Real time expression and in silico characterization of pea genes involved in salt and water-deficit stress

Muhammad Farooq (9argus8@gmail.com)
COMSATS Institute of Information Technology - Abbottabad Campus: COMSATS University Islamabad - Abbottabad Campus https://orcid.org/0000-0001-9438-4188

Rafiq Ahmad
COMSATS University Islamabad - Abbottabad Campus

Muhammad Shahzad
COMSATS University Islamabad - Abbottabad Campus

Saad Ur Rehman
GPGC Mandian Abbottabad: Government Post Graduate College Mandian Abottabad

Yasar Sajjad
COMSATS University Islamabad - Abbottabad Campus

Amjad Hassan
COMSATS University Islamabad - Abbottabad Campus

Mohammad Maroof Shah
COMSATS University Islamabad - Abbottabad Campus

Amber Afroz
University of Gujrat

Sabaz Ali Khan
COMSATS University Islamabad - Abbottabad Campus

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Abstract

Background

To tolerate salt and water-deficit stress, the plant adapts to the adverse environment by regulating its metabolism and expressing certain stress-induced metabolic pathways. This research analyzed the relative expression of four pea genes (*proC*, *PAL1*, *SOD*, and *POX*) in three pea varieties (Climax, Green grass, and Meteor) under different levels of salt and water-deficit stress.

Methods and Results

Results from RT-qPCR analysis showed increased expression of *proC*, *PAL1*, and *POX* genes, while *SOD* gene expression decreased under both stresses. Climax exhibited superior stress tolerance with elevated expression of *proC* and *PAL1*, while Meteor showed better tolerance through increased *POX* expression. Phylogenetic analysis revealed common ancestry with other species like chickpea, red clover, mung bean, and barrel clover, suggesting about the cross relationship among these plant species. Conserved domain analysis of respective proteins revealed that these proteins contain PLNO 2688, PLN02457, Cu-Zn Superoxide dismutase, and secretory peroxidase conserved domains. Furthermore, protein family classification indicated that the oxidation-reduction process is the most common chemical process involved in these stresses given to peas which validate the relationship of these proteins.

Conclusions

Salt and water-deficit stresses trigger distinct metabolic pathways, leading to the upregulation of specific genes and the synthesis of corresponding proteins. These findings further emphasize the conservation of stress-tolerance-related genes and proteins across various plant species. This knowledge enhances our understanding of plant adaptation to stress and offers opportunities for developing strategies to improve stress resilience in crops, thereby addressing global food security challenges.

Introduction

To tolerate salt and water-deficit stress, expression of complex physiological traits and biosynthetic or metabolic pathways is required for plants (Hossain, et al., 2017; Dos Santos et al., 2022). Both abiotic and biotic stresses result in the upregulation and downregulation of the electron transport chain. Under stress conditions, O_2 acts as the final electron acceptor and thus generating reactive oxygen species (ROS). ROS includes O , H_2O_2, OH , and $^1O_2$. Because of being a strong oxidizing agent, ROS is lethal for plant cells (Groß et al., 2013; Gupta and Huang, 2014; Hasanuzzaman et al., 2020; Sachdev et al., 2021). For detoxification of ROS, antioxidant enzymes, and non-enzymatic molecules are important plant defense mechanisms. Antioxidant enzymes comprise ascorbate peroxidase (APX), glutathione peroxidise (GPX) glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) (Gupta et al., 2005;

