

## Molecular cloning and expression analysis of a stress-responsive *WRKY* transcription factor gene, *BnWRKY57*, from *Brassica napus*

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### Abstract

WRKY transcription factors play important roles in regulation of various plant biological processes, including response to abiotic stresses. *WRKY* genes might be potential targets for transgenic breeding to enhance stress tolerance in rapeseed (*Brassica napus*). The present study aimed to clone and characterize *WRKY57* (*BnWRKY57*) gene derived from *B. napus* and to analyze patterns of *BnWRKY57* expression under drought and salt stresses in two *B. napus* cultivars with different levels of tolerance to drought and salt. The full-length coding region of *BnWRKY57* gene with 882bp long (GenBank Accession Number: MG699908) was cloned and sequenced. *BnWRKY57* gene encodes a hydrophilic polypeptide of 293 amino acids. It shared high homology with other known WRKY57s from Brassicaceae family. The promoter of *BnWRKY57* gene contained cis regulatory elements involved in response to phytohormones, light, biotic and abiotic stresses suggesting this gene may play a role to modulate different signaling pathways. Real time quantitative RT-PCR analysis revealed that *BnWRKY57* expression was responsive to drought and salt stresses. *BnWRKY57* gene showed different expression patterns between leaves and roots and also between the *B. napus* cultivars under stress conditions. Overall, the findings suggest that *BnWRKY57* gene may contribute to adaptive responses to drought and salt stresses in *B. napus*.

**Keywords:** Rapeseed; WRKY57; Drought; Salt stress; Promoter analysis.

**Abbreviations:** ABRE\_Abscisic acid Responsive Element; AP2/ERF\_APETALA2/Ethylene Response Factor; bZIP\_Basic Leucine Zipper; ChIP\_Chromatin immunoprecipitation; CRE\_Cis Regulatory Element; HSE\_Heat-Shock Element; MYB\_Myeloblastosis; MBS\_MYB binding site.

### Introduction

Drought and high salinity are critical environmental constraints that greatly limit plant growth and productivity. Plants utilize and coordinate a variety of molecular and cellular mechanisms to respond and cope with these harsh stresses. Transcriptional regulation is a fundamental process to control expression of genes involved in plant responses to environmental stresses. Identification and characterization of stress-responsive regulatory genes have revealed potential target genes to enhance plant stress tolerance through genetic engineering (Yang et al., 2010). Transcription factors are key components of cell regulatory machinery, as they act to modulate transcription of numerous downstream genes involved in diverse plant processes. Various families of transcription factors such as AP2/ERF, bZIP, MYB and WRKY significantly contribute to plant stress response and tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; Ren et al., 2010).

WRKY transcription factors are a large family of plant transcriptional regulators containing one or two WRKY domain with approximately 60 amino acids long (Eulgem et al., 2000).

WRKY domain is characterized by a highly conserved amino acid motif, WRKYGQK, at N-terminus together with a zinc-finger like motif at C terminus (Rushton et al., 2010). Arabidopsis WRKY proteins are classified into three groups based on number of WRKY motifs and features of Zinc-finger like motif (Eulgem et al., 2000). WRKY domain generally binds to W-box elements containing TTGAC(C/T) motif, present in promoter regions of target genes (Fan et al., 2015; Rinerson et al., 2015).

Plant WRKY transcription factors are known to function in various physiological processes, such as trichome development (Johnson et al., 2002; Huang et al., 2015), embryogenesis (Lagacé and Matton, 2004; Huang et al., 2015), seed development (Luo et al., 2005; Huang et al., 2015), dormancy (Pnueli et al., 2002; Huang et al., 2015), and senescence (Hinderhofer et al., 2001; Huang et al., 2015). Furthermore, previous studies have demonstrated essential roles of WRKYs in plant responses to internal and external stimuli. WRKY proteins are involved in plant defence responses against

bacterial, fungal, and viral pathogens (Eulgem and Somssich, 2007; Pandey et al., 2009; Huang et al., 2015; Jiang and Yu, 2016). They are also key components in a number of signal transduction processes mediated by plant hormones such as gibberellic acid (Zhang et al., 2004; Huang et al., 2015), abscisic acid (ABA) (Rushton et al., 2012; Huang et al., 2015), and salicylic acid (Du and Chen, 2000; Huang et al., 2015). For instance, Arabidopsis WRKY57 acts as a regulatory node in Jasmonic acid (JA)-induced senescence signalling pathways mediated by auxin and JA (Jiang et al., 2014).

It is also well documented that WRKY proteins are involved in response to abiotic stresses such as salinity, drought, and cold (Zhou et al., 2008; Ren et al., 2010; Huang et al., 2015). Several Arabidopsis WRKY genes are induced by environmental stresses (Seki et al., 2007; Ma et al., 2014; He et al., 2016). Previous findings indicated that *AtWRKY46* modulates osmotic stress responses and stomatal movement in Arabidopsis (Ding et al., 2014; He et al., 2016). In addition, Arabidopsis *WRKY63/ABO3* regulates responses to ABA and drought tolerance (Ren et al., 2010). Overexpression studies further supported involvement of WRKYs in conferring tolerance to abiotic stresses. Overexpression of the stress-induced *OsWRKY45* improved drought tolerance in Arabidopsis (Qiu and Yu, 2009). Transgenic Arabidopsis plants Overexpressing *TaWRKY2* or *TaWRKY19* from wheat showed enhanced tolerance to drought and salt stresses (Niu et al., 2012). Moreover, overexpression of Chrysanthemum genes *DgWRKY1* and *DgWRKY3* increased tolerance to salt stress in tobacco (Liu et al., 2013; Liu et al., 2014).

