

## Research Article

# Identification of Putative Osmotic Stress-Responsive Genes in Canola by *in Silico* Study of *Cis*-Regulatory Elements

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## Abstract

Osmotic stress is a primary or side-effect of different kinds of a biotic stresses, especially salt and drought stress, leads to a significant reduction in plant growth, productivity, and yield. Canola is one of the important oil crops cultivated all-over the world. In this paper, known *cis*-regulatory elements that regulate genes involved in molecular responding to salt, drought, and abscisic acid conditions were used in order to identify relevant genes with a similar function in canola. Of 62384 unigenes retrieved from Brassica Genome Gateway database, 29 putative osmotic stress responsive genes were identified. In order to evaluate accuracy of the identified genes, GOBP, GO Slim-plant, co-expression, and PPI analysis were performed. GOBP analysis enriched the most of the genes (95%) as "response to stimulus" and GO Slim-plant analysis enriched the genes into "response to stress" and "response to a biotic stress stimulus". Almost all identified genes had potential of expression under salt, drought, and abscisic acid treatments. For co-expression analysis, the gene expression profiling data were utilized and the results indicated that 25 of 29 identified genes had at least 0.46 correlations and 0.5 mutual connection. In predicted PPI network, there were interactions between proteins encoded by the identified genes. In this study, the identified osmotic stress responding genes could be introduced as candidate genes to be considered in genetic engineering of canola or would help to better understanding of molecular mechanisms of osmotic stress in canola at the cellular level.

**Keywords:** *Brassica napus*; Co-expression; Gene finding; Transcription

## Introduction

Using computer technology, known as the term bioinformatics, to collect, retrieve, and analyze scientific data in large scale allows biological researchers to perform experiments *in silico* - using different models and algorithms- and additional analysis of experimental results for further insights and details [1]. By emerging of sequencing technologies, genetic information was generated for model and non-model plants, providing information of identifying specific genes from well-studied plants such as *Arabidopsis* and applying relevant genes in other less understood plants [2]. However, identification of genes that are induced in response to a specific stimulus or in response to changes of environmental conditions have been remained one of the challenging areas of computational genomics [3]. Relying on the principal that a large number of genes are regulated at transcription level through interactions taking place between Transcription Factors (TF) and consensus *cis*-regulatory elements within promoter regions of genes [4,5], theoretically, makes it possible to gain specific genes that their expression are induced by a particular condition through searching *cis*-regulatory elements known and annotated for a particular condition [6]. Several studies have been carried out by similar approaches and their results confirmed with experimental works [2, 6-8]. Zhang et al. [6] reported using of a *cis*-regulatory based method to find genes that are induced by Abscisic Acid (ABA), a phytohormone, and a biotic stress and verified results obtained by

this approach using RT-PCR, with the accuracy of 67.5%. Mark stein et al. [9] in *Drosophila* and We nick et al. [10] in *C.elegans* used well-defined *cis*-regulatory elements for targeting a set of genes. Along with known *cis*-regulatory elements-based computational methods, the co-expression analysis of co-regulated genes with common *cis*-regulatory elements could further validate and complete the obtained information about targeted genes. Many microarray data have been produced from different experimental works and deposited on various databases that could be explored for predicting co-expressed genes, transcriptional regulation, and determining biological process using an appropriate bioinformatics tool [11-13].

In osmotic stress conditions, plants are unable to take up adequate water to maintain cellular processes. Osmotic stress is created as a consequence of imposing to some a biotic stresses, especially salt and drought stress [14]. In molecular level, osmotic stress triggers signal transduction pathways and induces a set of genes that some are common for salt, drought, and temperature stress [15,16]. The evolutionary process has created diverse molecular mechanisms in plants to cope with a biotic stresses. Plants exposed to osmotic stress trigger complex physio-biochemical reactions which are driven by a set of genes [17].

Canola (*Brassica napus* L.) is one of the widely cultivated oil crops for producing vegetable oil. This plant tolerates osmotic stress; however, its growth, yield, and performance are adversely

**Table 1:** Putative osmotic stress responsive genes identified using detection of cis regulatory elements at the promoter region of the genes.