Rajput et al., 2021). The antioxidant system plays its role to strengthen plant tolerance under salt stress (Ahmad et al., 2012; Hasanuzzaman et al., 2020) and drought stress (Yasar et al., 2014), while plants having higher antioxidants level showed greater resistance to damage caused by oxidative stress (Ahmad et al., 2012; Rajput et al., 2021). Several transcription factors regulate stress-related genes (Joshi et al., 2015, 2016). Similarly, the formation of non-enzymatic antioxidant molecules is also reported in salt stress (Gupta et al., 2005; Hasanuzzaman et al., 2020). The plants stress related defense mechanisms are controlled by multiple gene families which can be grouped into three classes. The first class includes those genes which play a role in the direct protection of cellular membranes and major proteins. Osmo protectants (*P5CS; P5CDH*), late embryogenesis abundant (LEA) proteins, metabolites (glycine betaine, proline, *etc.*), free radical scavengers, heat shock proteins, and chaperons belong to the first class of genes (Joshi et al., 2015, 2016). The second class has ion channels and membrane transporters like *SOS1* and *NHX1* regulating the uptake of water and ions. In the third class, several transcription factors like bZIP and DREB regulate stress-related genes (Joshi et al., 2015, 2016). Plants can regulate the expression of different genes that play a role in salt stress and drought stress tolerance (Carraro and Di Iorio, 2022) by modifying morphological, biochemical, physiological, molecular, and cellular pathways (Chen et al., 2014). These mechanisms include compatible solute synthesis, ion transport system, senescence response, hormonal regulation, antioxidant enzyme synthesis, and Ca^{2+} signaling pathways. These signaling pathways are controlled by several genes and transcriptional factors (Gupta and Huang, 2014).

Phylogenetic analysis of gene sequences of plant species has great potential for addressing a variety of questions and accordingly adds significant insights into biology (Hall et al., 2002; Doyle *et al.*, 2003). It helps in better understanding of phylogenetic characteristics of plants (Soltis and Soltis, 2003). The importance of allocating model organisms to the proper phylogenetic context will surely give a superior perception and understanding of the process of evolution (Soltis and Soltis, 2003). For comparative genomics, phylogenetic analysis act as a foundation (Soltis and Soltis, 2000; Walbot, 2000; Kellogg, 2001; Hall et al., 2002; Mitchell-Olds and Clauss, 2002; Pryer et al., 2002; Doyle and Luckow, 2003). The specific domain of a protein represents the unit of conserved function that can form a tertiary structure and can exist, function, and evolve separately from the rest of the amino acid chain of the protein. These domains have an independent ability to fold into three-dimensional organizations and are also stable independently. Many structural domains can be found in most proteins. Different types of proteins may contain one similar domain (Ribeiro et al., 2019) and these domains are conserved at structural and sequence levels (Rasouli et al., 2013). Domains are used as building blocks in the evolutionary process and these are recombined in different arrangements to make proteins with different functions (Aziz and Caetano-Anolles, 2021). These are stable independently and through genetic engineering can be swapped to make chimeric proteins (Ribeiro et al., 2019). New sequences are generated by nature using domains as raw material and can be considered genetically mobile units (Ren et al., 2008). But most of these domains have not yet been characterized according to their functions (El-Gebali et al., 2019).

The tribe Fabeae (formerly Vicieae) of the family Fabaceae holds several of humanity's most influential leguminous crops. It is necessary for a better perception of the origin and diversity of legume crops to reconstruct the phylogenetic connection among these species (Smykal *et al.*, 2011). Pea (*Pisum sativum* L.) belongs to the world's oldest domesticated crops (Zohary and Hopf, 2000; Smykal *et al.*, 2017). At the global level, it belongs to the third most grown food crop and is a good protein source for humans and livestock. Currently, it is cultivated in temperate zones around the globe. It was used by Mendel (1866) and Knight (1799) in the initial and first studies of plant genetics. Due to the huge size of its genome (4.45 gigabases, GB) and the presence of highly repetitive sequences (Macas et al., 2007) a significant part of current advancement in genomics and molecular genetics is not applied to *P. sativum* (Smykal *et al.*, 2011). The current study was thus conducted to analyze the relative expression and *in silico* characterization of pea genes that are involved in providing tolerance under salt stress and water-deficit stress.

Materials and methods

Experimental setup

The salt stress and drought stress studies were conducted as pots experiment in a greenhouse at COMSATS University Islamabad-Abbottabad Campus.