Previous reports have demonstrated the role of Arabidopsis *WRKY57* in abiotic stress tolerance. Activated expression of *WRKY57* enhanced drought tolerance of Arabidopsis by increasing ABA levels and by positively regulating the expression of stress-responsive genes (Jiang et al., 2012). ChIP assays illustrated that Arabidopsis *WRKY57* could directly bind the W-box within the promoter sequences of the stress-responsive genes, *RD29A* and *NCED3* (Jiang et al., 2012). Transgenic rice (*Oryza sativa*) plants harbouring Arabidopsis *WRKY57* exhibited improved tolerance to drought and salt stresses, indicating potential role of *WRKY57* in crop improvement (Jiang et al., 2016).

Rapeseed (*Brassica napus*) is one of the world's leading oilseed crops. Rapeseed production is substantially affected by adverse environmental factors (Diepenbrock, 2000; Raymer, 2002; Xie et al., 2007). Development of rapeseed cultivars with enhanced tolerance to environmental stresses is, therefore, of great importance in rapeseed breeding programs. Knowledge of molecular regulatory mechanisms involved in abiotic stress responses may potentially be used for molecular breeding toward improved stress tolerance in rapeseed. *WRKY* genes might be potential targets for molecular breeding of Brassica crops against abiotic and biotic stresses. Despite the importance of WRKYs in response to abiotic stresses, there are few reports describing the roles of *B. napus* WRKY transcription factors in mediating responses to stresses. 13 *BnWRKY* genes have been identified that are responsive to both fungal pathogens and hormone treatments (Yang et al., 2009). Furthermore, the expression of 26 *BnWRKY* genes under multiple stresses revealed that most of the genes were

induced by drought, salinity and low temperature (He et al., 2016).

To date, a comprehensive view of *B. napus* *WRKY57* (*BnWRKY57*) is still lacking. The present study aimed to perform structural and functional characterization of *BnWRKY57* gene. The coding sequence of *BnWRKY57* was isolated, cloned and sequenced for the first time. Afterwards, the expression of *BnWRKY57* gene in response to drought and salt stresses was analyzed in two *B. napus* cultivars with different levels of tolerance to drought and salt. Finally, the promoter region of *BnWRKY57* was analyzed to identify putative cis-regulatory elements that may be involved in regulation of *BnWRKY57* transcription.

## Results

### Molecular cloning and characterization of *BnWRKY57* gene

The full-length coding sequence of *BnWRKY57* gene was amplified using the specific primers. A single PCR product of expected size (around 900bp) was obtained and then cloned followed by sequencing. *BnWRKY57* coding sequence (GenBank Accession Number: MG699908) was 882bp in length encoding a deduced protein of 293 amino acid residues with a predicted molecular weight of 32.552 kDa and isoelectric point of 6.26. The *BnWRKY57* deduced protein contained a single WRKY DNA binding domain (from 151<sup>st</sup> to 208<sup>th</sup> amino acid residue) indicating that *BnWRKY57* protein is a member of WRKY transcription factor family (Fig. 1A). SOPMA analysis showed that the overall folding of *BnWRKY57* protein is basically built with alpha helix (25.26%) and random coils (60.07%) that are connected with extended strands (10.92%) and  $\beta$ -turns (3.75%) (Fig. 1B). Hydropathicity analysis revealed that *BnWRKY57* contained many hydrophilic amino acids suggesting that it is a hydrophilic protein (Fig. 2). According to a web bioinformatic tool, Distill, *BnWRKY57* protein was localized in nucleus, indicating it plays regulatory roles mainly in cell nucleus.

*BnWRKY57* shared highest homology (94%) with *Brassica rapa* *WRKY57* at the nucleotide level. In addition, *BnWRKY57* deduced protein showed high amino acid sequence identity with WRKY57 proteins from other Brassica species including *Brassica rapa* (91.9%), *Brassica campestris* (91.3%) and *Brassica oleracea* (89.6%).

The genomic structure of *BnWRKY57* gene, similar to its Arabidopsis counterpart, contained three exons with sizes of 368, 141, and 373 nucleotides, respectively (Fig. 3). The structural analysis revealed that *BnWRKY57* gene is located in the chromosome 7 of *B. napus* A genome originated from its progenitor, *B. rapa*. Syteny analysis showed that *B. rapa* and *B. oleracea*, the putative donors of *B. napus* A and C genomes, have orthologs of *BnWRKY57* located in the chromosomes 7 and 6 of their genomes, respectively.

A phylogenetic tree was reconstructed based on the amino acid sequences deduced from *WRKY57* genes from various plant species (Fig. 4). The results showed that *WRKY57* genes from species of Brassicaceae family were grouped together supported by a high bootstrap value. Indeed, *WRKY57* genes of

