

Research Article

Computational identification of six salt stress responsive genes in *Cynara scolymus* L.

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Citation

Iftekhar Ahmed Baloch, Saima Johar, Bibi Hani Mengal, Ambreen Khadija Alvi, Shehnaz Shair Qambrani, Muhammad Naeem Shawani and Bibi Sadia. Computational identification of salt stress responsive genes in *Cynara scolymus* L. Pure and Applied Biology. Vol. 9, Issue 3, pp1723-1736. <http://dx.doi.org/10.19045/bspab.2020.90183>

Received: 05/04/2019

Revised: 03/04/2020

Accepted: 08/04/2020

Online First: 09/04/2020

Abstract

Cynara scolymus L. is a perennial plant belonging to family Asteraceae. It has many traditional and medicinal values and is also used as a source of vegetable in many countries. This plant thrives well in saline areas and is considered to be moderately salt tolerant but very little information is available about its salt stress tolerance mechanism. In this study six (6) salt stress responsive genes (Four up-regulated i.e. CSD1, RCI3, AFB3, HVA22A and two down-regulated i.e. CSD2, MSD1) were identified in *Cynara scolymus* L. by using homology search approach with the help of various bioinformatics tools/resources (BLASTn, BLASTp, ORF finder, CD search, Clustal Omega, ProtParam and Phyre2). Furthermore, the secondary and tertiary structures of reference and target genes were predicted and compared for confidence and similarities in structure. All selected salt stress responsive genes were predicted in *Cynara scolymus* through computational analysis of 36,323 ESTs. The EST based identification of these genes is a confirmation of their expression and these findings will help us understand the salt stress tolerance mechanism in this important plant.

Keywords: Bioinformatics; *Cynara scolymus*; Salt stress genes

Introduction

Cynara scolymus L. (*C. scolymus*) is a perennial plant commonly known as Globe Artichoke and belongs to family Asteraceae. It is a rich source of minerals, fiber and phenolics [1, 2]. Its leaf extract is used as antimicrobial, hepatoprotective and for cholesterol reducing purposes. The eatable

parts of *C. scolymus* are the delicate internal leaves and the receptacle called as "heart" [3]. It assumes an essential part in humans' daily nutritional value, mainly in the Mediterranean regions [4]. *C. scolymus* has a small amount of fat and large amount of minerals like potassium (K), sodium (Na), phosphorus (P), vitamin C, polyphenols and

flavones [3, 5]. Many other *Cynara* species are grown in different areas of the world i.e. in U.S, principally in California, in South America, North Africa, Near East and China [4, 5].

The condition in which plants grow in higher than normal quantity of salt is called salinity or salt stress condition. Salt stress is an important abiotic stress which affects the metabolic and physiological responses of the plants. It is one of the main abiotic stresses faced by plants. Plants identify the stress signals and pass them to the cellular apparatus and then the adaptation is fulfilled by the regulation of gene expression [6]. *C. scolymus* L. grows in high salinity and drought conditions [7]. Since it is a medicinal plant, researchers have also studied the effect of salinity on its phenolic composition and antioxidant activity [8].

Salt stress genes have been extensively reported in model plants i.e. *Oryza sativa* and *Arabidopsis thaliana*. Since many salt tolerant plants express their salt tolerance genes, here we hypothesize that such salt stress responsive genes may also be present in *Cynara scolymus*. This research is an attempt to identify and annotate some salt stress responsive genes (Four up-regulated i.e. CSD1, RCI3, AFB3, HVA22A and two down-regulated i.e. CSD2, MSD1) in *C. scolymus* by using different bioinformatics tools (BLASTn, BLASTp, ORF finder, CD search, Clustal Omega, ProtParam and

Phyre2). A series of steps were performed to find the salt stress responsive genes in *C. scolymus*.

Materials and methods

A total of six (6) salt stress responsive genes (CSD1, RCI3, AFB3, HVA22A, CSD2 and MSD1) were selected from the model plant *Arabidopsis thaliana* through literature survey [9-12]. An attempt was made to identify the potential orthologs of these reference genes in *C. scolymus*. The main steps of methodology are discussed below.

Retrieval of selected gene sequences and their Open Reading Frames (ORFs).

Messenger RNA (mRNA) sequences of salt stress genes of the *Arabidopsis thaliana* were fetched by using gene database publically available at National Center for Biotechnology Information (NCBI) and saved in FASTA format for downstream analysis. FASTA sequence of each gene was subjected to ORF finder tool publically available at NCBI. Longest open reading frames of each gene were saved. The nucleotide and protein sequences of these genes were used to find their potential orthologs in *Cynara scolymus*. Gene symbol, Accession No of Reference Genes (from *Arabidopsis thaliana*), Longest ORF of Reference Gene (from *Arabidopsis thaliana*) and Accession No of target Genes (predicted in *C. scolymus*) are shown in (Table 1).

Table 1. Gene symbol, Accession No and ORFs of Reference (*Arabidopsis thaliana*) and accession no of target Genes (predicted in *Cynara scolymus*).