Plant materials and growth conditions

The seeds of pea varieties Meteor, Green grass, and Climax with varying salt (Shahid et al., 2012) and drought tolerance potential (Khan et al., 2013) were collected from Kisan Agro Seeds shop, Mansehra. Surface sterilization of seeds was done with 30% commercial bleach for 10 min. For the germination, 5 seeds of each pea variety for each control and treatment with replications for salt stress and drought stress experiment were soaked overnight in warm water and finally planted in the pots containing soil. Physio-chemical analysis of the soil used for sowing the seeds was done as reported in our other article (Farooq *et al.*, Submitted).

Number and level of salt and water-deficit treatments

Saturation percentage was utilized for the development of artificial salinity in the pots, *i.e.*, Control without salt treatment, Treatment 1: 50 mM salt treatment, Treatment 2: 75 mM salt treatment, and Treatment 3: 100 mM salt treatment. There were a total of 12 pots with five seeds in each pot. Field capacity (FC) was used for the development of artificial drought treatments in the pots, *i.e.*, Treatment 1 (Control; water application 100% of FC), Treatment 2 (water application 75% of FC), and Treatment 3 (water application 50% of FC). There were a total of 9 pots including all the treatments and control with five seeds in each pot. Drought treatment was started after 5 days of germination till harvesting.

Gene expression studies

Selection of genes involved in salt and water-deficit stresses

Pea genes involved in biosynthetic pathways of proline, flavonoids, and enzymatic antioxidant enzymes including SOD and peroxidase (POX) that provide tolerance against salt stress and water-deficit stress were selected for current analysis. The sequences of these genes were retrieved from NCBI and then online software Primer3Plus was used to design specific primers of these four selected pea genes (Table 1).

Table 1
Genes and primer sequences of pea genes involved in biosynthetic pathways and enzymatic antioxidant system under salt stress and water-deficit stress along with the housekeeping gene *Actin.*

Accession ID		Primers	Tm(°C)
X62842.1	Proline (*proC*)	F: 5' TACCCTCTTCCCGCATCGTA 3'	60.1
		R: 5' CTGCAGCAGGTGTATTTGGC 3'	59.8
D10002.1	Flavonoids (*PAL1*)	F: 5' GACACACTTGGGGGTGCTAT 3'	59.9
		R: 5' CGGGCTAACCTCAACAACAT 3'	60.0
AB087845.1	Superoxide dismutase (*SOD*)	F: 5' CTGTTGGGGTGTCCTGAGAT 3'	60.0
		R: 5' GTTTGTGGTGTCTCCCAAGG 3'	60.4
AB193820.1	Peroxidase (*POX*)	F: 5' ATGCCCTAAGGGTGGAAGTG 3'	59.4
		R: 5' TGTTCCCAGTAAGCACACCA 3'	59.2
X90378.1	*Actin*	F: 5' CCACTTCTGCAGAGCGAGAA 3'	60.0
		R: 5' CGGAGATTCCATGCCGATCA 3'	60.0

F: Forward primer, R: Reverse primer.

RNA extraction and quantification

For RNA extraction the leaf samples from each plant variety including all the treatments and controls were collected after two weeks of salt stress and drought stress treatments. CTAB (Cetyl trimethyl ammonium bromide) protocol was used for RNA extraction as reported by (Barbier et al., 2019) with minor modifications. A plant leaves sample (100 mg) was pooled from 5 plants and homogenized using a pestle and mortar in liquid nitrogen and transferred to a 1.5 mL Eppendorf tube. CTAB-β-mercaptoethanol (1%) (600 µL) solution preheated for 1 hour (hr) at 65°C was subsequently added to the Eppendorf tube and allowed incubation at room temperature for 15 min. Centrifugation was allowed at 4°C (12000 rpm) for 15 min. The supernatant was pipetted out in a newly labeled Eppendorf tube and added with 600 µL of chilled chloroform-isoamyl alcohol (24:1) solution. Centrifugation was performed at

12000 rpm (4°C) for 15 min. The supernatant was pipetted out in a newly labeled Eppendorf tube and added with ¼ 10M lithium chloride of total supernatant volume. The sample was kept at -20°C for overnight and the next day again centrifugation was achieved at 12000 rpm (4°C) for 15 min. As the pellet was formed at the bottom of the Eppendorf tube so supernatant was discarded and 70% ethanol was added to the pellet. Again, centrifugation was carried out at 6500 rpm (4°C) for 5 min. The pellet was allowed to air dry, while the supernatant was discarded. Finally, the pellet was suspended in 50 µL of DEPC water and kept at -80°C for further analysis.