(A) WRKY57 amino acid sequence

```
MTDPEDPDLNEDSAWRQLTAPDSVFFNRDTSSILSDFGWDLHASSSDNHHNLRFDPCLP  
PTSTVPSSVTTTATPDPIPASSHSSTVAAATASVVSTSNPNSATSSSSEGPTDNSTPKTP  
ETPKKEKKQAQKRIRQPRFAFMTKSDVDNLEDGYRWRKYGQKAVKNSSFPRSYYRCTNSR  
CTVKKRVERSSDPSIVITTYEGQHCHQTVGFPRGGIFPAHDPHNFTSHHLLPPLPNPY  
YYQELLHQLHREITSSPQLPQSTTDDGHFAVPSINQPEEGLLGDIVPQTMRNP
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(B)

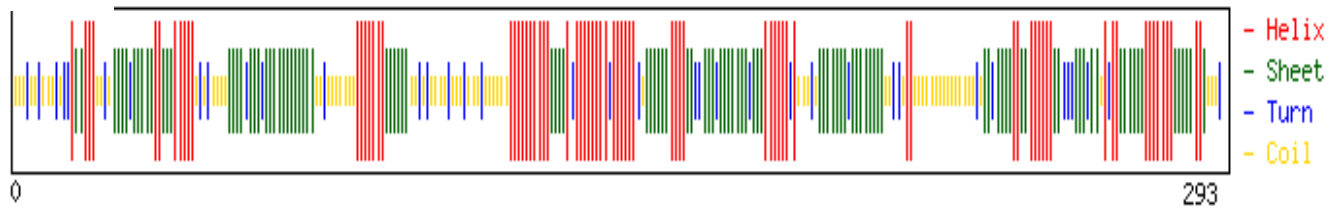


Fig 1. (A) The deduced protein of *BnWRKY57* gene containing 293 amino acids. The highlighted amino acid sequence (from 151<sup>st</sup> to 208<sup>th</sup> amino acid residue) represents a WRKY DNA binding domain. (B) The secondary structure of *BnWRKY57* protein. Alpha helix, extended strand, beta turn and random coil are shown in red, green, blue and yellow vertical lines, respectively.

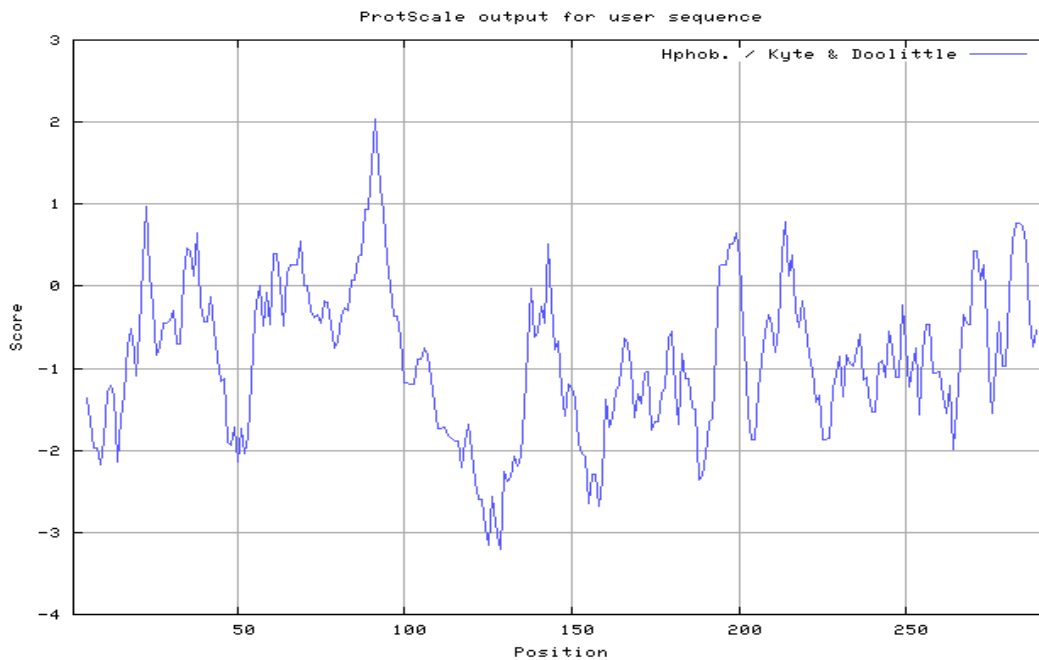


Fig 2. Hydropathicity plot for *BnWRKY57* protein according to Kyte and Doolittle method. Amino acid positions are presented on the X-axis. The hydropathicity scores for individual amino acids are on the Y-axis.



Fig 3. Schematic diagram of the genomic structure of *BnWRKY57* gene. Exons and introns are shown in blue and red boxes, respectively.

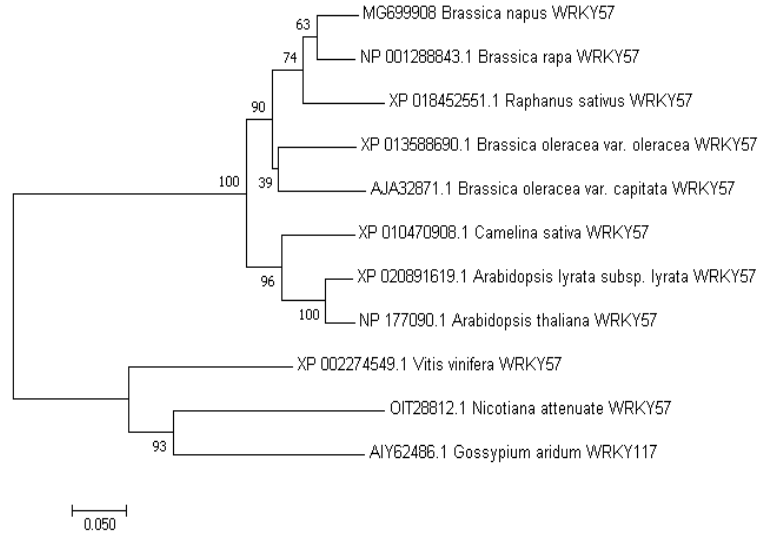


Fig 4. Neighbour-joining phylogenetic tree based on aligned amino acid sequences deduced from WRKY57 genes from Brassicaceae family and other plant species. GenBank accession numbers of individual genes are shown in the phylogenetic tree. Values at the nodes indicate bootstrap support (%) out of 1000 replicates.

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-1500
+ CCCTAAATAAAGATAAAAATCTAAAGTAATTATAACACTCAGACAAGAAAAAAGATTAGCAAAATTT
- GGGATTTATTTCTATTTTAGATTTTCATTAATATGTGAGTCTGTTCTTTTTTCTAATCGTTTATAA

TC-rich repeats
+ AAGTATCGGAATTTGGCTCCAGTTTTTGTTCCTTAAGTGCACATACATGCAATAACTTTAATAAAGTC
- TTCATAGCCTTAAACGAGGTGC AAAAACAAGGAATTCACGTGATGTACGTTATGAAATTAATTCAG
+ AGCAGATATATTAAAGTTAAGTTACATCAAAATTTATGCTATACATTTATACCTTAAGAAATCTGCAATCCG
- TCGCTATATATTTCAATTCATGTAGTTTTTAAAGCGATGTAAATGGAATCTTTAGACGTTAGGC
+ TCCGGTGTAGAGACCCATGATCAACAATGAATAATATGTAGAGATACTGCTTACAACCCATTTGTGCC
- AGCCCACTACTCTGTGGTACTAGTTGTTACTTATATACATCTCTATAGACGAATGTGGGTAACACCGG
+ ATATAATTTTCCGGCTGCAATATCTTCTGGAATGAGTTAGCACCTCCTTAGCTATGTCACCAAGATTA
- TATATTAAGGCGCAGCGTTAAGAGACCTTACTGATCTGGAGAGATCGATAGACGTTGTTAAT
+ TGATGTTTTGCTATAAGGCGACCTGCTTGGAGAAATATCATACAAGAGACATGCATATATGTCATTTT
- ACTACCAACGATATTTCCGGTGGACCACTCTTATATAGTATGTTCTTCTGACGTATATACAGTAAAA

TC-rich repeats
+ ATCTTGAANAATAGCATATATAAGCACATCCCTGTATACGTAATATATGAGTTTCTGCTGATGC
- TAGAACTTTTATATCGTATATATCTGTTAGGGACATAGACTTATATAACCAAGACGACTACG
+ GAATGGTGTATAATTTGTCATGTATTAATATAGCTTCTTCTTATAGCATATGTTCCAAAGTCAATATT
- CTTACCACATATAAACAGTACACTAATTAATATCGAAAGAAAGAAATCGTATACAAGGTTGAGTTATAA

TC-rich repeats
+ GTACGTAAGATGCTCATATATTTCTTCTTATTCACTTTCTCAACCTTTTTTCTTCTGCTGCTTTAG
- CATGCATTTACAGTATATAAAGAAAGAAATAGTCAAAGAGTGGGAAAAAGAAAGACGACGAAATTC

GARE - motif
+ CTAAACAGAAAGCAATTTTCTCCTGTACCTAACATATCACCCCTCAAACTTATACAACATATATATGC
- GATTTTGTCTTTCTGTAATAAGGACACTGGATGTATAGTGGGAGTTTTGAATAGTTGTATATATACG
+ CGCATGCATATGCATCTCATGTACACGTACATATAACAGATGTATGCACAGGACACATAGTATGTCAG
- GCGTACGTATACGTAGAGTACATGAGCACTATATATATGTTCAACTAGTGGTCTCTGTTATCATGACT

ABRE
+ CATGCAGTTGAGTTAAATTAATACAGAAAATTAAGTTTTGATATAAATGTAAGGGGAGATCCAG
- GTACGTCAACTCAATTTAATTTATGTGCTTTTAAATTCACACATATATTTACATTTTCCCTCTAGTGC

MBS
+ CATCAGAGTGACACTGAGTGGAGTGTGAGATTCGTTAATTAATTTGTTGACATTTCTATAATCTTGC
- GTAGTCTCACTGTGACTCACTCATCAACTCTAAGCAATAATATAAACACTGTAAGATATATAGAACG
+ CGACCCATTTCCGGCTCATCTTACCTCACATAAGTAGCCATATATATGCTTATAGCAAGAGGAGTAG
- GCTGGTTAAGCCGAGTAGAGATGAGATGATATCTGGAATATATAGCGAATATPCTGTTCTCTATTC
+ ACAGAGGCACACTATACACAGTGTGATCCTAATGCAATCACTAGTTAATAATAATAATATATAGA
