Molecular Biology Research Communications 2013;2(3):63-71 MBRC

Original Article Open Access

Isolation of *Brassica napus MYC2* gene and analysis of its expression in response to water deficit stress

Massumeh Aliakbari, Hooman Razi*

Department of Crop Production and Plant Breeding, College of Agriculture, Shiraz University, Shiraz, Iran

A B S T R A C T

Manipulation of stress related transcription factors to improve plant stress tolerance is a major goal of current biotechnology researches. *MYC2* gene encodes a key stress-related transcription factor involved in Jasmonate (JA) and abscisic acid (ABA) signaling pathways in Arabidopsis. *Brassica napus*, as a globally important oilseed crop, is a close relative of Arabidopsis. In the present study, a 960bp cDNA fragment of *B. napus MYC2* (*BnMYC2*) was isolated, cloned and sequenced. The deduced amino acid sequence of the *BnMYC2* cDNA fragment showed high homology with *Arabidopsis thaliana MYC2* and the putative *Brassica oleracea MYC2*, implying the conserved functions among these orthologous genes. The expression analysis by a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) revealed that *BnMYC2* is a drought inducible gene. A different expression profile of *BnMYC2* was observed between drought tolerant and sensitive *B. napus* cultivars. The drought tolerant cultivar showed a higher accumulation of *BnMYC2* transcript in response to water deficit stress during the studied time course. This result indicates that *BnMYC2* may contribute to drought tolerance in *B. napus*.

Key words: *Brassica napus*; *MYC2* transcription factor; Semi-quantitative RT-PCR; Water deficit stress

INTRODUCTION

Among various environmental limiting factors on plant growth and development, drought is the most serious constraint causing major yield loss of crop plants worldwide. Drought triggers a range of physiological and molecular processes in plants including the accumulation of the phytohormone ABA and the differential expression of many functional and regulatory genes [1].

Deciphering the functions of stress-responsive genes helps to make clear stress tolerance mechanisms. With the advancement of high-throughput technologies such as microarray analysis, several hundred stress-induced genes have been detected as potential

* Address for correspondence: Department of Crop Production and Plant Breeding, College of Agriculture, Shiraz University, Shiraz, Iran. Tel: 0711-6138375 Fax: 0711-2286134
E-mail: razi@shirazu.ac.ir

pISSN 2322-181X eISSN 2345-2005

targets for genetic manipulation. Of those, many are regulatory genes such as transcription factors and protein kinases [2]. The identification and genetic transfer of key regulatory genes involved in drought tolerance is now a promising strategy to minimize the adverse effects of drought.

Arabidopsis thaliana has been widely used to discover the genes and gene regulatory networks involved in drought stress response and tolerance. Functional analyses of drought-induced genes have revealed the existence of at least two ABA-independent as well as two ABA-dependent regulatory pathways in Arabidopsis [3, 4]. Each pathway contains transcription factors which regulate the expression of downstream target genes. One of the ABA-dependent pathways is mediated by the basic leucine zipper transcription factor's ABFs [1, 5]. MYB and MYC transcription factors act to modulate the downstream cascade in the other distinct ABA-dependent pathway [1, 5].

The MYC family members are basic helix-loop-helix (bHLH) transcription factors with diverse functions. In Arabidopsis, *AtMYC2* (At1g32640) is an ABA and drought-responsive gene identified as a transcriptional activator in ABA signaling [6]. Under drought stress conditions, *AtMYC2* and *AtMYB2* (a MYB-type drought-responsive transcription factor), cooperatively induce the transcription of *rd22*, the downstream drought-responsive gene, through binding specifically to the MYC and MYB recognition sites within the *rd22* promoter [7]. Transgenic plants overexpressing both *AtMYC2* and *AtMYB2* exhibited an ABA hypersensitive phenotype with improved tolerance to osmotic stress [7]. Moreover, *AtMYC2* transcription factor functions as a key regulator of transcriptional activity of many JA- responsive genes [8, 9, 10, 11]. Genetic analyses have demonstrated that *AtMYB2* mutants show less sensitivity to ABA and JA mediated responses [12]. Other studies have shown the involvement of *AtMYC2* in light and circadian clock signaling [13, 14]. As a result of *AtMYC2* functional versatility, this gene is recently recognized as a master regulator involved in different aspects of JA signaling pathway as well as interactions between JA and other phytohormones in Arabidopsis [15].