Gene symbol	Accession No of Reference Gene (<i>Arabidopsis thaliana</i>)	Longest ORF of Reference Gene (<i>Arabidopsis thaliana</i>)	Accession No of target EST (<i>Cynara scolymus</i>)
CSD1	NM_100757.4	+3	GE599954.1
RCI3	NM_100405.4	+1	GE578194.1
AFB3	NM_101152.5	+1	GE583634.1
HVA22A	NM_118628.4	+2	GE589804.1
CSD2	NM_128379.4	+2	GE599319.1
MSD1	NM_111929.4	+1	GE594969.1

Homology search

Basic Local Alignment Search Tool (BLAST) is an important tool for ortholog search. The available 36,323 Expressed Sequence Tags (ESTs) of *Cynara scolymus* were computationally screened to search the selected genes. The nucleotide ORF sequences of *Arabidopsis thaliana* genes were subjected to nucleotide Blast (BLASTn) against ESTs of *Cynara scolymus* and amino acid sequences of ORFs were subjected to protein Blast (BLASTp). On the basis of query coverage and percentage identity the best matched ESTs and protein sequences were selected and their sequences were saved in FASTA format.

Finding conserved domains

Best matched ESTs of *Cynara scolymus* and genes of the model plant *Arabidopsis thaliana* were subjected to Conserved Domain Database (CD Search) to detect the presence and position of conserved domains which perform functions in saline conditions. Conserved domains of the *Arabidopsis thaliana* genes were compared with the conserved domains of best matched ESTs of *Cynara scolymus* by this method.

Phylogenetic analysis and Percent Identity Matrix of MSD1 gene

Freely available Multiple sequence alignment (MSA) tool, Clustal Omega was used for phylogenetic analysis of MSD1 gene. A Cladogram was constructed for MSD1 due to its conserved nature in many plants. Amino acid sequences of newly predicted MSD1 gene in *C. scolymus* was compared with that of *Camelina sativa*, *Papaver somniferum* and *Arabidopsis thaliana*. Percent Identity Matrix was also calculated for each MSD1 gene sequence. The results were saved.

Deduction of secondary and tertiary protein structures

The physical properties including Theoretical PI, Instability Index, Aliphatic Index and

GRAVY of the deduced protein structures of the target *C. scolymus* ESTs and *Arabidopsis thaliana* reference genes were obtained using online tool ProtParam from the ExPasy server [13]. Whereas, the secondary and tertiary structures were obtained from Phyre2 online protein analysis server [14]. The deduced secondary and tertiary structures were compared for confidence and similarities in structure.

Results

Identification of new conserved salt stress genes in *C. scolymus*

All selected salt stress responsive genes were identified in *Cynara scolymus* through computational analysis of 36,323 ESTs. Homology search of each gene showed less query coverage and identity when compared with nucleotides (blastn) and comparatively higher query coverage and identity when compared with protein sequences (blastp). Query coverage of nucleotide and protein sequences of CSD1, RCI3, AFB3, HVA22A, CSD2 and MSD1 are shown in (Fig. 1a & b).

Identification of similar conserved domains in predicted genes

CD search results of newly predicted conserved genes in *Cynara scolymus* demonstrated that all predicted genes share almost similar conserved domains with their corresponding reference genes. Similarities of conserved domains are shown in (Fig. 2a to f).

Phylogenetic analysis and Percent Identity Matrix of MSD1 genes

Phylogenetic analysis of the protein coding sequence (amino acid) of MSD1 gene with its orthologs in *Camelina sativa*, *Papaver somniferum* and *Arabidopsis thaliana* showed that MSD1 gene of *Cynara scolymus* is more close to *Papaver somniferum* as compared to *Camelina sativa* and *Arabidopsis thaliana* (Fig. 3a & b). Percent Identity Matrix (as predicted by Clustal Omega) for

MSD1 gene of each plant is shown in (Table 2).

Deduction of secondary and tertiary structures of proteins

In current analysis the deduced secondary and tertiary structures of proteins also showed similar physical properties including molecular stability, aliphatic index and GRAVY.

The results in (Table 3) showed that the *C. scolyumus* target ESTs coded for proteins showed homology to the respective *Arabidopsis* reference genes. All deduced proteins of target ESTs except GE578194.1 showed similar stability / instability to their respective *Arabidopsis* reference counterparts. Values of aliphatic indices deviated within the range of ± 11 to the reference protein values which indicated comparable contribution of the aliphatic side

chains to the stability of the protein molecules. All target EST deduced proteins showed comparable polarity to the reference sequence proteins except for the EST sequence GE583634.1, which gave a very low positive GRAVY value.

The deduced secondary and tertiary structures of target ESTs selected from *Cynara scolyumus* were comparable to the predicted structures obtained from the respective *Arabidopsis* reference genes. The three dimensional models for GE578194.1, GE594969.1, GE583634.1 and GE599954.1 were predicted with 100% confidence by the Phyre2 software with a query coverage range from 88 – 99 % in the structures. A comparison of deduced tertiary structures of both *Cynara scolyumus* and *Arabidopsis thaliana* are given in (Fig 4a-l).