- TGCTTCCGTGATGATATGTGGTACGCACTAGGATACGTTAGTATCAATTAATATATATATATCT
+ TATAGATAGCCAGAGGCAATCATCCACTATTCGTGTATGCTTATCCAGCTTTTATTATGTGCTCAG
- ATATCTATCTCCGCTCCGCTAGTAGTGTATAGGACATAGCTAATAGGTCGAAATATATATACAGAGTC
+ TAAAAATGAATAATGAAAAGCACTAGCCGACATATCGATCAAGTATCAAAATGTTGTTGATCACGG
- ATTTTACTTATTAACCTTTTCTGATCGGCTGTATAGCTAGTCTACATAGATTTAACAACAACATAGTGC
+ GATAGATATATGACTGATTAATCAATGATACACTATACACTATGATTTCTACATATATACATCAGG
- CTATCTATATACTCAGGAGGATAGTACTAATGTGAATGTGATAGCTAAGAGGATATATAGTATGTC

MBS TC-rich repeats
+ TAGACTTGCTTCTGCAAGCATGGAAATTTCTCCCACTTTAAGATATCTCTGATTTGGTTTAAATTT
- ATCTGAACGAAGACAGTTCTGTAATTTAAAGAGGAGTAAATTTCTATAGAGAATTAACCAAAATTTAA

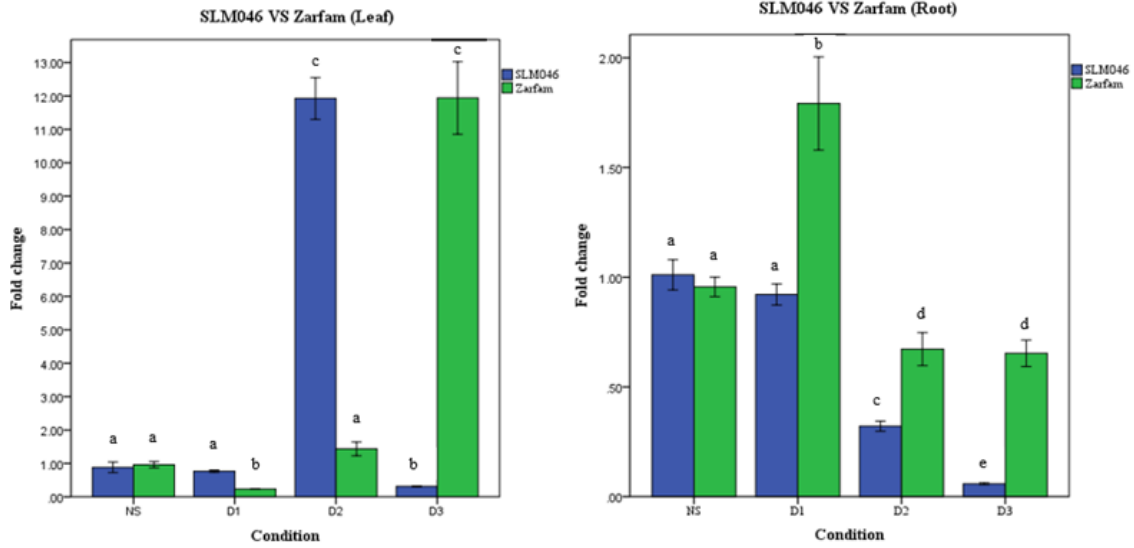
HSE AuxRR-core
+ TCAGACTAGTAAGAAAGTAAACCTTCATGCATCTGTGCTGATTTAAAGTAATAAACCTTGTCAACCAA
- AGTCTGATCATTTCTTCAATGGGAAGTACGTAGACACCAGTAAATTTCAATTTGGGAACGATTGGTT

TCA-element ABRE
+ AAAATATAATTTTCCATCTTTTTATAGAATTCACCTGACCGGTATATATAATATAGACAAGCACT
- TTTTATATAAAGATAGAAAATATCTTAAGTGAAGCTGCTGATATATATATATCTGTCTCGTGA

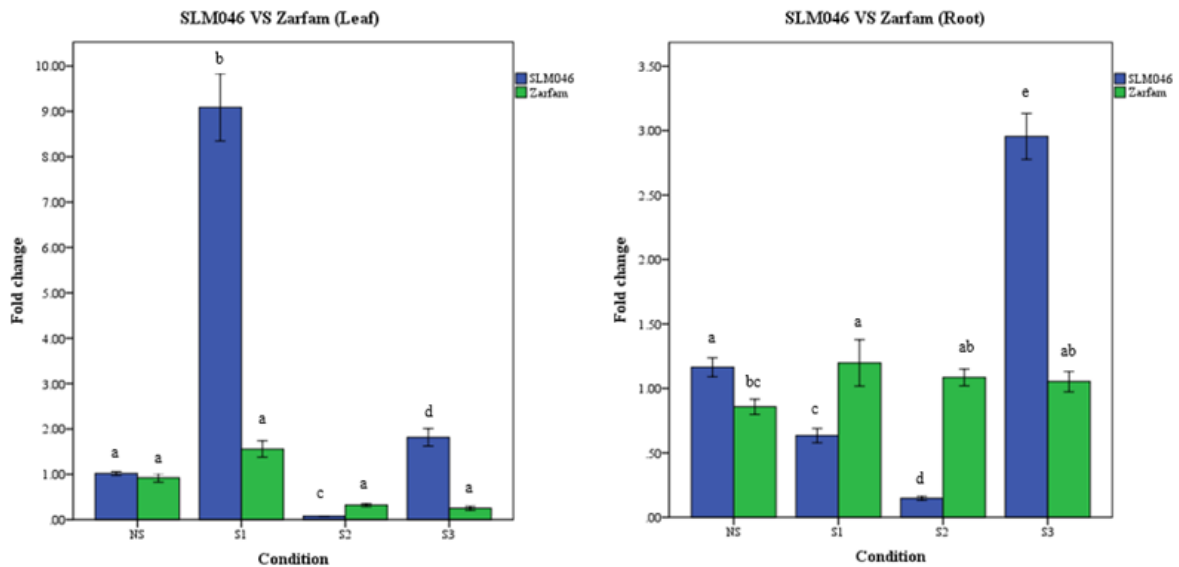
MBS
+ AACAAATAAATAATAGGAGCTTTACC +1
- TTGTTAATTTTATATATCTCGAAATGG ATGACCGATCC

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Fig 5. 1500 bp upstream of the start codon of *BnWRKY57* gene. The putative CREs involved in response to phytohormones and abiotic stresses are shown in the promoter region.



**Fig 6.** Expression of *BnWRKY57* gene in *B. napus* cultivars (SLM046 and Zarfam) under drought stress. A: *BnWRKY57* expression in leaves. B: *BnWRKY57* expression in roots. The expression data were determined by real time quantitative RT-PCR. The drought stress treatments include the time that soil moisture reached to 40% of field capacity (D1) as well as maintaining that for 6 h (D2) and 24 h (D3). NS denotes non-stress condition. Different letters indicate significant differences according to LSD test ( $P < 0.05$ ).



**Fig 7.** The expression of *BnWRKY57* gene in *B. napus* cultivars (SLM046 and Zarfam) under salt stress. A: *BnWRKY57* expression in leaves. B: *BnWRKY57* expression in roots. The expression data were determined by real time quantitative RT-PCR. The salt stress treatments include 3 h (S1), 24 h (S2) and 7 days (S3) after adding salt solution. NS denotes non-stress condition. Different letters indicate significant differences according to LSD test ( $P < 0.05$ ).

**Table 1.** Cis regulatory elements (CREs) identified in the promoter region of *BnWRKY57* gene.

CRE	Function	Number of occurrences
3-AF1 binding site	Light responsive element	1
A-box	Regulatory element	1
AAGAA-motif	Unknown	2
ABRE	Abscisic acid responsive element	2
ACE	Light responsive element	1
ARE	Anaerobic inducible element	2
AuxRR-core	Auxin responsive element	1
Box 4	Light responsive element	2
Box III	Protein binding site	1
CAAT-box	Cis-acting elements /enhancer	25
CCGTCC-box	Meristem specific activation element	1
G-box	Light responsive element	3
GAG-motif	Light responsive element	1
GARE – motif	Gibberellin responsive element	1
GCN4-motif	endogenous expression element	1
HSE	Heat stress responsive element	1
I-box	Light responsive element	1
MBS	Drought inducible element	3
Skn-1-motif	Endosperm expression element	3
TATA-box	Core promoter element	32
TCA-element	Salicylic acid responsive element	1
TCT- motif	Light responsive element	3
As-2-box	shoot-specific expression and Light responsive element	1
Chs-CMA2a	Light responsive element	1

**Table 2.** List of primers used for isolation and expression analysis of *BnWRKY57* and *Bnelf1α* genes.

Gene	Primer	Amplicon length on cDNA(bp)
<i>BnWRKY57</i>	BWRKY-F 5'-ATGACCGATCCTGAAGATCCC-3'	882
	BWRKY-R 5'-GGAGCTTTACCTCAAGGATTGC-3'	
<i>BnWRKY57</i>	BWRT-F 5'-GCTACTGCTTCTGTTGTCTCG-3'	121
	BWRT-R 5'-CCTGCTTCTCTCTTCTTTGG-3'	
<i>Bnelf1α</i>	EIF-F 5'-AGCCGCAAGTCTCTCTCAG-3'	186