The concentration and purity of the RNA samples were assessed using UV Spectrophotometric (Mapada UV 1200 approach at the 260 nm wavelength. 2 µl of RNA sample was diluted in 1998 µl of double distilled water. For quantifying RNA, optical density readings were observed at wavelengths of 260 nm and 280 nm while distilled water was utilized as a blank. The RNA concentration was quantified at the wavelength of 260 nm, while RNA purity was calculated by the ratio of 260 and 280 nm.

$$RNAConc\left(\frac{ng}{\mu \mathrm{l}}\right) = \mathrm{OD260xDFX40}$$

Where DF = volume of RNA / total volume

Complementary DNA (cDNA) synthesis

Enzynomics cDNA synthesis kit (TOPsccriptTM) was used for cDNA synthesis. RNA extract was added with autoclaved distilled water and allowed 5 min incubation at 70°C. After adding Oligo dT primers, the mixture was kept on the ice, and the master mix was made by using reverse transcriptase enzyme (200 U/µl), RNase inhibitor (40 U/µl), dNTPs mixture (each 2mM), and TOPscriptTM RT-buffer (10X). The quantity of master mix used for each sample was 5.5 µl and the final volume was made 20 µl. After the addition of the master mix into each sample, it was homogenized and incubated twice for 1hr (50°C) and 5 min (95°C). cDNA was kept at -80 $^{\circ}C$ for further analysis.

Quantitative Real-Time PCR (RT-qPCR) analysis

Quantitative real-time PCR using cDNA as a template was used to analyze the gene expression. Threshold cycle (Ct) values were scored when the amplicon number measured was directly proportional to the starting amount. Amplification was carried out utilizing Thermo Scientific Maxima SYBR Green q RT-PCR Master Mixes in "Low profile PCR tubes". The reaction mixture (10 µL) contained 1 µL cDNA, 5 µM each pair of specific primers, and 2 µL RT-qPCR Master Mix (having Hot Start Taq DNA Polymerase and buffer). Agilent real-time PCR system was used for the amplification of all q RT-PCR reactions. The procedure of q RT-PCR was as follows: 95°C for 5 min (denaturation); 95°C for the 30s (denaturation), 30s for annealing temperature that differed according to the primer pair, and 72°C for 30s (polymerization). In each run of q RT-PCR gene of interest and reference gene were tested.

Gene's relative expression (Fold) was calculated by the 'Delta, Delta Ct method' (Livak and Schmittgen, 2001) using the equation:

$R = 2[Ct\ Sample - Ct\ Control]$

$R = 2^{-\Delta Ct}$

$R = 2^{-[\Delta Ct\ Sample - \Delta Ct\ Control]}$

$R = 2^{-\Delta\Delta Ct}$

Housekeeping or reference genes are utilized for the normalization of RT-qPCR. These genes show constitutive expression in several different types of cells and tissues and show minimum or no change in their expression in experimental conditions (Reboucas *et al.*, 2013). Actin was previously utilized as a reference gene in plant gene expression studies (Wang *et al.*, 2017; Knopkiewic & Wojtaszek, 2019). In the present research, *the Ps-Actin* gene was used as a reference gene.

Phylogenetic analysis and in silico characterization of the selected genes

Phylogenetic and conserved domains analysis

Pea genes were analyzed for phylogenetic analysis in closely related crops having similarity percent identity 80% and above. The sequences of genes and proteins of the pea were downloaded from the NCBI Genbank database. Phylogenetic analysis was carried out by generating the FASTA sequence of the particular gene using the gene accession number at the NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/). The basic local alignment search tool (BLAST) of the NCBI was used to search identical/similar sequences against the query. In the next step, the five most similar sequences to the query sequence were selected based upon a percent ID of 80% and above in closely related crops. Finally, CLUSTAL W was used for multiple sequence alignment for the generation of the phylogenetic tree (https://www.genome.jp/tools-bin/clustalw).