Relatively little is known about *MYC2* orthologs in economically important crop species. *MYC2* orthologs of tobacco species control the expression of nicotine biosynthesis genes in roots [16]. Putative *MYC2* orthologs with roles in biotic and abiotic stress tolerance have been reported in maize [17], banana [18] and rice [19, 20]. So far, no orthologs have been detected in rapeseed (*Brassica napus*) which is one of the most important oilseed crops worldwide. The present study aimed to isolate and sequence a cDNA fragment of the *B. napus MYC2* (*BnMYC2*) gene. The expression of *BnMYC2* in response to water deficit stress in two *B. napus* cultivars with different levels of tolerance to drought was also analyzed.

MATERIALS AND METHODS

Plant materials and water deficit treatment: Seeds of two winter-type *B. napus* cultivars, Karun (originated from France) and Zarfam (originated from Iran), were sown in pots with sterilized soil and grown under greenhouse conditions with 16 hours daylight and 25°C day temperature. The results of a previous field experiment had shown that Karun

was a more drought tolerant cultivar than Zarfam [21]. Plants with three true leaves were subjected to water deficit stress imposed by withholding water for four days. The leaf samples were collected separately at different time points including 0 (control), 9, 12, 24 and 36 hours after stress.

Isolation and sequencing of the partial cDNA of *BnMYC2*: Total RNA was extracted from leaf samples of Karun cultivar using a RNX-Plus kit (Cinnagen) according to the manufacturer's instructions. RNA quantity and quality was checked by agarose gel electrophoresis and spectrophotometry (Nanodrop). One microgram of total RNA was then used to synthesize first strand cDNA using a First Strand cDNA Synthesis kit (Fermentas) following the manufacturer's protocol. Since there is much similarity between *B. napus* and *A. thaliana* exon regions, the forward primer, *AtMYC2* F (5´-CTTGGTTTCGATGAC GCAGAGC-3´) and the reverse primer, *AtMYC2* R (5´- GACGCAATCGCTTACATCAA CG-3´) were designed from the exon region of the *A. thaliana MYC2* gene. The PCR reaction mixture (20 µl) contained 1µl of the first strand cDNA, 0.25 mM of each dNTP, 0.4 mM of each primer, 2mM of MgCl2, 1x PCR buffer and 1U of Taq DNA polymerase. PCR reaction was performed as follows: initial denaturation at 95°C for 2.5 min followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, and then a final extension at 72°C for 2.5 min. The PCR product was detected by agarose gel electerophoresis. It was then purified and cloned into pTZ57R/T vector (Fermentas) and finally sequenced by SeqLab (Germany) using standard M13 forward and M13 reverse primers.

Sequence analysis: A sequence homology search was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The amino acid sequence was deduced from the sequenced cDNA of *BnMYC2* using the Expasy Translate tool (http://web.expasy.org/ translate). Multiple amino acid alignment was carried out using Clustal Omega (http:// www.ebi.ac.uk/Tools/msa/clustalo/). Pfam (pfam.sanger.ac.uk/) was used to identify the functional domain within the *BnMYC2* protein.

Semi-quantitative RT-PCR analysis : Semi-quantitative RT-PCR was used to detect the expression levels of *BnMYC2* under water deficit stress. Total RNA was isolated from the leaves of Karun and Zarfam cultivars using a RNX-Plus kit (Cinnagen).The cDNAs were synthesized from equal amounts of DNase-treated RNA samples using a First Strand cDNA Synthesis kit (Fermentas) following the manufacturer's instruction. The cDNAs were amplified for 28 cycles using the forward primer mentioned earlier, *AtMYC2* F and the reverse primer, *BnMYC2*R (5´- CGACGTTGGTGCTGGAGATTTAC-3´) designed from the sequenced fragment of *BnMYC2*. RT-PCR reactions were also performed for the control gene, *B. napus* actin (*BnActin*), using the specific primers BnACTF (5´-ACACTGGTGTCATGGTTGGGA-3´) and BnACTR (5´-AGACGGAGGATAGCGTGA GG-3´). RT-PCR products were analysed by 1% agarose gel electrophoresis. Three independent experiments were conducted. The amplicons were quantified by the Total Lab software, which provides quantitative estimates of the amplicon band intensities by

converting them into corresponding numerical values. The expression levels of *BnMYC2* were normalized relative to the amount of *BnActin* expression.