	EIF-R 5'-TTCATCTCAGCAGCCTCTCTCG-3'	

Brassica species formed a monophyletic group suggesting they have been probably originated from a common ancestral gene. Moreover, *WRKY57* genes of Brassica species fell into a subgroup separated from their Arabidopsis orthologs. The phylogenetic reconstruction also uncovered that *BnWRKY57* was most closely related to *B. rapa WRKY57*.

#### Analysis of *BnWRKY57* promoter

The 1.5kb upstream promoter region of *BnWRKY57* gene was analyzed using PlantCARE database to identify putative CREs. A total of 95 CREs from 25 different types were identified within the *BnWRKY57* promoter sequence (Table 1). The positions of some CREs are shown in Fig. 5. *BnWRKY57* promoter contained several hormone-responsive CREs associated with activity and regulation of abscisic acid, gibberellin, auxin and salicylic acid implying that *BnWRKY57* may be involved in hormone crosstalk. Moreover, a number of putative CREs involved in tissue-specificity, defence, light response and anaerobic induction were found in the *BnWRKY57* promoter. The CREs related to responses to heat and drought stresses (HSE and MBS motifs) were also detected in the *BnWRKY57* promoter, indicating that the gene is involved in plant responses to environmental stresses.

#### Expression pattern of *BnWRKY57* under drought stress

The expression pattern of *BnWRKY57* was examined in response to drought stress using real time quantitative RT-PCR (qRT-PCR) in leaves and roots of the *B. napus* cultivars. The different expression profiles were observed for the gene not only between the cultivars but also between leaves and roots during drought stress period. In leaves of the drought tolerant cultivar (SLM046), *BnWRKY57* expression was more quickly induced in response to drought stress and showed the maximum level of transcript accumulation at 6 hours after maintaining soil moisture at 40% of field capacity (D2) (Fig. 6A). Moreover, *BnWRKY57* expression exhibited a significantly higher level of induction in leaves of the drought tolerant cultivar, SLM046, compared to that of the other *B. napus* cultivar (Zarfam). *BnWRKY57* expression was significantly downregulated in roots of SLM046 during drought stress period, whilst it significantly increased in roots of Zarfam at the first drought stress treatment (D1) (Fig. 6B) and then showed a significant downregulation at 6 (D2) and 24 (D3) hours after maintaining soil moisture at 40% of field capacity.

#### Expression pattern of *BnWRKY57* under salt stress

The expression pattern of *BnWRKY57* was analysed under salt stress condition using qRT-PCR in leaves and roots of the *B. napus* cultivars. The results revealed significant differences for the expression of *BnWRKY57* between the cultivars and also between leaf and root tissues. *BnWRKY57* expression rapidly and sharply increased in leaves of the *B. napus* salt tolerant cultivar, SLM046, and reached its maximum level at three hours following salt stress (S1) in which the transcript accumulation of *BnWRKY57* was almost nine times higher than that of non-stress conditions (Fig. 7A). *BnWRKY57* expression was then significantly downregulated and showed the minimum transcript level at 24 hours (S2) after salt stress application. *BnWRKY57* expression was also induced in leaves of the *B. napus* salt sensitive cultivar, Zarfam, at S1, however much less induction occurred in comparison with that of SLM046 (Fig. 7A). *BnWRKY57* expression significantly declined in roots of SLM046 at 3 (S1) and 24 (S2) hours following salt stress, and then showed a dramatic upregulation as the transcript accumulation of *BnWRKY57* exhibited an almost 3-fold increase at 7 days post salt stress (S3) compared to non-stress conditions (Fig. 7B). On the other hand, *BnWRKY57* expression was slightly induced in roots of Zarfam by salt stress, and showed no significant changes during salt stress (Fig. 7B).

