzaman et al., 2020). The antioxidant defense system includes enzymatic and non-enzymatic components for ROS inhibition in various intracellular organs like chloroplasts, peroxisomes, plasma membranes, and endoplasmic reticulum (Sharma et al., 2012). Enzymatic antioxidants contain enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX), Monohydro ascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione S-transferases (GST). Non-enzymatic antioxidants include ascorbic acid, glutathione, carotenoids, tocopherols, proline, glycine, and glutathione (Pandey et al., 2017).

Superoxide dismutase enzymes act as antioxidants and preserve cellular components against ROS (Alscher et al., 2002). When an O$_2$ molecule absorbs an electron from the transfer chain, it is reduced to O$_2^-$. Superoxide is a disruptive factor for enzymes, DNA, and lipid oxidation (Smirnoff, 1993). Catalase is an enzyme that catalyzes the production of O$_2$ and H$_2$O$_2$ from superoxide and consequently reduces its adverse effects. Catalase acts as a network interacting with several processes. Fundamentally, proteins act as a network interacting with each other (Taghvaei et al., 2019). MicroRNAs are one of the most critical post-transcriptional regulatory elements. These 21 to 22 nucleotide sequences regulate the activity of the gene by complementing with their mRNA. In other words, the complete binding with the target region leads to cleavage, and in the case of incomplete binding, it prevents the translation of the target gene (Bartel, 2004).
It is required to examine protein interactions and genes and other factors involved in stress-responsive systems to produce stress-tolerant plants. Rapeseed has a complicated genome as a result of its evolutionary history, making it an attractive genome for plant breeding research (Raza et al., 2020b). Abiotic stresses are the most crucial factors affecting rapeseed’s yield. So, it is essential to investigate cold tolerance mechanisms and the role of cold-tolerant genes in rapeseed. In the plant’s response to cold stress, the antioxidant defense system is of great importance. Valuable and comprehensive studies can be done using bioinformatics studies, protein network analysis, and microRNAs investigation as the most important post-transcriptional gene regulators. Therefore, the present study was conducted to clarify the relationships and interactions in the antioxidant defense system of rapeseed and the altered gene expression behavior under cold stress in sensitive and tolerant cultivars.

2 Materials and methods

2.1 Investigation of protein interactions

In the present study, to investigate the interactions of proteins in the antioxidant defense system of rapeseed, their information and genetic identity were taken using the UniProt database. Afterward, it was used 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 of 0 to 1, the minimum required interaction score was set to the highest (0.7) (Szklarczyk et al., 2019; Taghvaei et al., 2019).

2.2 Phylogenetic tree construction

To investigate and compare the evolutionary relationships of different types of catalase and superoxide dismutase enzymes between Brassica napus and Arabidopsis thaliana, the existing sequences of Catalase 1 (CAT1) and Superoxide dismutase [Cu–Zn] 1 (CSD1) were taken from the NCBI database. ClustalW was used for the alignment of the sequences (Thompson et al., 1994). The results of alignment were used to construct phylogenetic trees by MEGAX software (Kumar et al., 2018) using the Neighbor-joining (NJ) method and Bootstrap analysis with 1000 replications (Gupta et al., 2019).

2.3 Prediction of microRNAs

To identify microRNAs targeting BnCAT1 and BnCSD1 genes, psRNATarget online database 2017 was used (the expectation parameter was set to 4). Hence, all identified plant microRNA sequences (miRBase Release 22.1, October 2018) were used to search for BnCAT1 and BnCSD1 genes. In this study, microRNAs sequences were given to the software to find targeting the genes. This platform is specifically designed to predict microRNAs and target genes in plants (Dai et al., 2018). Besides, Cytoscape software version 3.8 was used to map the relationship between the predicted microRNAs and the studied genes (Shannon et al., 2003).