No	Accession number <sup>1</sup>	Description <sup>2</sup>	Detected motif <sup>3</sup>	Motif annotation <sup>4</sup>
1	AT3G50830.1	Cold-regulated 413 plasma membrane protein 2	CGACACGT	ABA, DREB1Aox, Drought
2	AT2G21490.1	Probable dehydrin LEA	CGTGACGT	ABA, DREB1Aox
3	AT1G54410.1	Dehydrin HIRD11	TCGCCACG	ABA
4	AT3G01520.1	Universal stress protein A-like protein	AGACACGT	ABA
5	AT2G47710.1	Adenine nucleotide alpha hydrolases-like protein	AGCCACGT	ABA
6	AT3G11930.3	AT3G11930 protein	AAGCCACG	ABA
7	AT3G11930.4	Universal stress protein-like protein	AAGCCACG	ABA
8	AT5G66400.1	Dehydrin Rab18	GGACACGT	ABA, DREB1Aox, Drought
9	AT3G11930.2	AT3g11930/MEC18.3	AAGCCACG	ABA
10	AT1G62740.1	Hsp70-Hsp90 organizing protein 2	ATGACACG	ABA
11	AT5G17390.1	Adenine nucleotide alpha hydrolases-like super family protein	TAATTACG	ABA
12	AT1G01170.1	At1g01170	GACACGTA	ABA, DREB1Aox
13	AT4G37220.1	Cold-regulated 413 plasma membrane protein 4	AAGCCACG	ABA
14	AT1G29390.1	Cold-regulated 413 inner membrane protein 2, chloroplastic	CTGACGTG	ABA
15	AT1G68300.1	Adenine nucleotide alpha hydrolases-like super family protein	ACGTGTCC	ABA, DREB1Aox, Drought
16	AT4G35770.1	Rhodanese-like domain-containing protein 15, chloroplastic	CTGACGTG	ABA
17	AT1G09210.1	Calreticulin-2	TACACGTG	ABA, DREB1Aox
18	AT4G34190.1	Stress enhanced protein 1, chloroplastic	ACGTGTCC	ABA, DREB1Aox, Drought
19	AT4G21320.1	Protein HEAT-STRESS-ASSOCIATED 32	CGTGCCAT	ABA, DREB1Aox
20	AT2G41430.1	Protein EARLY RESPONSIVE TO DEHYDRATION 15	TACACGTG	ABA, DREB1Aox
21	AT1G07600.1	Metallothionein-like protein 1A	ATGCCACG	ABA, DREB1Aox
22	AT3G46550.1	Fasciclin-like arabinogalactan protein 4	CGCGTGAA	Drought
23	AT5G08670.1	ATP synthase subunit beta	ACGTGTCC	ABA, DREB1Aox, Drought
24	AT5G55070.1	Dihydropyridyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 1, mitochondrial	CACGTGTA	ABA, DREB1Aox
25	AT2G21970.1	Stress enhanced protein 2, chloroplastic	ACGACACG	ABA, DREB1Aox, Ethylene, Drought
26	AT5G17000.1	AT5g16970/F2K13_120	CGTGACGT	ABA, DREB1Aox
27	AT1G01470.1	Probable desiccation-related protein LEA14	GACCGACT	DREB1Aox, ABA
28	AT5G24270.1	Calcineurin B-like protein 4	ACGTGACA	ABA
29	AT2G01980.1	Sodium/hydrogen exchanger 7	TACACGTG	ABA, DREB1Aox

<sup>1</sup>Accession number is from TAIR, <sup>2</sup>Description provided from UniProtKB, <sup>3</sup>Motifs collected from PLACE, Plant CARE, and DoOP, <sup>4</sup>Annotations is from Plant PromoterDB.

affected [18]. Identification of osmotic stress responding genes not only introduces candidate genes for the goals of genetic engineering but also provides a background to understand details of molecular mechanisms of plants. The experimental works are time- and cost-consuming process to be repeated for all plants. Fortunately, bioinformatics has provided different computer-based approaches for using handy information or related experimental studies to study other plants. In this work, known *cis*-regulatory elements were utilized for identification of putative osmotic stress responding genes in canola. It is obvious that reaching osmotic stress-tolerant plants depends on exact and detailed knowledge of the underlying adaptive mechanisms.