In silico characterization of selected proteins used in this study was carried out by using gene Accession ID to retrieve the desired Protein ID from the NCBI website (Table 2). Using Protein ID FASTA sequence of desired protein was retrieved. Finally, analysis was carried out for conserved domains of selected proteins of a pea to that of other related crops through alignment at the NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Table 2
Pea biosynthetic pathways and enzymatic antioxidant system genes involved in salt and water-deficit stress and their corresponding protein IDs.

Metabolite/enzyme	Gene accession IDs	Protein IDs
Proline	X62842.1	CAA44646.1
Flavonoids	D10002.1	BAA00886.1
Superoxide Dismutase	AB087845.1	BAC81657.1
Peroxidase	AB193820.1	BAD97439.1

Classification of protein families based on CDD

After the identification of conserved domains, classification of domains was performed. The classification leads to the families of the domains. The family classification was done by the INTERPRO online server (https://www.ebi.ac.uk/interpro/).

Statistical analysis

Pearson correlation analysis was carried out among the scored traits using SPSS software.

Results

Expression of the selected genes under salt stress

Proline biosynthesis pathway gene *proC* (accession number X62842.1) showed increased relative expression in the Climax variety as compared to Green grass and Meteor in all the salt treatments. The maximum relative expression was observed in Climax in 100 mM salt treatment, *i.e.*, 2.26 fold. Meteor showed a minimum relative expression in 100 mM salt treatment, *i.e.*, 1.52 fold. Green grass showed 1.56-fold greater relative expression in 100 mM salt treatment than control (Fig. 1). Flavonoids biosynthesis pathway gene *PAL1* (accession number D10002.1) showed no obvious change in the relative expression at 50 mM and 75 mM salt treatments in all three pea varieties. However, Climax showed a maximum (2.98 folds) relative expression and Meteor showed a minimum (1.81 folds) relative expression as compared to control at 100 mM salt treatment (Fig. 1). The superoxide dismutase (accession number AB087845.1) biosynthesis gene showed decreased relative expression under all the salt treatments in all three varieties. Salt treatment at a concentration of 100 mM caused pronounced effects on the relative expression of all three varieties being less than 0.30 folds as compared to the control Peroxidase (accession number AB193820.1) biosynthesis gene showed a maximum relative expression in Meteor in 100 mM salt treatment, *i.e.*, 1.52 fold as compared to control. Green grass showed a minimum relative expression at 100 mM treatment, *i.e.*, 1.34 fold, while Climax showed 1.36 fold expression in the same treatment as compared to the controls (Fig. 1).

Correlation among genes under salt stress

Table 3 shows the Pearson correlations among studied parameters at $P \leq 0.01$ and $P \leq 0.05$ under salt stress. Relative expression analysis of *SOD* showed a significantly negative correlation with the expression of *P5CR*, *PAL1*, and *POX*, while expression analysis of *P5CR*, *PAL1*, and *POX* showed a significantly positive correlation with each other.

Table 3
Pearson correlation among *P5CR*, *PAL1*, *SOD* and *POX* under salt stress.

	P5CR	*PAL1*	*SOD*	*POX*
P5CR		.908**	− .768**	.694*
PAL1	.908**		− .696*	.713**
SOD	− .768**	− .696*		− .832**
POD	.694*	.713**	− .832**	