2.4 Plant materials and treatment conditions

Two cultivars of canola, Sarigol (sensitive) and Zarfam (tolerant) were selected (Safaei et al., 2018). The cultivars’ seeds were obtained from the seed and plant improvement institute (Karaj, Iran). The seeds were sterilized by immersing them in 70% ethanol for 1 minute and in sodium hypochlorite solution (5%) for 1 minute, then washing with deionized sterile water (Gao et al., 2016). The seeds were then placed in a growth chamber at 25°C (Biotechnology Laboratory of the Faculty of Agriculture, University of Gullan, Iran). Afterward, healthy germinated seeds were transferred to pots for hydroponic cultivation. A closed hydroponic system and Hoagland nutrient solution at half concentration were used for plant cultivation (Epstein and Bloom, 2004). Two rapeseed plant cultivars, “Zarfam” (a cold-tolerant cultivar) and “Sarigol” (a cold-sensitive cultivar), were culture-grown under a 16-h light (25°C)/8-h dark (22°C) photoperiod (photo intensity 6000 Lx). Plants were exposed to 4°C for 0, 4, 8, 12, 24, and 48 h to analyze the antioxidant genes of rapeseed under cold stress. In the case of controls, plants were exposed to 25°C for 0, 4, 8, 12, 24, and 48 h. The treated and untreated juvenile leaves were then harvested into liquid nitrogen and stored at −80°C (Megha et al., 2018).

2.5 Extraction of RNA and synthesize of cDNA

RNA extraction was performed with TRIZol reagent (Invitrogen, USA) 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. DNase I.Rnase-free (Sinaclon, Iran) was used to remove genomic DNA contamination. Consequently, the First Strand cDNA Synthesis kit (Sinaclon, Iran) was used to make cDNA. After cDNA synthesis, the tube was transferred to a −80°C freezer.
2.6 Design of specific primers

To design the primers, *BnCAT1*, *BnCSD1*, and *BnActin* (as a reference gene) sequences were obtained from the NCBI database. The primers were designed using Oligo 7 and Primer3 software. Furthermore, the sequence of the primers and their melting temperature were evaluated by Oligo analysis and finally checked for specificity in NCBI Primer-BLAST (Tab. 1).

2.7 qRT-PCR analysis of gene expression

The expression of candidate genes was examined by real-time PCR in Zarfam (tolerant) and Sarigol (sensitive) cultivars. The qRT-PCR was performed using an SYBR Green qPCR 2X (Sinaclon, Iran). 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 qRT-PCR results were normalized as follows: sample CT values were determined and standardized based on the actin gene control prime reaction, and the 2^{-ΔΔCT} method was applied to calculate the relative changes in gene expression from qRT-PCR experiments (Schmittgen and Livak, 2008). Statistical analysis was performed using a split-plot design in time by SAS software, Version [9,4].

3 Results and discussion

3.1 Protein-protein interactions

This study investigates the protein interactions between enzymes involved in the rapeseed’s antioxidant system (especially catalase and superoxide dismutase). Therefore, the STRING platform was used, in which nodes represent proteins and connections representing known or predicted and direct or indirect interactions. The relationship between the nodes was based precisely on the present information extracted from relevant databases and articles. By default, this network specifies all protein interactions with the minimum required interaction score of 0.4. However, a stricter coefficient (0.7) was used in the present study to increase the interactions’ accuracy. Subsequently, the interactions between three CAT and eight SOD enzymes showed 11 nodes and 45 edges (Fig. 2), all located in the network. Results indicated that these two groups of antioxidants were closely related to each other.

As shown in Figure 2, there was a direct interaction between CAT and CSD enzymes. Besides, the relationship between CAT and CSD enzymes and known enzymes of the rapeseed’s antioxidant system was examined, including catalase enzymes, superoxide dismutase enzymes, APX enzymes, MDHAR, DHAR, GST, and glutathione reductase (GR). As a result, 35 nodes and 192 edges were observed (Fig. 3), and all proteins were located in the network. As shown in Figure 3, the CAT1 enzyme interacted directly with the SOD, GR, and APX1 groups. The CSD1 enzyme interacted directly with both the catalase group and the GR and ascorbate peroxidase (APX6, APX2, APX1) enzyme groups. These results indicated the extensive and dependent relationship of enzymes involved in the plant’s antioxidant defense system.