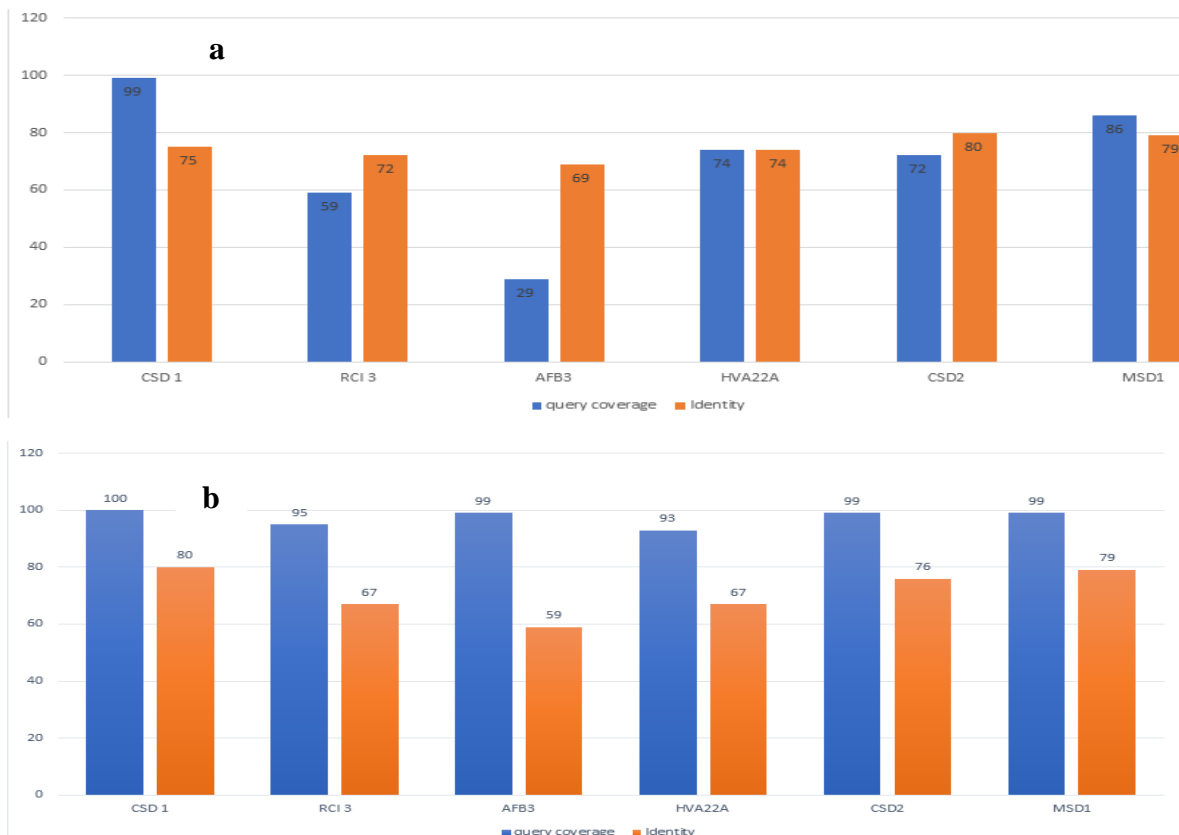


Figure 1(a). BLASTn results showing Nucleotide query coverage and identity of 6 salt stress genes in *C. scolyumus*. **(b)** BLASTp results showing amino acids query coverage and identity of 6 salt stress genes in *C. scolyumus*

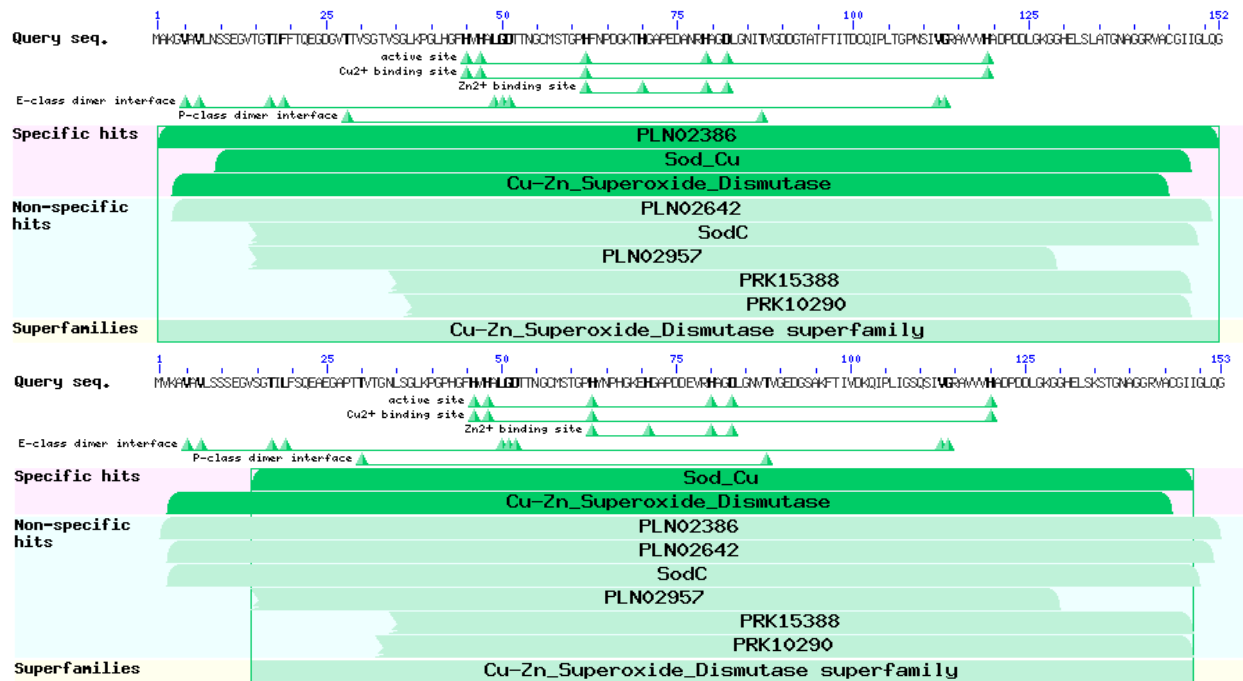


Figure 2a. Comparison of conserved domains found in CSD1 genes of *Arabidopsis thaliana* (above) and *Cynara scolymus* (below) showing presence of similar domains predicted by CD search