Results

Isolation and sequence analysis of the *BnMYC2* cDNA: Using the *AtMYC2* specific primers and *B. napus* cDNA as a template, the RT-PCR reaction amplified the expected single band. Cloning and sequencing of the RT-PCR product confirmed the detection of a *B. napus MYC2* ortholog, designated as *BnMYC2*. The *BnMYC2* cDNA fragment consisted of 960bp (Fig.1). This cDNA sequence has been submitted to GenBank under the accession number HF674727. Blast homology search revealed that the putative *Brassica oleracea MYC2* [Bo*MYC2* (EF423803)] and *AtMYC2* (NM-102998) were the most similar genes to *BnMYC2*. *BnMYC2* showed 92% and 82% nucleotide identity to Bo*MYC2* and *AtMYC2* , respectively.

```
ttggtttcgatgacgcagagcttcgcttgcggttctggattggcgggtaaagcgttatcg
 L  V  S  M  T  Q  S  F  A  C  G  S  G  L  A  G  K  A  L  S
acaggtaacgtagtttgggtttatgggtcggatcagttatccggatcgggttgtgagcgg
 T  G  N  V  V  W  V  Y  G  S  D  Q  L  S  G  S  G  C  E  R
gcgaagcaaggaggagtgtttgggatgcaaaccatcgcgtgtatcccttcggcgaacgga
 A  K  Q  G  G  V  F  G  M  Q  T  I  A  C  I  P  S  A  N  G
gttgttgaactcgggtcaacggagcagatcccaccaagttcggatcttatgagcaaggtg
 V  V  E  L  G  S  T  E  Q  I  P  P  S  S  D  L  M  S  K  V
cgagtacttttcaatttcgacgttggtgctggagatttaccgggtcttaactggaacctt
 R  V  L  F  N  F  D  V  G  A  G  D  L  P  G  L  N  W  N  L
gacccgactcaaggcgaaaacgatccgtctatatggattaatgacccgattggagcaccc
 D  P  T  Q  G  E  N  D  P  S  I  W  I  N  D  P  I  G  A  P
gagccgggtaacggagctccgagctctttctccaagctttttgccaagtcgatccagttt
 E  P  G  N  G  A  P  S  S  F  S  K  L  F  A  K  S  I  Q  F
gaaaatggtggtagttcaagcaccatcatcggaaacccgaatccggattcggctccaagc
 E  N  G  G  S  S  S  T  I  I  G  N  P  N  P  D  S  A  P  S
ccggttcattcccagacccagaatccaaaattcagcaacaatttctcccccgaattaaat
 P  V  H  S  Q  T  Q  N  P  K  F  S  N  N  F  S  P  E  L  N
ttctccacgtcgagcaccaccttggtgaaacccagaccccgagagatattgagcttcggc
 F  S  T  S  S  T  T  L  V  K  P  R  P  R  E  I  L  S  F  G
aatgaggataaacggagctccatgaacccggatccgagttccaattcgggtcagactcag
 N  E  D  K  R  S  S  M  N  P  D  P  S  S  N  S  G  Q  T  Q
ttagagaataacacaaagaagttcatagatgacaaggttctatctttcggaaccggcgga
 L  E  N  N  T  K  K  F  I  D  D  K  V  L  S  F  G  T  G  G
ggagaatccgatcactccgacctagaagccttcatcgtgaaagaaatcccggagaaacgt
 G  E  S  D  H  S  D  L  E  A  F  I  V  K  E  I  P  E  K  R
cccaagaaacgcggaagaaaaccggccaacggtagagaagagccgcttaaccacgtcgaa
 P  K  K  R  G  R  K  P  A  N  G  R  E  E  P  L  N  H  V  E
gcggagagacagagacgggagaaactaaaccagcgattctacgcgttacgtgcggttgta
 A  E  R  Q  R  R  E  K  L  N  Q  R  F  Y  A  L  R  A  V  V
ccaaacgtctccaaaatggacaaagcttctttgctcggagacgcaatcgcttacatcaac
 P  N  V  S  K  M  D  K  A  S  L  L  G  D  A  I  A  Y  I  N
```