#### Discussion

*WRKY* genes constitute a large and important gene family in higher plants. Accurate annotation of structure and function of these transcription factor genes is an essential step for further in-depth understanding plant regulatory pathways. In the present study, we isolated, cloned and sequenced the coding region of *B. napus WRKY57* encoding a protein of 293 amino acids. *BnWRKY57* exhibited a high degree of similarity with *WRKY57* proteins from Arabidopsis and Brassica species implying they may have conserved functions. Increasing evidence suggests that *WRKY* genes are involved in various biological processes, including plant development, and responses to biotic and abiotic stresses (Eulgem and Somssich, 2007; Rushton et al., 2010; Luo et al., 2013; Liu et al., 2016; Zhao et al., 2015). Phylogenetic relationships revealed that *WRKY57* genes from diploid Brassica species (*B. rapa* and *B. oleracea*) and the amphidiploid *B. napus* fell into the same clade supporting the view reported by Rana et al. (2004) that gene duplication events occurred before the divergence of the diploid Brassica species.

The promoter analysis suggested that *BnWRKY57* gene expression is regulated by modulators, such as phytohormones, light, drought. In addition, *BnWRKY57* gene with diversified CREs may play as a mediator to link different signalling pathways; for instance, between phytohormones and abiotic stress signalling pathways. Plant hormones, as essential endogenous signal molecules, can regulate cellular processes, plant growth, and development under stress conditions (Grove et al., 1979; Kermodé, 2005; Ryu and Cho, 2015). Previous findings indicated that the expression of *WRKY* genes is affected by hormone treatments (Yang et al., 2009; Jiang et al., 2014). The expression analysis showed that *BnWRKY57* gene was significantly induced by drought stress. The different expression patterns of *BnWRKY57* gene between the cultivars during drought stress were somewhat associated with the level of drought tolerance of the cultivars. Accordingly, a strong and quick upregulation of *BnWRKY57* expression was observed in leaves of the drought tolerant cultivar (SLM046) compared to the drought sensitive cultivar (Zarfam). Similar to *BnWRKY57*, *B. rapa WRKY57* was upregulated up to 4 hours after drought stress treatment and then showed downregulation (Abdul Kayum et al., 2015). Arabidopsis *WRKY57* in transgenic rice plants enhanced tolerance to drought and salt stresses (Jiang et al., 2016). It has been previously reported that activated expression of Arabidopsis *WRKY57* enhanced adaption to drought tolerance by elevation of ABA levels that is closely involved in drought stress responses and by positively regulating the expression of stress-responsive genes (Jiang et al., 2012). Interestingly, the promoter region of *BnWRKY57* contained ABRE motif that is involved in ABA responsiveness and in early response to abiotic stresses leading to activate a broad range of adaptation mechanisms (Corrêa et al., 2008). Furthermore, the presence of HSE and MBS motifs in the *BnWRKY57* promoter was in accordance with the responsiveness of *BnWRKY57* expression to abiotic stresses.