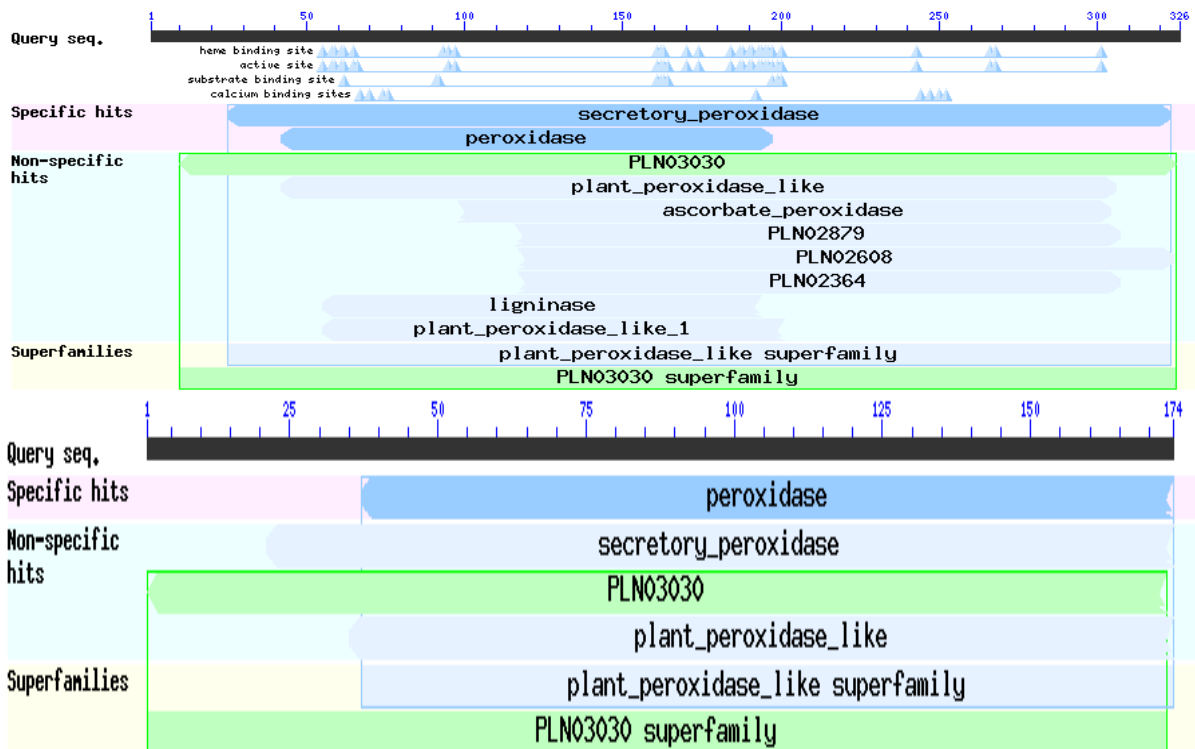


Figure 2b. Comparison of conserved domains found in RCI3 genes of *Arabidopsis thaliana* (above) and *Cynara scolymus* (below) showing presence of similar domains predicted by CD search