Figure 1. The partial cDNA sequence and deduced amino acid sequence of *BnMYC2* gene (GenBank accession number: HF674727). The highlighted sequence represents the helix-loop-helix DNA binding domain.

The deduced protein of the *BnMYC2* fragment contained 320 amino acid residues (Fig.1). The amino acid alignment showed high homology among *BnMYC2*, *AtMYC2* and the putative Bo*MYC2* (Fig.2). *BnMYC2* exhibited high levels of identity to *AtMYC2* (84%) and the putative Bo*MYC2* (90%) at the amino acid level. *BnMYC2* protein contained a helix-loop-helix DNA binding domain (from 275th to 320th amino acid residues) which is a common feature of the MYC family proteins.

Figure 2. Multiple alignment of the deduced amino acid sequences of *AtMYC2* (NP-174541), the putative Bo*MYC2* (ABS11038) and *BnMYC2* (CCQ71910). (*), (:) and (.) represent identical, conserved and semi conserved residues, respectively.

Expression analysis of the *BnMYC2* gene in response to water deficit stress:The expression pattern of the *BnMYC2* gene was analyzed in response to water deficit stress by semi-quantitative RT-PCR in a time course study. For this purpose, the gene specific RT-PCR amplified the 281bp *BnMYC2* cDNA fragment. As shown in Fig.3, the expression of *BnMYC2* was detectable in the leaves of both drought-tolerant (Karun) and –sensitive (Zarfam) cultivars under stress-free conditions; however, the basal level of the *BnMYC2* expression was about two times higher in the drought tolerant cultivar. Water deficit stress induced the expression of the *BnMYC2* gene at a significant level in both cultivars, although different expression profiles were observed between the cultivars (Fig. 4).

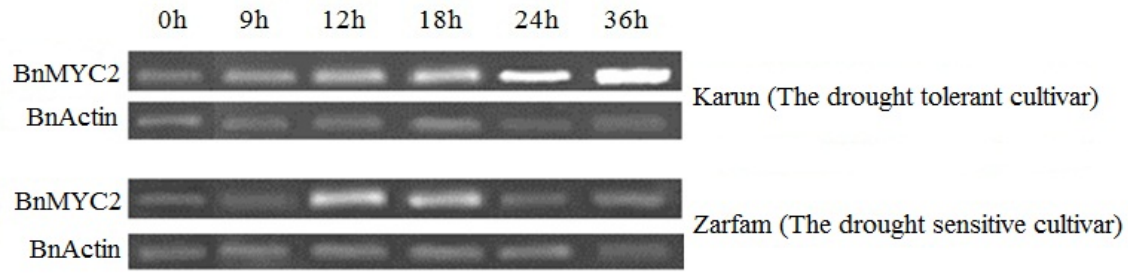

Figure 3. Expression profile of *BnMYC2* gene at the different time points following water deficit stress in the drought tolerant cultivar (Karun) and the drought sensitive cultivar (Zarfam) determined by semi-quantitative RT-PCR. *BnActin* was used as the control gene.