*BnWRKY57* gene was also induced by salt stress, however it showed different expression profiles between the salt tolerant and sensitive *B. napus* cultivars. This finding was in consistent with the expression pattern of *B. rapa WRKY57* in response to salt stress (Abdul Kayum et al., 2015). Additionally, the expression analysis of 26 *BnWRKY* genes uncovered that most of the genes responded to drought and salt stresses (He et al., 2016). It has already reported that *WRKY57* may promote tolerance to osmotic and salt stresses in Arabidopsis by acting as a positive regulator in response to hyperosmotic stress (Jiang et al., 2012). Indeed, this study has provided evidence implying that *BnWRKY57* gene may be involved in adaptive responses to abiotic stresses in *B. napus*, although it is essential to perform overexpression and knockout experiments to resolve the direct involvement of this gene in abiotic stress tolerance mechanisms.

## Materials and Methods

### ***Cloning and sequencing of B. napus WRKY57 gene***

Total RNA was extracted from leaves of *B. napus* cv. SLM046 using column RNA isolation kit (DENA Zist Asia, Iran) according

to manufacturer's instruction. RNA quantity and integrity were checked by agarose gel electrophoresis and spectrophotometry. DNA contamination was removed from RNA using Fermentas DNaseI treatment (ThermoFisher Scientific, USA). First strand cDNA was synthesized from total RNA using Fermentas First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA). The full-length coding sequence of *BnWRKY57* was amplified by PCR using the primers BWRKYF and BWRKYR (Table 2). The primers were designed based on a *B. napus* ortholog of *WRKY57* identified by searching *B. napus* genome sequence (<http://www.genoscope.cns.fr/spip/>) against Arabidopsis *WRKY57*.

The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 90 s; and finally, 72°C for 10 min. The PCR product was analysed by agarose gel electrophoresis (1% w/v). Subsequently, the PCR product was purified and cloned into pTZ57R/T vector (InsTAclone PCR Cloning kit, ThermoFisher Scientific, USA). Transformation of competent cells of *E. coli* strain DH5α was performed by heat-shock method (Froger and Hall, 2007). Finally, the cloned PCR fragment was sequenced. The coding sequence of *BnWRKY57* was submitted in GenBank under the accession number MG699908.

### ***In Silico sequence analysis***

The coding sequence of *BnWRKY57* was subjected to similarity searches against non-redundant nucleotide and protein databases using BLASTN and BLASTX (E-value $\leq 10^{-5}$ ), respectively, available at NCBI website (<http://www.ncbi.nlm.nih.gov/blast>). The exon-intron structure and synteny of *BnWRKY57* were determined using Blat tool of GenoScope (<http://www.genoscope.cns.fr/spip/>), the *B. napus* genome sequence database. The amino acid sequence of *BnWRKY57* was deduced and the corresponding primary structure parameters such as molecular weight and isoelectric point were obtained using the ExPasy website (<https://www.expasy.org/tools/>). Hydropathicity of the deduced *BnWRKY57* protein was computed by ProtScale (<https://web.expasy.org/protscale/>) according to Kyte and Doolittle method (Kyte and Doolittle, 1982). Secondary structure of *BnWRKY57* was analysed using SOPMA tool ([https://npsa-prabi.ibcp.fr/cgi-bin/secpred\\_sopma.pl](https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl)). *BnWRKY57* subcellular localization was predicted by Distill (<http://distill.ucd.ie/distill/>). Pfam (<http://pfam.xfam.org/>) (Finn et al., 2016) was used to identify functional domains within the *BnWRKY57* protein.

Phylogenetic analysis was performed based on the amino acid sequences deduced from *WRKY57* genes from Brassicaceae family and other plant species using neighbour-joining method with 1000 bootstrap replicates implemented by MEGA software Version 7 (Kumar et al., 2016). To characterize the promoter region of *BnWRKY57* gene, 1500 bp upstream of the start codon, as a putative promoter region, was obtained from GenoScope (<http://www.genoscope.cns.fr/spip/>). The promoter sequence was searched for cis-regulatory elements (CREs) using PlantCARE