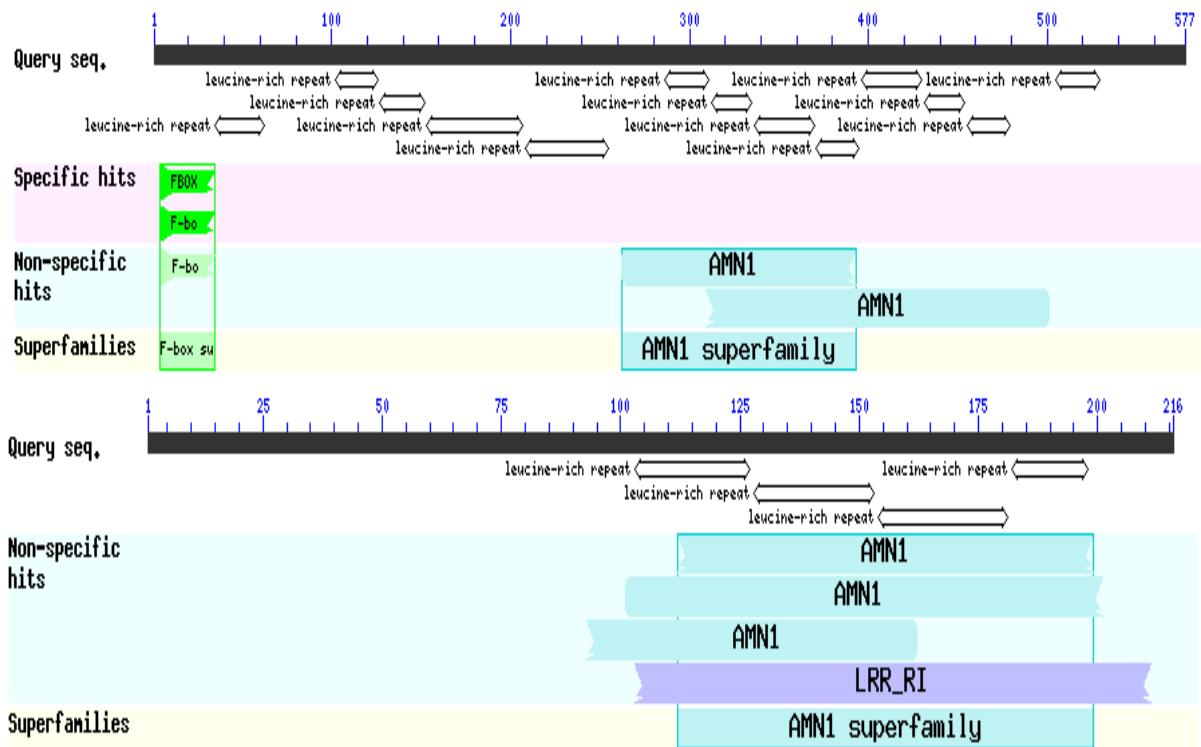


Figure 2c. Comparison of conserved domains found in AFB3 genes of *Arabidopsis thaliana* (above) and *Cynara scolymus* (below) showing presence of similar domains predicted by CD search

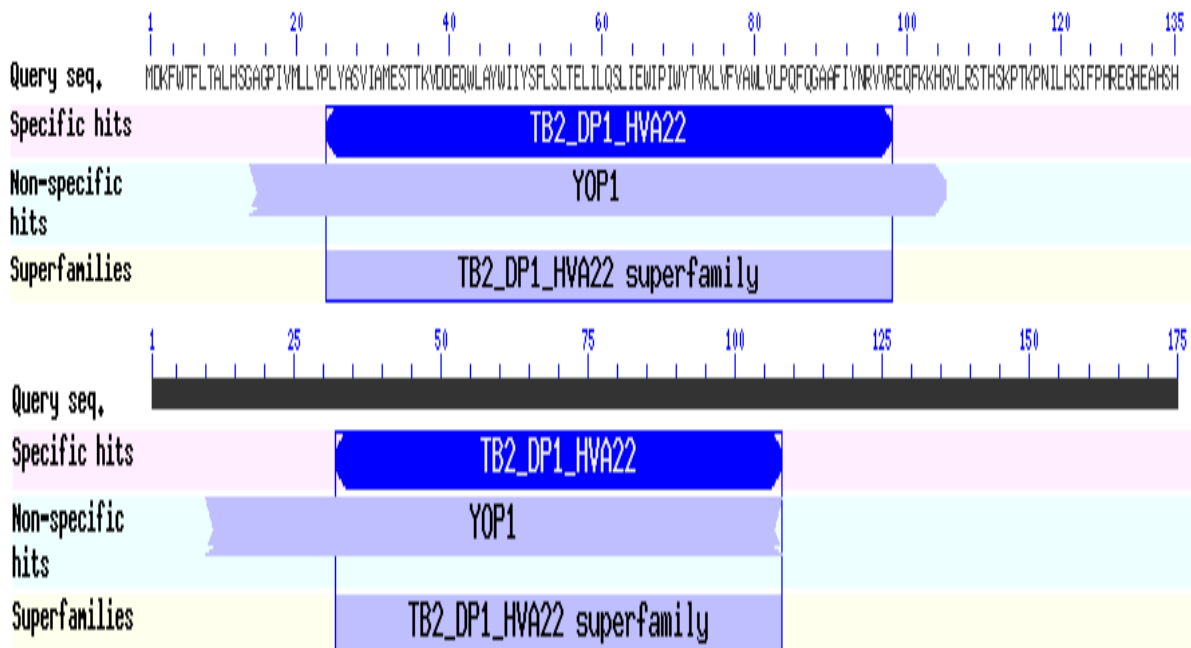


Figure 2d. Comparison of conserved domains found in HVA22A genes of *Arabidopsis thaliana* (above) and *Cynara scolymus* (below) showing presence of similar domains predicted by CD search