3.2 Evolutionary analysis

Arabidopsis shares a recent common ancestry with many species, including various oil-producing crops, most of which are Brassica species (Parkin et al., 2005). Rapeseed originated from interspecific hybridization between *B. rapa* and *B. oleracea*. After this genome collision, *B. napus* sustained
vast genome restructuring via homoeologous chromosome exchanges, resulting in widespread segmental deletions and duplications (Lee et al., 2020). The evolutionary relationships were investigated using known sequences in the NCBI database (BnSODs, BnCATs, AtSODs, and AtCATs). It was observed that the BnSODs and BnCATs showed a phylogenetic relationship with AtSODs and AtCATs in each group (Fig. 4). However, in recent studies, genome-wide analysis of the Brassica napus genome was performed. As a result, they classified BnCATs into four groups, including 14 BnCATs and BnSODs into three groups, including 31 BnSOD genes (14 BnCSDs, 11 BnFSDs, and six BnMSDs). Besides, they found the BnSODs and BnCATs have a closer phylogenetic relationship with the Brassica oleracea (BolCATs and BolSODs) and Brassica rapa (BraCATs and BraSODs) in each group. Collinearity investigation exhibited strong orthologs of SOD genes and CAT genes among B. napus and three closely related species (B. rapa, B. oleracea, and A. thaliana) (Raza et al., 2021; Su et al., 2021).

Fig. 3. Protein-protein interaction network using the STRING database to investigate the relationships between catalase and superoxide dismutase enzymes with other rapeseed’s antioxidant groups. Colored nodes represent proteins, and colored lines represent their interactions with each other based on the references in the database.

Fig. 4. Constructed phylogenetic trees for two groups of enzymes in Brassica napus (Bn) and Arabidopsis thaliana (At): (a) catalase and (b) superoxide dismutase. Multiple alignment was performed using ClustalW software, and tree construction was performed using MEGA X software with NJ method and Bootstrap test with 1000 replications.
Table 2. Identified microRNAs affecting CAT1 gene using psRNATarget software with the expected value 4.