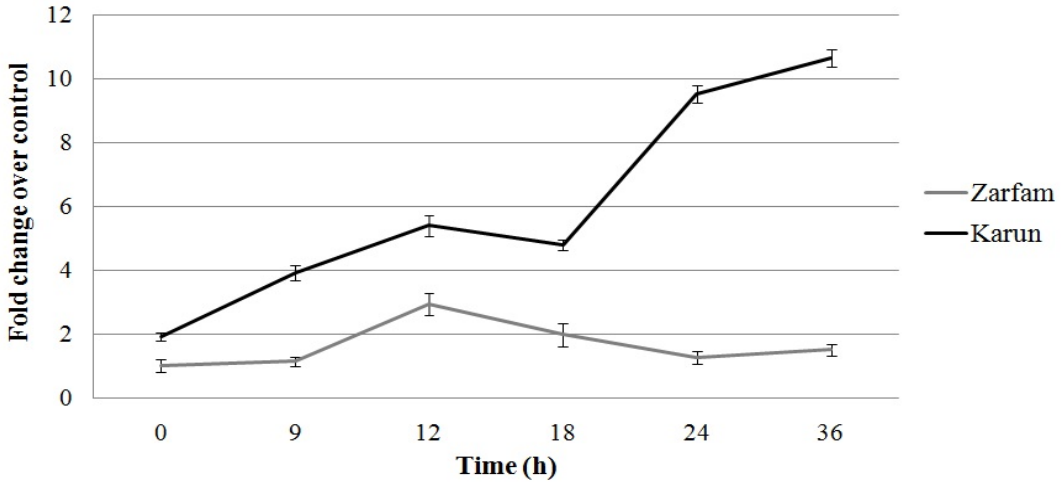

Figure 4. Comparison of the quantified expression profiles of *BnMYC2* in response to water deficit stress between two *B. napus* cultivars, Karun and Zarfam. The expression levels of *BnActin* were used for normalization. The data represent the mean±SE of three replicates.

In the drought tolerant cultivar, *BnMYC2* was significantly upregulated at 9 hours after water deficit treatment and then continued to increase at the 12h treatment. The *BnMYC2*

expression showed a dramatic increase at 24 hours following the water deficit stress, reaching its highest level of expression at the 36h water deficit treatment in which the amount of *BnMYC2* transcript was over five times higher than that of the non-stress conditions.

The drought sensitive cultivar, Zarfam, had a lower level of *BnMYC2* transcript compared to the tolerant cultivar at all time points. The *BnMYC2* expression represented a gradual increase at 9 hours after water deficit stress in the Zarfam cultivar. Maximum expression of *BnMYC2* was observed at the 12h water deficit treatment in which the level of *BnMYC2* transcript was nearly three times higher than that of non-stress conditions. After that, the expression level of *BnMYC2* significantly decreased although it still remained higher than the non stress treatment.

Discussion

B. napus production is adversely influenced by drought in arid and semi-arid regions. Hence, identification and characterization of key regulatory genes involved in drought tolerance are of great importance for *B. napus* genetic improvement. The close phylogenetic relationship between Arabidopsis and Brassica genus makes possible the use of sequences of Arabidopsis genes to identify and isolate orthologous genes in *B. napus*. The Arabidopsis *MYC2* gene, as one of the key components of JA and ABA signaling pathways, plays an important role in abiotic stress tolerance [6, 7]. This is the first report of its kind on the isolation of *B. napus MYC2* and the analysis of its expression in response to water deficit stress.

We obtained a 960bp cDNA fragment of *BnMYC2* encoding 320 amino acids. The large similarity (over 80%) between the amino acid sequences of *BnMYC2* and the other Brassicaceae *MYC2* orthologs may reflect the conserved functions of these genes. Similar to *A. thaliana MYC2* [6] and the rice putative ortholog of *MYC2* [19], *BnMYC2* is a drought-responsive gene. However, it is obvious that many drought responsive genes do not necessarily contribute to drought tolerance; rather, their response reflects drought stress damage. Therefore, we compared the expression pattern of *BnMYC2* in two *B. napus* cultivars with contrasting response to drought to test whether *BnMYC2* gene is associated with drought tolerance. The significant difference observed in the expression of *BnMYC2* between the drought tolerant and sensitive cultivars can be linked to their response to water deficit, as the drought tolerant cultivar exhibited the higher extent of up-regulation in response to water deficit stress during the study. As a result, *BnMYC2* may contribute to drought tolerance in *B. napus*.

This study has provided basic information about the *BnMYC2* gene and its role in drought stress response and tolerance. However, further functional investigations are required to show the conclusive role of *BnMYC2* in drought tolerance.