## Materials and Methods

### Input data

In order to identify and study osmotic stress-responsive genes in canola, we downloaded annotated brassica unigene set from Brassica

Genome Gateway (<http://brassica.nbi.ac.uk>). Of 62384 unigenes, 246 unigenes annotated as responding ones to various stresses such as salinity, drought, and oxidative were selected for further analysis.

### Identification of osmotic stress-responsive genes

Transcriptions start site (TSS) and the promoter region for all of these genes were obtained from PlantPromoterDB (<http://ppdb.agr.gifu-u.ac.jp/>). The *cis*-regulatory elements - exclusive for abscisic acid, salinity and drought- from PLACE [19,20], Plant CARE [21,22], and DoOP [23] were used to find out an occurrence of motifs at the promoter regions. The presence of motifs with abscisic acid, salt, and drought annotation at the promoter region of a gene was considered as a marker for determining the putative osmotic stress responsive role for that gene. This consideration was based on the ration that a large portion of the gene expression control takes place at the transcriptional level. After identification of osmotic stress-responsive genes, Gene Ontology (GO) enrichment analysis of osmotic stress-

**Table 2:** GOBP description of 20 enriched genes from 29 identified putative osmotic stress responsive genes.

GO-ID	GOBP term	p-value*	No. of gene	Cluster frequency
6950	Response to stress	3.5765E-16	17/20	85%
50896	Response to stimulus	1.6381E-15	19/20	95%
9628	Response to abiotic stimulus	1.7045E-8	10/20	50%
42221	Response to chemical stimulus	6.9138E-6	9/20	45%
9415	Response to water	2.5095E-5	4/20	20%
42535	Hypotonic salinity response	1.7926E-3	1/20	5%
6971	Hypotonic response	1.7926E-3	1/20	5%
9651	Response to salt stress	3.8769E-3	3/20	15%
6970	Response to osmotic stress	4.7795E-3	3/20	15%

responsive genes was carried out for determining their biological processes using The Biological Networks Gene Ontology tool (BiNGO), a plug in Cytoscape [24], and Protein-Protein Interaction network (PPI) plus metabolic pathway using STRING 10.0 (<http://string-db.org>).

### Gene expression analysis

In-silico gene expression analysis of identified osmotic stress-responsive genes were performed using Genevestigator [25] available at <https://genevestigator.com/gv/>. The expression profile analysis and co-expression correlation of the genes based on the microarray data was done for well-studied *Arabidopsis* under perturbation conditions for further evaluation of the identified genes involved in response to osmotic stress.

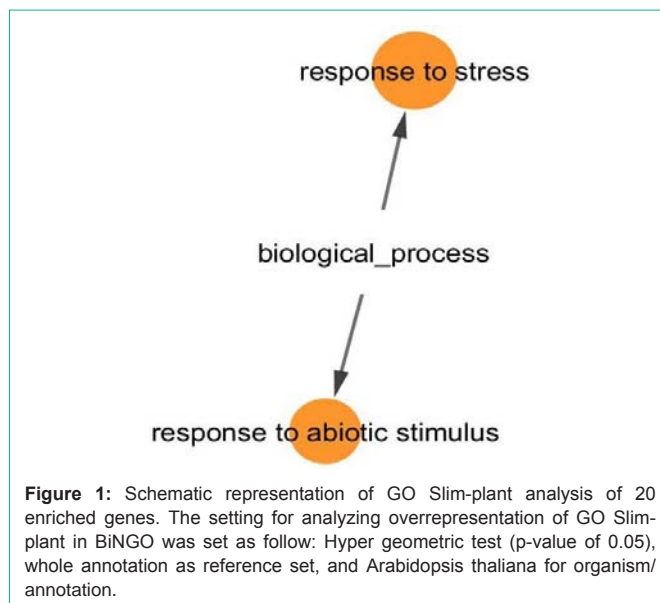
## Results and Discussion

### Identification of osmotic-stress responsive genes and GO enrichment analysis

Identification of putative osmotic stress responsive genes was done for canola using *cis*-regulatory motifs known to regulate genes involved in salt and drought stress. These stresses cause plants to meet osmotic stress. In addition, *cis*-regulatory motifs involved in abscisic acid response were used. Abscisic acid mediates sensing water shortage around the root. Of 246 collected unigene, at least one of *cis*-regulatory motifs was found at the promoter region of 29 unigenes. Since many of the genes had unknown protein annotation, the accession number and description were provided from UniProtKB for each gene (Table 1).