| miRNA_Acc. | Target_Acc. | Expectation | Target_start | Target_end | Inhibition | Target_Desc. |
|------------|-------------|-------------|--------------|------------|-----------|--------------|
| mtr-miR5228 | XM_013797970.2 | 2.5 | 509 | 529 | UCUGGUGUACAACUUGAUGGA | Translation CAT1 |
| bdi-miR7735-5p | XM_013797970.2 | 3.5 | 1400 | 1422 | UUGUUUUCCUUCUGCACUCCCGGC | Cleavage CAT1 |
| bra-miR5717 | XM_013797970.2 | 3.5 | 1799 | 1819 | GUUUGGAUUGUUUGCCUUGGC | Cleavage CAT1 |
| gma-miR5039 | XM_013797970.2 | 3.5 | 1727 | 1747 | CCCUUUUAAUCGUUGCAUGAA | Cleavage CAT1 |
| ata-miR2118a-3p | XM_013797970.2 | 4 | 687 | 708 | GGGAAUGGGAACAUGGAGGAA | Cleavage CAT1 |
| ata-miR2118b-3p | XM_013797970.2 | 4 | 687 | 708 | GGGAAUGGGAACAUGGAGGAA | Cleavage CAT1 |
| ata-miR393-5p | XM_013797970.2 | 4 | 251 | 271 | UUCCAAAGGGAUCGCAUUGAU | Cleavage CAT1 |
| bdi-miR5179 | XM_013797970.2 | 4 | 1053 | 1073 | UUUUGCUCAAGACCGCGCAAC | Cleavage CAT1 |
| gma-miR393h | XM_013797970.2 | 4 | 250 | 271 | UUCCAAAGGGAUCGCAUUGAU | Cleavage CAT1 |
| gma-miR393j | XM_013797970.2 | 4 | 250 | 271 | UUCCAAAGGGAUCGCAUUGAU | Cleavage CAT1 |
| gma-miR393k | XM_013797970.2 | 4 | 250 | 271 | UUCCAAAGGGAUCGCAUUGAU | Cleavage CAT1 |
| gma-miR5668 | XM_013797970.2 | 4 | 902 | 922 | AGCAAUGGAAUUAUAGACUGC | Translation CAT1 |
| gra-miR7486c | XM_013797970.2 | 4 | 962 | 985 | UUUGUCCACGUGAACAGAAAACGC | Cleavage CAT1 |
| mtr-miR5268a | XM_013797970.2 | 4 | 691 | 714 | CCAGAGUGGAAUGAAGAUAUGGUU | Cleavage CAT1 |
| mtr-miR5268b | XM_013797970.2 | 4 | 691 | 714 | CCAGAGUGGAAUGAAGAUAUGGUU | Cleavage CAT1 |
| osa-miR1860-3p | XM_013797970.2 | 4 | 411 | 432 | AUCUUGGAAGAGAUGGUGGUAU | Cleavage CAT1 |
| osa-miR5179 | XM_013797970.2 | 4 | 1053 | 1073 | UUUUGCUCAAGACCGCGCAAC | Cleavage CAT1 |
| osa-miR5514 | XM_013797970.2 | 4 | 1559 | 1579 | UCCCAGAGCUUUGGCCGUCGC | Cleavage CAT1 |
| smo-miR1091 | XM_013797970.2 | 4 | 225 | 245 | CGGCAGUGAGGGAGGAUUUGC | Cleavage CAT1 |
| stu-miR482e-5p | XM_013797970.2 | 4 | 664 | 684 | AGUGGGUGGUGUGGUAAGAUU | Cleavage CAT1 |
| tae-miR9677b | XM_013797970.2 | 4 | 1365 | 1385 | CAGGGCGGGGAACAGGUGGCC | Cleavage CAT1 |
| zma-miR166j-5p | XM_013797970.2 | 4 | 1551 | 1572 | GGUUUGUUUGUCUGGUUCAAGG | Cleavage CAT1 |
| mRNA_Acc. | Target_Acc. | Expectation | Target_start | Target_end | Target_aligned_fragment | Inhibition | Inhibition_Desc. | Target_inhibition_desc. |
|-----------|-------------|-------------|--------------|-----------|------------------------|------------|------------------|------------------------|
| osa-miR3979-5p XM_013861011.2 | 3 | 637 | 282 | 282 | UCUCUCUCUCUCCCUUGAAGGC | Translation | CSD1 | |
| ath-miR8167a XM_013861011.2 | 3.5 | 282 | 282 | 282 | AGAUGUGGAGAUCGUGGGGAUG | Cleavage | CSD1 | |
| ath-miR8167b XM_013861011.2 | 3.5 | 282 | 282 | 282 | AGAUGUGGAGAUCGUGGGGAUG | Cleavage | CSD1 | |
| ath-miR8167c XM_013861011.2 | 3.5 | 282 | 282 | 282 | AGAUGUGGAGAUCGUGGGGAUG | Cleavage | CSD1 | |
| ath-miR8167d XM_013861011.2 | 3.5 | 282 | 282 | 282 | AGAUGUGGAGAUCGUGGGGAUG | Cleavage | CSD1 | |
| ath-miR8167e XM_013861011.2 | 3.5 | 282 | 282 | 282 | AGAUGUGGAGAUCGUGGGGAUG | Cleavage | CSD1 | |
| stu-miR398b-3p XM_013861011.2 | 3.5 | 129 | 129 | 129 | UUGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| stu-miR8002-3p XM_013861011.2 | 3.5 | 419 | 419 | 419 | AUUCCAUUAUUAUCAAGAAAAAAG | Cleavage | CSD1 | |
| ahy-miR398 XM_013861011.2 | 4 | 129 | 129 | 129 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| aly-miR398a-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| aly-miR398b-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| aqc-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| ath-miR398a-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| ath-miR398b-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| ath-miR398c-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| bdi-miR7777-3p.2 XM_013861011.2 | 4 | 7 | 7 | 7 | UGAGAUGGUGUCUGUUGAAGG | Translation | CSD1 | |
| bol-miR398a-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| bra-miR398-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| cme-miR398b XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| csi-miR398 XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| ghr-miR398 XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| gma-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| gma-miR398b XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| gra-miR398 XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| gra-miR7502d XM_013861011.2 | 4 | 482 | 482 | 482 | CUUUUAACAGUAGAAUUUGAUGGA | Cleavage | CSD1 | |
| hme-miR-14 XM_013861011.2 | 4 | 649 | 649 | 649 | UCAGUCUUUUUCUCUCUCCUAU | Cleavage | CSD1 | |
| mda-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| mtr-miR398a-3p XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| osa-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| ptc-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| rco-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| ssl-miR398 XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| tcc-miR398b XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
| vvi-miR398a XM_013861011.2 | 4 | 128 | 128 | 128 | UGUGUUCUCAGGUCACCCCU | Cleavage | CSD1 | |
3.3 MicroRNAs targeting BnCAT1 and BnCSD1