Acknowledgements

This research was funded by Shiraz University. The support of the Institute of

Biotechnology of Shiraz University is gratefully acknowledged.

Conflict of Interest: Author has no financial or any non-financial competing interests.

REFERENCE

1. Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. J Exp Bot 2007;58:221-227.
2. Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K. Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr Opin Biotech 2006;17:113-122
3. Nakashima K, Yamaguchi-Shinozaki K. Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants Physiol Plantarum 2006;126:62-71.
4. Seki M, Umezawa T, Urano K, Shinozaki K. Regulatory metabolic networks in drought stress responses. Curr Opin Plant Biol 2007;10:296-302.
5. Bartels D, Sunkar R. Drought and salt tolerance in plants. Crit Rev Plant Sci 2005;24:23-58.
6. Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K. Role of Arabidopsis MYC and MYB homologs in drought and abscisic acid-regulated gene expression. Plant Cell 1997;9:1859-1868.
7. Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. Arabidopsis *AtMYC2* (bHLH) and *AtMYB2* (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 2003;15:63-78.
8. Boter M, Ruíz-Rivero O, Abdeen A, Prat S. Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 2004;18: 1577-1591.
9. Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners JM, Kazan K. *MYC2* differentially modulates diverse jasmonate-dependent functions in Arabidopsis. Plant Cell 2007;19:2225-2245.
10. Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R. JASMONATE-INSENSITIVE 1 encodes a MYC transcription factor essential to discriminate between different Jasmonate regulated defense responses in Arabidopsis. Plant Cell 2004;16:1938-1950.
11. Verhage A, Vlaardingerbroek I, Raaijmakers C, Dam NM, Van Dicke M, Van Wees SCM, Pieterse CMJ. Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory. Front Plant Sci 2011;2:1-12.
12. Yadav V, Mallappa C, Gangappa SN, Bhatia S, Chattopadhyay S. A basic helix-loop-helix transcription factor in Arabidopsis, *MYC2*, acts as a repressor of blue light mediated photomorphogenic growth. Plant Cell 2005;17:1953-1966.
13. Gangappa SN, Prasad VBR, Chattopadhyay S. Functional interconnection of *MYC2* and SPA1 in the photomorphogenic seedling development of Arabidopsis. Plant Physiol 2010;154:1210-1219.

14. Shin J, Heidrich K, Sanchez-Villarreal A, Parker JE, Davis SJ. Time for coffee represses *MYC2* protein accumulation to provide time-of-day regulation of jasmonate signaling. Plant Cell 2012;24:2470-2482.
15. Kazan K, Manners JM. *MYC2*: the master in action. Mol Plant 2012;6:686-703.
16. Todd AT, Liu E, Polvi SL, Pammett RT, Page JE. A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in *Nicotiana benthamiana*. Plant J 2010;62:589-600.
17. Engelberth J, Contreras CF, Viswanathan S. Transcriptional analysis of distant signaling induced by insect elicitors and mechanical wounding in *Zea mays*. PLoS One 2012;7:e34855
18. Zhao ML, Wang JN, Shan W, Fan JG, Kuang JF, Wu KQ, Li XP, Chen WX, He FY, Chen JY, Lu WJ. Induction of jasmonate signaling regulators Ma*MYC2*s and their physical Interactions with MaICE 1 in methyl jasmonate-induced chilling tolerance in banana fruit. Plant Cell Environ 2013;36:30-51.
19. Seo JS, Joo J, Kim MJ, Kim YK, Nahm BH, Song SI, Cheong JJ, Lee JS, Kim JK, Choi YD. OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. Plant J 2011;65: 907-921.
20. Miyamoto K, Shimizu T, Mochizuki S, Nishizawa Y, Minami E, Nojiri H, Yamane H, Okada K. Stress-induced expression of the transcription factor ReRJ1 is tightly regulated in response to jasmonic acid accumulation in rice. Protoplasma 2013;250: 241-249.
21. Aliakbari, M. Isolation and characterization of *MYC2* transcription factor gene in a drought tolerant rapeseed cultivar. MSc Thesis 2010; Shiraz University.