GO analysis in which whole genome annotation assumed as reference, demonstrated that 20 identified genes out of 29, were significantly enriched as putative osmotic stress responsive genes at p-value of 0.05. According to Gene Ontology-Biological Process (GOBP) analysis, all enriched genes had GOBP term that both main effects and secondary effects of them are the imposing osmotic stress on plants (Table 2).

The majority of genes were enriched as response to stimulus (cluster frequency of 95%) and response to stress (cluster frequency of 85%). Furthermore, a few of genes enriched as a specific GOBP including AT5G24270.1 for hypotonic and hypotonic salinity response, AT4G35770.1 and AT5G24270.1 for response to osmotic/



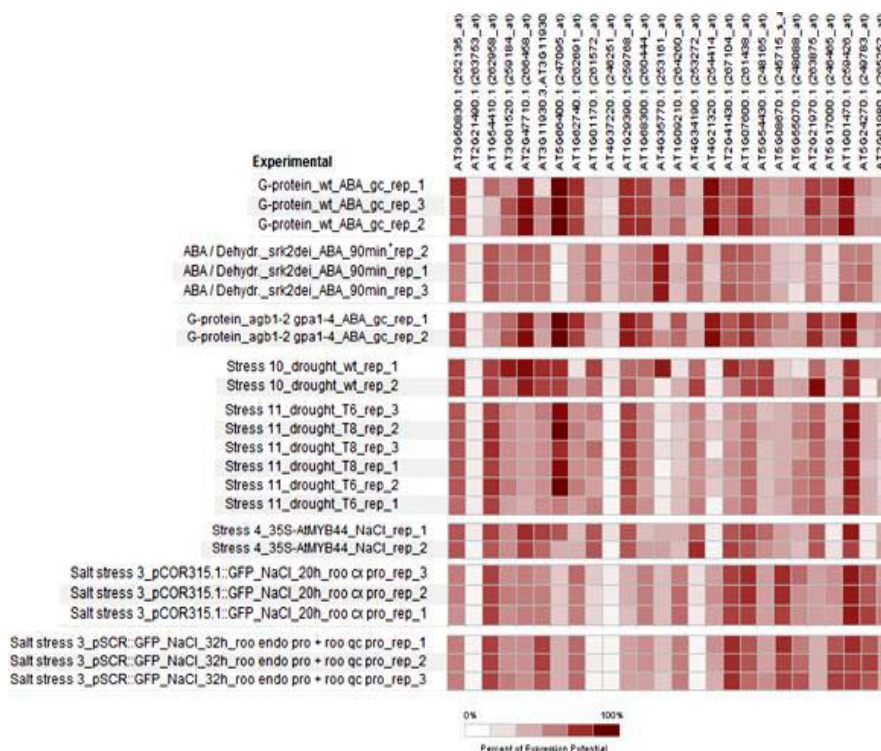
salt stress. GO Slim-plant analysis enriched genes into two categories: (1) response to stress with p-value of 2.0406E-16 and 17/20 number of gene (cluster frequency of 75%) and (2) response to a biotic stimulus with p-value of 7.2183E-7 and gene number of 10/22 (cluster frequency of 45.4%) (Figure 1).

The results of STRING 10.0 based on PFAM Protein Domains and INTERPRO Protein Domains and Features databases indicated that 3 domains including UspA (pathway ID: IPR006016), universal stress protein A (pathway ID: IPR006015 and PF00582) and Rossmann-like alpha/beta/alpha sandwich fold (pathway ID: IPR014729) were significantly enriched for proteins AT1G68300.1, AT2G47710.1, AT3G11930.3, AT5G17390.1.

The results of GOBP and GO Slim-plant analysis illustrate a reliable interference for putative osmotic stress responsive genes determined by *cis*-regulatory element detection method. Four enriched genes were further confirmed through the results of STRING 10.0 which indicated presence of specific domains known for involving in response to stress. The Universal Stress Protein A Domain (USPA) is widespread in prokaryotes and eukaryotes appearing to be part of an ancient protein that have been implicated for serving a function in the cell while organism encounters different kinds of environmental stimuli [27,28].

Binding transcription factors on the regulatory region of a gene affects its structure which in return leads RNA polymerase activation and initiation of transcription. More than 50 families of such transcription factors have been identified in plants, which are contributors in about all aspects of cellular activities under different conditions [29,30].