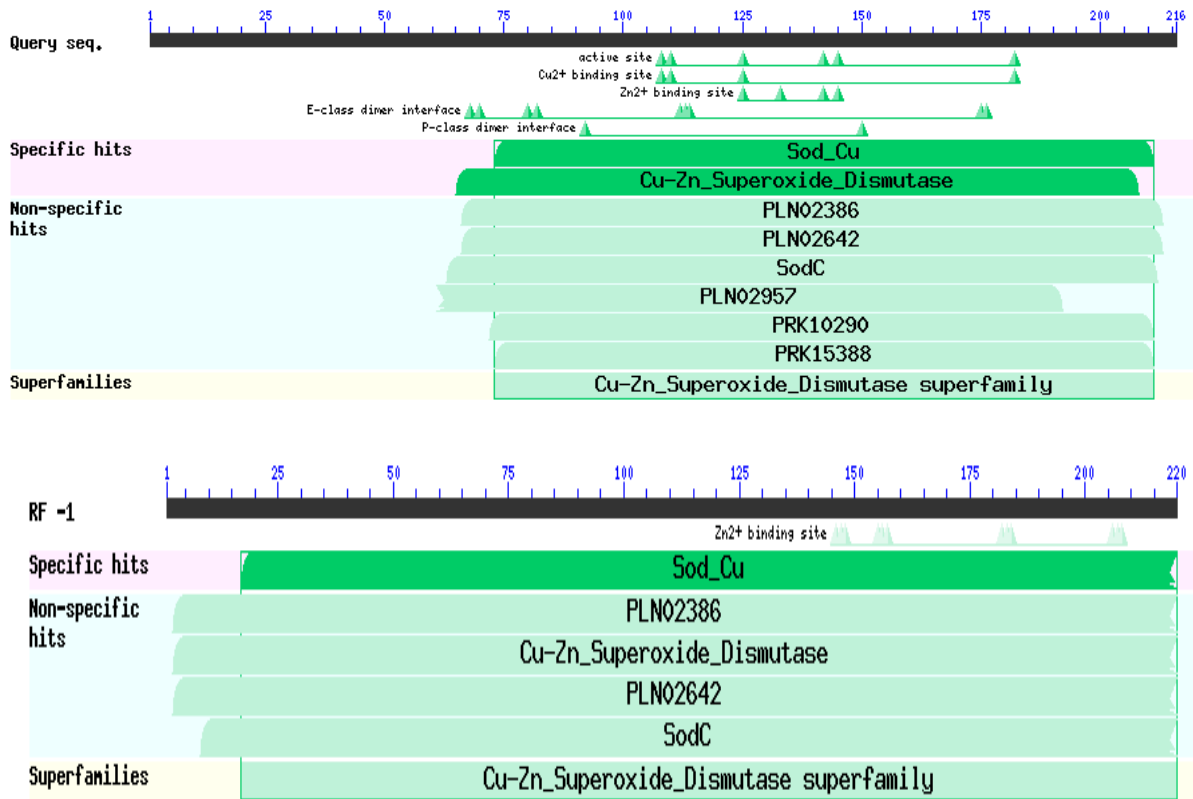


Figure 2e. Comparison of conserved domains found in CSD2 genes of *Arabidopsis thaliana* (above) and *Cynara scolymus* (below) showing presence of similar domains predicted by CD search

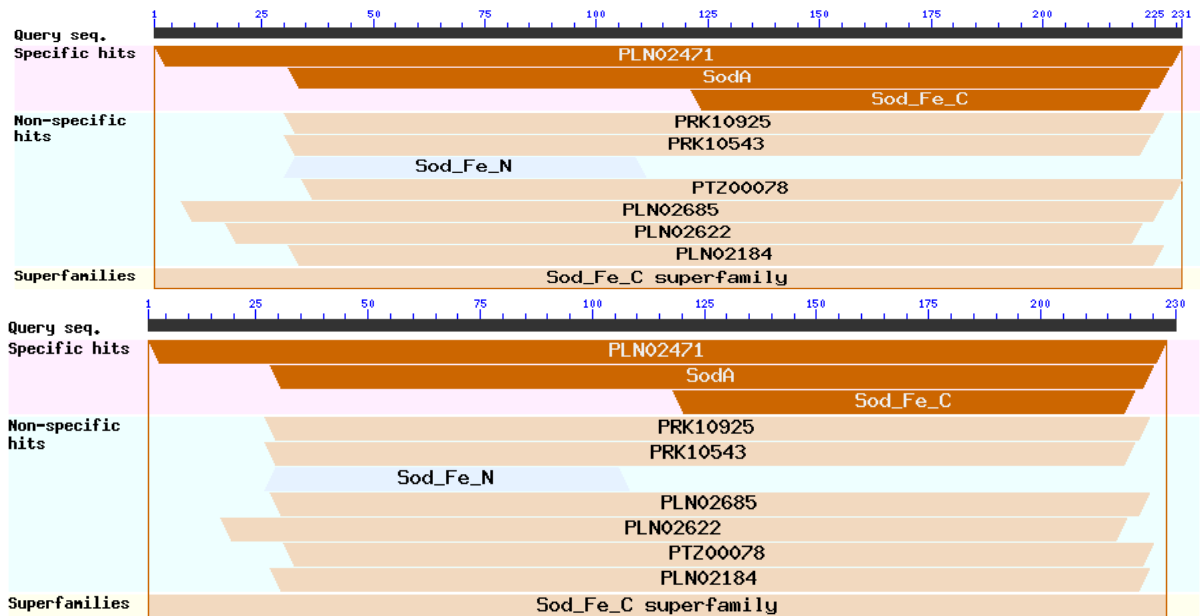
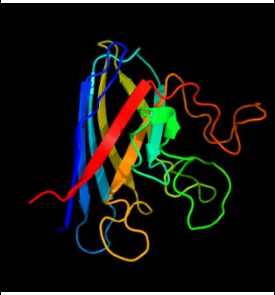
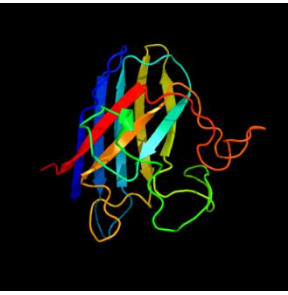
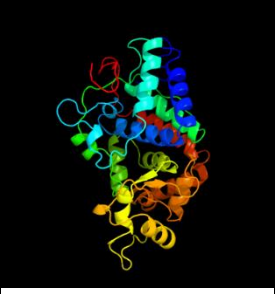