(<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al., 2002).

### **Expression profiling of *BnWRKY57* under drought and salt stress conditions**

The *B. napus* cultivars, SLM046 and Zarfam, with distinct responses to abiotic stresses (Rameeh et al., 2012; Darjani et al., 2013; Mirzaee et al., 2013; Shamloo-Dashtpagerdi et al., 2018), were employed to perform drought and salt stress experiments. The drought stress experiment was performed to analyse expression changes of *BnWRKY57*. Plants were grown in plastic pots filled with sterilized sandy-clay soil under growth room conditions (16h light/8h dark; 24 °C day/8 °C night). Plants with five true leaves were exposed to drought stress by withholding water to reach soil moisture to 40% of field capacity (FC). Leaf and root samples were collected under non-stress together with three drought stress treatments including when soil moisture reached to 40% of FC as well as maintaining that for 6 and 24 hours. The non-stress condition was conducted by regularly irrigating plants to keep soil moisture around FC during the experiment.

The salt stress experiment was also conducted to assay expression levels of *BnWRKY57*. Surface-sterilized seeds of the *B. napus* cultivars were sown in sandy soil under a long-day photoperiod (16 h light/8 h dark) with 24 and 8 °C day and night temperature, respectively. 10-day-old seedlings were then transferred to a hydroponic system supplemented with half-strength Hoagland's nutrient solution (pH≈6.0) under growth room conditions. Salt stress was applied to plants with five true leaves by adding 150 mM NaCl solution to the medium. Samples of leaves and roots were collected under non-stress as well as three salt stress treatments including 3 hours, 24 hours and 7 days after adding salt solution.

qRT-PCR was used to assess mRNA expression levels of *BnWRKY57* in leaves and roots of the *B. napus* cultivars during drought and salt stresses. Total RNA extraction and cDNA synthesis were done as described earlier for isolation of *BnWRKY57*. qRT-PCR was performed on an ABI StepOne real-time PCR machine (Applied Biosystems, USA) by using RealQ PCR Master Mix kit (Ampliqon, Denmark) according to the manufacturer's instructions. The thermal cycling conditions were as follows: 15 min at 95 °C, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. qRT-PCR reactions were also performed for the reference gene, *B. napus Elongation Factor 1α* (*Bnelf1α*; GenBank Accession number: FJ529181) for normalization of expression data. The specific primers for *BnWRKY57* (BWRTF and BWRTF) and *Bnelf1α* are listed in Table 2. Three independent biological samples were used to do qRT-PCR. Each reaction was also repeated two times as technical replicates. The relative expression levels of *BnWRKY57* gene were calculated using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). LSD statistical test was performed to determine significant differences between the expression levels.

### **Conclusion**

This study is the first report on cloning and molecular characterization of the full-length coding sequence of *B. napus* transcription factor gene, *BnWRKY57*, aimed at providing a framework for future studies regarding *BnWRKY57* functions. The expression patterns of *BnWRKY57* gene of the two *B. napus* cultivars under drought and salt stress conditions along with analysis of the *BnWRKY57* promoter suggested that this gene may serve as a regulator involved in responses to drought and salt stresses. Further in-depth studies are required to decipher the roles of *BnWRKY57* gene in stress regulatory pathways.

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