Binding transcription factors to *cis*-regulatory elements is regular point to regulate a gene expression [31]. It has been suggested that the genes with related functions are mostly regulated are by similar sets of transcription factors, so it is possible to identify the functions of genes using known *cis*-regulatory elements from known and annotated genes [6]. Li et al [7] in their study for genome-wide identification of



**Figure 2:** Heat-map representation of the expression potential of identified putative osmotic stress responsive genes. The accession numbers of the genes from TAIR were introduced into Genevestigator software and presence of expression potential investigated under various perturbations that result in osmotic stress in plants. The results of this software is based on the experimentally works on Arabidopsis thaliana deposited in different databases. Some of results are indicated here in which darker colour depicts more potential of presence in a given condition.

osmotic stress responsive genes used known *cis*-regulatory elements to train Artificial Neural Network (ANN) modelling. They confirmed osmotic stress responsive genes identified by this way using GO analysis and experimental RT-PCR analysis. In other study, Sharma [8] in silico demonstrated that those genes that are co-expressed under osmotic stress share similar *cis*-regulatory elements. They concluded that the co-expressed genes are co-regulated with the same regulatory system under osmotic stress. Furthermore, according to annotations given to detected motifs, the all identified genes had Abscisic Acid (ABA) annotation (Table 1). The protective role of abscisic acid have been indicated in response to osmotic stress, in addition, it is demonstrated that a biotic stress responsive genes are also induced by abscisic acid treatments [15,32-34]. In plants, one of the important pathways of sensing osmotic stress as consequence of salt, drought, and temperature stress is ABA-dependent pathway in which the central role of four AREB/ABF transcription factors including AREB1, AREB2, ABF3, and ABF1 well characterized. These transcription factors control most downstream genes of ABA-dependent pathway under osmotic stress through binding to specific motifs on the promoter region of genes [32]. Dehydration conditions occur in plants because of salinity and share mostly similar regulatory elements and signal transduction pathways with abscisic acid dependent pathways [35].

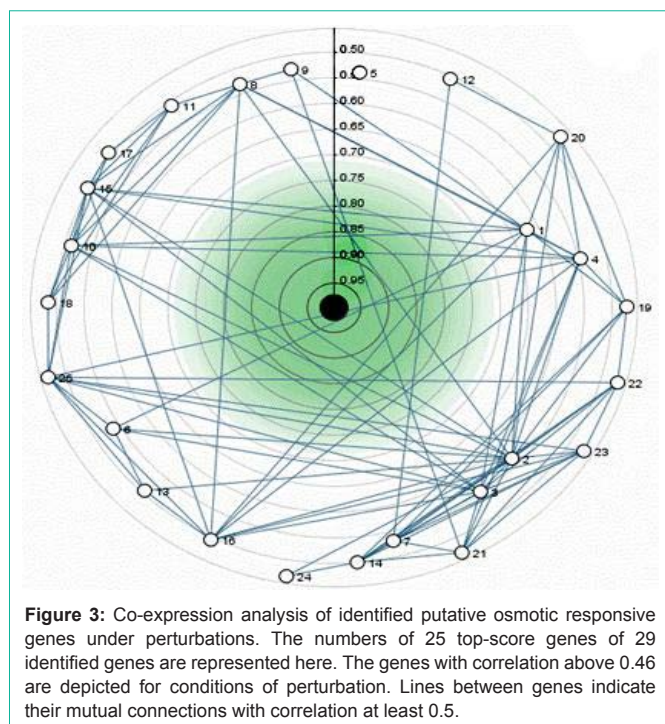
**Analyzing the expression of the identified osmotic stress responsive genes**

We checked the in silico identified osmotic stress responsive genes, in addition to GO analysis, by Genevestigator software. First,

all of the identified genes investigated for the presence of expression potential under salt, drought, and ABA treatments. Then, they have been investigated for co-expression analysis under stressful conditions. The results of expression potential indicated acceptable results for almost all identified genes except AT5G66400.1 that represented weak potential for being expressed under salt, drought, and ABA treatments (Figure 2). In addition, the genes AT4G37220.1 and AT4G35770.1 did not show expression potential under salt and drought stress, but showed strong expression under ABA treatments. These three genes indicated strong expression under cold stress. Cold stress is one of the stresses that lead to water deficit conditions in plants, thereby influencing plant growth and development deleteriously [36,37]. In molecular level, it also has the common regulatory system and cross-talk with salt and drought stresses [37].