Regulation of gene expression at the post-transcriptional and post-translational levels carries a significant role in plant response to stress. MicroRNAs are one of the post-transcriptional regulators of gene expression, which perform an essential role in responding to abiotic stresses. MicroRNAs exert their regulatory activity by cleaving target mRNAs or inhibiting translation. In other words, the presence of complementary bases between the target gene and miRNA is a key factor in miRNA's functional mechanism. According to previous studies, complete complementarity of bases between target genes and miRNA leads to cleavage of the target gene. An incomplete pairing of bases prevents translation of the target gene transcript (Bartel, 2004). MicroRNAs are evolutionarily conserved in plant species and usually belong to conserved evolutionary families (Millar and Waterhouse, 2005; Moran et al., 2017). Therefore, microRNAs identified in other plant species can be used for preliminary and bioinformatics studies. psRNATarget was developed to identify plant sRNA targets by (1) analyzing complementary matching between the sRNA sequence and target mRNA sequence using a predefined scoring schema and (2) by evaluating target site accessibility (Dai et al., 2018).

Fig. 5. Relationships between microRNAs affecting BnCAT1 and BnCSD1 genes were mapped using Cytoscape software. The figure on the right represents the BnCAT1 gene, and the left figure represents the BnCSD1 gene. The red color indicates microRNAs that block gene translation, the rhombus shapes represent microRNAs observed in rapeseed under cold stress (Megha et al., 2018), and the green color indicates individually located microRNAs, other groups with the same color represent the microRNA family.

MicroRNAs targeting BnCAT1 and BnCSD1

The results indicated that the BnCSD1 gene was regulated by 35 types of microRNAs in plants. Most of them (24) belonged to the well-known and highly conserved family of miR398, followed by the miR8167 family. Nevertheless, miR398 was identified as a regulator for this gene in rapeseed. Among these microRNAs, only two of them regulated gene activity by inhibition of translation. In the recent study, 30 miRNAs from seven families targeting 13 BnSODs genes were bioinformatically identified in B. napus. Their results showed that the bna-miR159 family targeted BnCSD7, BnCSD14, and bna-miR166 family targeted BnCSD10, and bna-miR172 family targeted BnCSD2, and bna-miR397 family targeted BnCSD10 (Su et al., 2021). In previous studies, miR398 and miR8167 families have been mentioned as the Cu/Zn-SOD’s primary regulators (Gupta et al., 2019). However, miR398 has been identified in several studies as one of the most effective microRNAs in the cold (Sunkar and Zhu, 2004; Liu et al., 2008; Cao et al., 2014; Wang et al., 2014; Sun et al., 2015; Megha et al., 2018). As well, the examination of the BnCAT1 gene revealed that this gene was targeted by 23 regulatory microRNAs. On the contrary, the diversity of microRNAs targeting this gene was more than the BnCSD1 gene. However, the highest abundance of microRNAs targeting BnCAT1 was related to the miR393 family. According to the previous research, this conserved microRNA family’s role in responding to cold stress had been identified (Sunkar and Zhu, 2004; Zhang et al., 2014; Koc et al., 2015; Liu et al., 2017). Also, in the recent study, five members of the bna-miR166 family and one member of the bna-miR393 family targeting three CAT genes (BnCAT4, BnCAT6, and BnCAT8) were bioinformatically identified in B. napus (Raza et al., 2021). Furthermore, in other research, expression change of miR166 in cassava under cold and drought stresses conditions and in Chinese cabbage under heat stress conditions was observed (Li et al., 2017). On the other hand, the role of miR398, miR5717, and miR393 in rapeseed, in response to cold stress, has been proven by miRNA sequencing (Megha et al., 2018).
3.4 Morphological changes in plants at 4°C

The previous study on Zarfam and Sarigol cultivars at 4°C found that freezing temperature stress has specific morphological effects on the Sarigol cultivar. In Sarigol seedlings, dehydration and wilting of plants were observed in the 4–6 h after cold treatment (−4°C) and continued until the end of 24th h. However, in Zarfam seedlings after cold treatment, no significant difference was observed compared to normal growth conditions (Safaei et al., 2018). This phenotypic change could reveal the Zarfam cultivar’s resistance and the Sarigol cultivar’s sensitivity to cold stress.