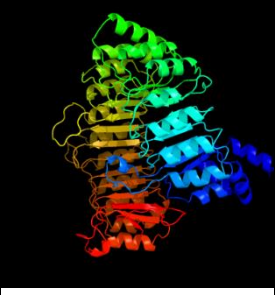
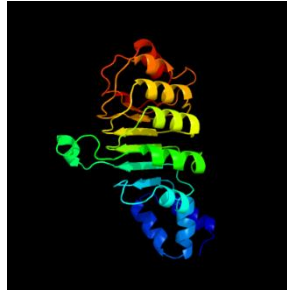


Figure 2f. Comparison of conserved domains found in MSD1 genes of *Arabidopsis thaliana* (above) and *Cynara scolymus* (below) showing presence of similar domains predicted by CD search

Table 3. Comparison of molecular structures of deduced proteins from *Arabidopsis thaliana* reference genes and *Cynara scolymus* target ESTs obtained by ProtParam

Parameters	<i>Arabidopsis thaliana</i> Reference Genes						<i>Cynara scolymus</i> Target ESTs					
	NM_100757.4	NM_100405.4	NM_101152.5	NM_118628.4	NM_128379.4	NM_111929.4	GE599954.1	GE578194.1	GE583634.1	GE589804.1	GE599319.1	GE594969.1
Gene Symbol/BlastP Result	CSD1	RCI3	AFB3	HVA22 A	CSD2	MSD1						
Open reading frame	+3	+1	+1	+2	+2	+1	+1	+2	+3	+3	-3	-2
Start ORF	153	61	382	205	137	76	34	212	204	96	501	32
Stop ORF	611	1041	2115	612	787	771	495	736	851	623	283	724
ORF length	459	981	1734	408	651	696	462	525	648	528	219	693
# of AA	152	326	577	135	216	231	153	174	216	175	72	230
Mol. Wt	15097.68	34905.70	64906.18	15753.41	22243.95	25443.94	15351.05	18532.54	24712.52	20068.94	8250.63	25729.34
Instability index	14.73	36.16	45.62	31.36	27.46	29.27	21.49	48.88	54.79	39.79	24.24	33.97
Aliphatic Index	76.32	88.28	98.37	106.15	86.20	89.48	79.61	99.20	94.72	99.20	79.72	88.70
GRAVY	-0.076	0.006	-0.069	0.194	-0.011	-0.338	-0.178	0.301	0.003	0.115	-0.029	-0.327
Secondary Structure												
Disordered residues (%)	24	21	9	7	43	19	20	24	6	10	19	22
Alpha helix (%)	0	48	36	92	4	60	0	59	39	87	49	58
Beta strand (%)	39	0	12	0	38	10	43	0	8	0	22	9
TM helix (%)	-	5	-	45	-	-	-	9	-	39	-	-
Tertiary Structure												
Residues	152	301	569	12	154	202	152	153	210	56	47	202
Confidence of Model	100	100	100	30	100	100	100	100	100	51	44.4	100
Coverage	100	92	99	9	71	87	99	88	97	32	65	88
<i>Arabidopsis</i> reference genes tertiary structure analogues						<i>Cynara scolymus</i> EST tertiary structure analogues						

	<p>a) Accession: NM_100757.4 PDB header: oxidoreductase Chain: B: PDB molecule: superoxide dismutase PDB Title: Crystal structure of superoxide dismutase from p.2 Atrosanguina</p>		<p>b) Accession: GE599954.1 PDB header: oxidoreductase Chain: B: PDB Molecule:superoxide dismutase; PDBTitle: crystal structure of superoxide dismutase from p.2 Atrosanguina</p>
	<p>c) Accession: NM_100405.4 PDB header: oxidoreductase Chain: A: PDB Molecule:Royal palm tree peroxidase; PDB Title: crystal structure of highly glycosylated peroxidase from royal palm2 tree</p>		<p>d) Accession: GE578194.1 Fold: Heme-dependent peroxidases Superfamily: Heme-dependent peroxidases Family: CCP-like</p>
	<p>e) Accession: NM_101152.5 PDB header:signaling protein Chain: E: PDB Molecule: transport inhibitor response 1 protein; PDB Title: mechanism of auxin perception by the tir1 ubiquitin ligase</p>		<p>f) Accession: GE583634.1 PDB header:signaling protein Chain: E: PDB Molecule: transport inhibitor response 1 protein; PDBTitle: mechanism of auxin perception by the tir1 ubiquitin ligase</p>

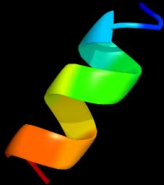
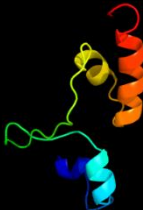
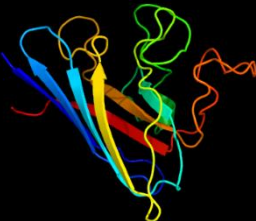