Since co-expression and mutual connection of genes could be a reliable indicator of their involvement in response to a particular condition, we analyzed co-expression of identified genes to check out in silico prediction. All identified putative osmotic stress responsive genes indicated correlation above 0.46 and mutual connection of at least 0.5 (Figure 3). The results of co-expression analysis were confirmed using produced Protein-Protein Interaction (PPI) network by STRING 10.0. Most of identified genes’ corresponding proteins showed interaction between themselves (Figure 4).

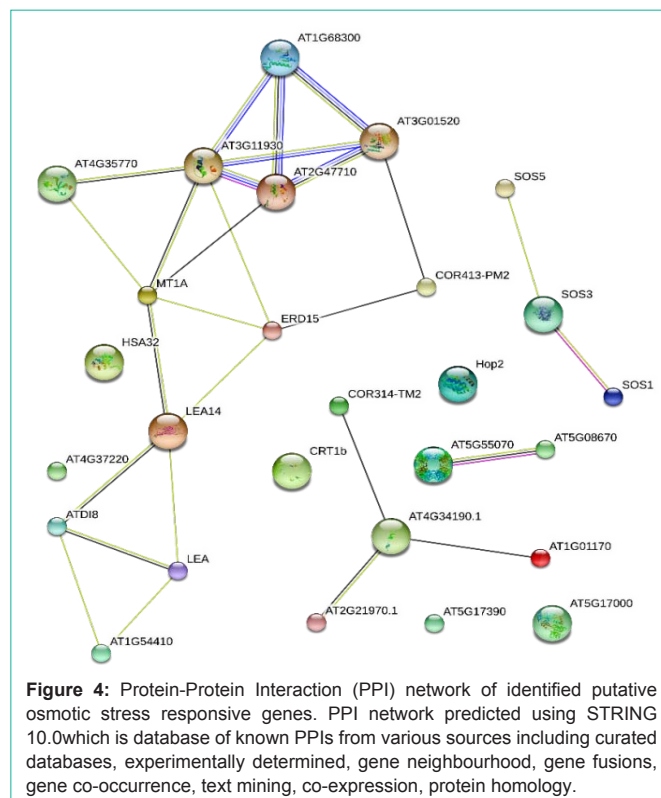
Different correlation calculations have been used for categorizing gene expression data. Co-expression analysis, indeed, reveals response of genes to a particular stress or more stresses with



some similar consequences. Furthermore, in order to confirm co-expressed genes, their co-regulation might be evaluated for having similar *cis*-regulatory elements [8,38]. Therefore, to check out assumed functionally related genes with same co-regulation pattern, comparing the results with stress microarray data could be useful. The stress microarray data have deposited on various databases and are accessible with different co-expression analysis tools. According to the results, all identified genes represented acceptable correlation based on Pearson's correlation coefficient (Figure 3). The co-expression analysis results demonstrated the accuracy of the *cis*-regulatory elements detection. For this aim, functional relationship of proteins encoded with the identified genes could further confirm the participation of them in response to particular stresses. STRING v10 [39] provides user friendly interface by which could easily study proteins of interest based on physical and functional association from various known predicted interactions scattered over databases. As shown in (Figure 4), more than 63% of the identified genes showed interaction with each other in protein level. These data illustrated accordance of co-expression results with their functional property.

## Conclusion

Based on the rationale that the large proportion of gene expression regulation occurs at transcriptional level, we identified 29 putative osmotic stress responsive genes out of 62384 unigene using known *cis*-regulatory elements of inducible genes against drought/salt stress and ABA treatment. GO analysis enriched the major of identified genes (95%) as responsive genes to stimuli and GO Slim-plant analysis enriched all genes into "response to stress" and "response to a biotic stimulus". For further evaluation, the accuracy of identified genes was checked out using Genevestigator software. Major part of the genes showed potential of expression under salt, drought, and ABA treatment as well as similar co-expression pattern with correlation



and mutual connection of more than 0.46 and 0.5, respectively. PPI network indicated that proteins encoded by identified genes had interactions with each other.

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