Observations in this study indicated that chilling temperature (4°C) did not have a phenotypically significant effect on both rapeseed cultivars. Nevertheless, slight dehydration was observed at the beginning of stress in sensitive cultivar (Sarigol). However, this temperature did not have a noticeable effect on the plant phenotypically and did not lead to plant death within 48 h (Fig. 6). However, examining the response at the molecular level can reveal more differences.

3.5 The expression patterns of the BnCSD1 and BnCAT1

Among antioxidant enzymes, SOD is the first line of cellular defense against ROS that primarily scavenges superoxide radicals and converts them to hydrogen peroxide (Kim et al., 2010). The expression patterns of the BnCSD1 gene were examined under cold stress at 4°C for 48 h (Fig. 7). The results showed increased expression of this gene in both cultivars of rapeseed, Zarfam (tolerant), and Sarigol (sensitive). The present study observed that 4 h after exposure to stress, due to the stress and increased production of free radical superoxide, the expression level of the BnCSD1 gene in both cultivars increased. After 8 h, the sensitive plant could not maintain BnCSD1 expression. As a result, a decrease in gene expression was observed, and this amount remained almost steady for 12 h after stress. Conversely, the BnCSD1 expression level remained high in the tolerant cultivar, and 12 h after stress exposure, the highest expression level of this gene was observed. Initial rapid reaction to detoxification of ROS is necessary to maintain the Calvin cycle and transpiration (Yabuta et al., 2002). It seems that by producing high levels of BnCSD1 at the 12th h of stress exposure, the tolerant plant has counteracted quickly with the production of superoxide radicals. Finally, because of the plant’s stress adaptation, the BnCSD1 expression level was reduced and almost stabilized at 24 and 48 h. Nevertheless, at the same time, the amount of BnCSD1 in Sarigol increased. Therefore, it seems that the sensitive plant was still trying to cope with stressful conditions. Since SOD is produced in response to O2− free radicals, it appears that tolerant plants’ response to this free radical was more capable, stable, and rapid. These changes depend on the inhibitory power of the antioxidant system and the presence of superoxide free radicals in the two cultivars. In other words, the amount of free radical production may be different between the two cultivars. SOD activity was significantly increased in rapeseed under cold stress conditions (He et al., 2021). In tomato (Solanum lycopersicum), overexpression of the StSOD1 gene led to tolerance against 4°C stress (Che et al., 2020). Another study on the SOD gene’s expression profile also confirmed its increased expression under cold stress (Hu et al., 2019). A higher accumulation of SiCSD was observed in leaves rapidly exposed to cold (4°C) and drought stress, which may make an urgent mechanism to detoxify ROS and protect against environmental stress. Also, the csl1 and csl2 mutations resulted in resistance and increased H2O2 accumulation in rice (Li et al., 2019).

It has been revealed that expression of CAT and APX genes is induced after treatments with cold, oxidative stresses, and ABA (Du et al., 2008). BnCAT1 is a significant player in the scavenging of H2O2 produced under various environmental