	<p>g) Accession: NM_118628.4 PDB header:photosynthesis Chain: I: PDB Molecule: photosystem i reaction center subunit viii; PDB Title: crystal structure of plant photosystem i at 3 angstrom resolution</p>		<p>h) Accession: GE589804.1 Fold: Acyl carrier protein-like Superfamily: Retrovirus capsid dimerization domain-like Family: SCAN domain</p>
	<p>i) Accession: NM_128379.4 Fold:Immunoglobulin-like beta-sandwich Superfamily:Cu,Zn superoxide dismutase- like Family:Cu,Zn superoxide dismutase-like</p>		<p>j) Accession: GE599319.1 PDB header: transcription activator/dna Chain: D: PDB Molecule:iron-regulated transcriptional activator aft2; PDBTitle: crystal structure of aft2 in complex with dna</p>
	<p>k) Accession: NM_111929.4 PDB header: oxidoreductase Chain: B: PDB Molecule: superoxide dismutase [mn] 1, mitochondrial; PDB Title: crystal structure of manganese superoxide dismutase from arabidopsis2 thaliana</p>		<p>l) Accession: GE594969.1 PDB header:oxidoreductase Chain: C: PDB Molecule:superoxide dismutase [mn/fe]; PDB Title: superoxide dismutase from aeropyrum pernix k1, apo-form</p>

Fig 4 (a-l). Tertiary structure analogues obtained by Phyre2 online analysis server from deduced proteins of *Arabidopsis thaliana* reference genes and *Cynara scolymus* target ESTs

Discussion

Cynara scolymus L. thrives well in saline areas and is regarded as a moderately salt resistant crop, but very little information is available about its salt stress tolerance mechanism. However various researchers have worked out to comprehend the effect of salt stress on phenolic compounds and antioxidant activity of its leaves [8]. The microRNA (miRNA) analysis for *Cynara scolymus* has also been carried and many salt stress genes are reported to be targeted by miRNAs [15].

This study resulted in the bioinformatics identification of salt stress genes in this important plant. All selected genes have been predicted in ESTs; confirming their expression in *Cynara scolymus*. Results completely supported our hypothesis.

Many researchers have used bioinformatics approach to identify salt stress responsive genes and microRNAs in plants [16, 17]. EST analysis is an important approach to identify new conserved genes [18]. Researchers prefer to screen out the EST sequences (if available) to find out conserved sequences due to confirmation of their expression [19, 20]. To understand the transcriptional profiling of genes whose expression levels alter in response to salt stress, different EST/cDNA collections have been utilized in plants [21].

As compared to nucleotide sequences query coverage, protein sequence query coverage is greater due to codon degeneracy and due to the fact that DNA alignment doesn't take into account the redundancy of amino acid codons. Furthermore, DNA alignment also doesn't consider the more similar structures of some amino acids to others and the similar functional role they have in the protein e.g. Isoleucine and valine have similar structures, both have hydrophobic side chains and differ only by the addition of an extra carbon on isoleucine. A mutation from one to the other is not as likely to substantially change the

protein structure as some other mutations would, but a DNA sequence alignment treats this mutation the same as any other. Therefore both approaches were adopted in this research. The higher query coverage in case of BlastP as compared to Blastn for all six genes is upto the mark.

This research also investigated the presence of conserved domains on reference genes (*Arabidopsis thaliana*) and their predicted orthologs (*Cynara scolymus* L.) Presence of same conserved domains in all predicted genes provides a clue of similar function. All the predicted genes share similar super families and specific hits and therefore a high confidence level for the inferred function of the protein query sequence.

The amino acid sequence of a protein is known to determine the secondary and tertiary structure of a protein molecule [22]. In the current analysis the deduced secondary protein structures also show comparable physical properties including molecular stability, aliphatic index and GRAVY thus inferring similar results as above. This is also evident by the presence of trans-membrane helices in *Arabidopsis* reference genes RCI3 and HVA22A and their target ESTs in *Cynara scolymus* (Accession No.s GE578194.1 and GE589804.1). It is known that genes for proteins with structural and functional similarity have similarities in secondary structures leading to comparable three dimensional tertiary structures. This is relevant for similarity in their functional properties. Moreover, the tertiary structure analogs of *Cynara scolymus* EST Acession GE599954.1 (superoxide dismutase), GE578194.1 (heme-dependent peroxidase), GE583634.1 (transport inhibitor response 1 protein) and GE594969.1 (molecule superoxide dismutase) showed 100% confidence of model with analogs of their respective *Arabidopsis* reference genes. These evident similarities lead to the conclusion that the targeted ESTs possessed

similar structure and function as their reference genes. Thus, indicating the possibility of their orthologous origin [23]. Many scientists are pursuing similar in-silico (bioinformatics) approaches to identify genes related to stress tolerance in plants from available gene pool of plant genomes [24, 25]. This saves researchers from unwanted waste of time and potential funds. Due to the above findings it is plausible to say that *Arabidopsis* conserved domains can be used to predict orthologs in *Cynara scolymus* which can further be used to identify potential genes for salt tolerance in plants.

Conclusion

It is predicted that in *C. scolymus* CSD1, MSD1, CSD2, AFB3, RCI3 and HVA22A salt stress responsive genes are present which may potentially help this plant against salinity. Bioinformatics identification of salt stress responsive genes in *C. scolymus* and their molecular annotation will be a good contribution in understanding the acclimation process of this plant under saline conditions.

Authors' contributions

Conceived and designed the experiments

IA Baloch & S Johar,

Performed the experiments:

S Johar, BH Mengal & AK Alvi,

Analyzed the data: MN Shawani & SS Qambrani,

Wrote the paper:

IA Baloch & B Sadia.

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