![Fig. 6. Cold treatment (4°C) on two Brassica napus cultivars in a period of 48 h (16 h of light and 8 h of darkness). (a) left side: Zarfam genotype and (b) right side: Sarigol genotype.](image-url)
stresses (Xu et al., 2013). Examining the BnCAT1 gene expression patterns during cold stress of 4°C for 48 h showed the increase of this gene’s expression in both cultivars. Initially, 4 h after exposure to stress, a slight increase in the expression level was observed in both cultivars. Next, a decreasing trend was observed, especially in the tolerant cultivar. This decreasing trend continued up to 12 h after the stress exposure and even approached zero. Probably, because the amount of hydrogen radicals at the beginning of the stress is less than the number of superoxide radicals, BnCAT1 expression occurs to a lesser extent during this time period. In the tolerant plant, 12 h after cold stress, the highest amount of BnCSD1 expression level was observed, and the lowest amount of BnCAT1 expression was seen at the same time. Nevertheless, after 24 h, an increasing trend was observed again and achieved the highest value in 48 h (Fig. 7). However, this upward trend in the sensitive cultivar started earlier than the 12th h of stress treatment, and it increased and stabilized at 24th and 48th h. On the other hand, despite the rise of BnCSD1 expression level, which occurred mostly in the early hours, BnCAT1’s expression level increased in the later hours. This phenomenon can be due to two possible reasons: first, as a result of protein interactions revealed, other enzymes are directly related to catalase, such as APX and GR, and therefore probably at the beginning of the stress, these enzymes perform the activity of removing hydrogen peroxide. For example, an examination of Saccharum spontaneum CAT3, CAT1, and CAT2 genes revealed that they were significantly upregulated under chilling stress. GPX and GR genes were upregulated upon low-temperature stress (Selvarajan et al., 2018). Second, as mentioned, catalase is activated at high concentrations of H$_2$O$_2$; therefore, this substance’s concentration may have increased over time (SOD activity also produces H$_2$O$_2$). As a result, the plant raised the expression level of the catalase gene in the last hour. Considering the substrate for CAT activity is H$_2$O$_2$, a by-product of SOD action, their functions should be coupled to achieve a synergistic effect on stress tolerance (Xu et al., 2014). Published studies also confirmed that cold stress increased catalase and superoxide dismutase activity in B. oleracea (Soengas et al., 2018; Wojciechowska et al., 2013). Another study on sweet potato (Ipomoea batatas) indicated Overexpression of IbCAT2 gave salt and drought tolerance in Escherichia coli and Saccharomyces cerevisiae. The positive response of IbCAT2 to abiotic stresses implied IbCAT2 might carry out a significant function in stress responses (Huang et al., 2020). Abiotic stress disrupts the metabolic balance of plant cells, resulting in a loss of balance of ROS production and scavenging in the cytoplasm, chloroplasts and mitochondria in plants. Therefore, the physiological process of ROS toxic concentration in plants could be reduced by developing a complicated and efficient ROS scavenging and antioxidant defense system (Ritonga and Chen, 2020).

4 Conclusion

This study identified and investigated the antioxidant system’s possible interactions and microRNAs targeting BnCAT1 and BnCSD1 genes using online databases. The effect of cold stress (4°C) in 48 h on these genes’ expression was subsequently examined. The protein interactions revealed extensive and close interactions within the antioxidant system, and it appears that these systems act interdependently in response to stresses. In other words, not only did the CATs and CSDs enzymes have a relationship together, but they also interacted directly with APX and GR groups. MicroRNAs are also critical factors affecting genes’ expression during stress and play a fundamental role in regulating genes’ expression after transcription. This study also showed that BnCAT1 and BnCSD1 genes are targeted by many highly conserved microRNAs in plants. Using the results of this research can be performed a complete and comprehensive analysis in future research by investigating the expression behavior of these microRNAs under cold stress in a tolerant and sensitive cultivar of rapeseed. BnCAT1 and BnCSD1 genes’ expression increased during the stress in both sensitive and tolerant plants, but this increase was more noticeable in a tolerant cultivar. The highest expression of the BnCSD1 was observed in the early hours of stress exposure, especially at 12th h. In other words, the tolerant plant’s defense system seems to act faster and neutralize the superoxide radicals efficiently. However, the product of this reaction is the hydrogen peroxide radical. In contrast, the BnCAT1 responsible gene for maintaining H$_2$O$_2$ radicals had the highest expression level in the 48th h.
Therefore, cooperation BnCAT1 with BnCSD1 enzyme can improve an effective defense system facing free radicals. However, other components of the antioxidant defense system should be studied to understand these mechanisms better, such as APX enzymes and GR. They could be the subject of subsequent research; therefore, the interactive nature of these enzymes and the systems involved in responding to reactive oxygen species and cold stress could be better understood with such experiments.

**Supplementary Material**

**Table S1.** Identified microRNAs affecting CAT1 gene using psRNATarget software with the expected value 4.

**Table S2.** Identified microRNAs affecting CSD1 gene using psRNATarget software with the expected value 4.

The Supplementary Material is available at [http://www.ncbi.nlm.nih.gov/oclc/2021051/olm](http://www.ncbi.nlm.nih.gov/oclc/2021051/olm).

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