**. Correlation is significant at the 0.01 level. *. Correlation is significant at the 0.05 level.

Expression of the selected genes under water-deficit conditions

Proline biosynthesis pathway gene *proC* showed increased relative expression against increasing levels of water deficit conditions. The relative expression of the *proC* gene showed an increased level in Climax as compared to Green grass and Meteor at 75% and 50% water deficit conditions. The maximum (2.55 folds) relative expression was observed in Climax at 50% FC drought treatment and Meteor showed a minimum (1.53 folds) relative expression in the same treatment, while Green grass showed 2.1 folds relative expression at 50% FC drought treatment (Fig. 2). Flavonoids biosynthesis pathway gene *PAL1* showed a maximum relative expression in Climax, *i.e.*, 2.56 fold as compared to the control, while Green grass showed a minimum relative expression, *i.e.*, 1.83 fold as compared to control at 50% water deficit conditions. The superoxide dismutase biosynthesis gene showed decreased relative expression under all water deficit treatments in all three varieties. However, 50% water deficit treatment caused noticeable effects on the relative expression of all three varieties being less than 0.56 fold as compared to the control. Meteor showed a minimum relative expression, *i.e.*, 0.36 fold as compared to control, while Green grass showed a maximum relative expression, *i.e.*, 0.55 fold at 50% water deficit treatment (Fig. 2). Peroxidase biosynthesis gene showed a maximum relative expression in Meteor at 50% of water deficit conditions, *i.e.*, 1.43 fold as compared to the control, while Green grass showed a minimum relative expression, *i.e.*, 1.37 fold as compared to Climax and Meteor at 50% water deficit conditions.

Correlation among Genes under water-deficit conditions

Table 4 shows the Pearson correlations among studied parameters at P ≤ 0.01 and P ≤ 0.05 under drought stress. Relative expression analysis of *SOD* showed a significantly negative correlation with the expression of *P5CR*, *PAL1*, and *POX*, while expression analysis of *P5CR*, *PAL1*, and *POX* showed a significant and positive correlation with each other under drought stress.

Table 4
Pearson correlation among *P5CR*, *PAL1*, *SOD* and *POX* under drought stress.

	P5CR	***PAL1***	***SOD***	***POX***
P5CR		.941**	− .676*	.790*
PAL1	.941**		− .803**	.898**
SOD	− .676*	− .803**		− .852**
POD	.790*	.898**	− .852**	
**. Correlation is significant at the 0.01 level.*. Correlation is significant at the 0.05 level.				

Phylogenetic analysis of the selected salt and water deficit stress-related genes of pea

A phylogenetic analysis of selected genes was carried out (Fig. 3). The cladistics analysis showed *P. sativum* proline biosynthesis pathway gene *proC* shared common ancestry with *Cicer arietinum* having a common ancestor (Fig. 3A). The Flavonoid biosynthesis pathway gene *PsPAL1* showed common ancestry with *Trifolium pratense* (Fig. 3B). *PsSOD* showed common ancestry with *Vigna radiata*. *PsPOX* showed the same ancestry as *Medicago truncatula* (Fig. 3D).

Analysis of conserved domains of respective proteins

Domains are primarily identified as folding units that are structurally independent and are conserved functional units that could carry one or more motifs. These are conserved at sequence and structure levels (Rasouli et al., 2013). Here conserved domains were identified from NCBI CDD. Pyrroline-5-carboxylate reductase (P5CR) of *P. sativum* contained PLNO 2688 conserved domain in its amino acid sequence (Fig. 4A). *P. sativum* phenylalanine ammonia-lyase (PAL) contained PLN02457 conserved domain (Fig. 4B). *P. sativum* SOD contained Cu-Zn Superoxide dismutase conserved domain (Fig. 4C). *P. sativum* POX contained secretory peroxidase conserved domain (Fig. 4D).

Classification of proteins based on CDD

Family classification of proteins was carried out using the INTERPRO server; by the classification of the proteins, proteins can be easily related to domains through, which we can recognize the exact purpose of the proteins we can easily relate proteins to domain and can identify the exact purpose and function of

the proteins. In this study, the homologous families, biological process, and molecular function of the selected proteins were identified and classified based on their function and homologous superfamilies as shown in Table 5.

Table 5
Protein families and their biological processes and molecular functions involved in salt stress and water-deficit stress in pea.

Protein IDs	Protein family	Biological Process	Molecular Function
CAA44646.1	Pyrroline-5-carboxylate reductase	Proline biosynthesis oxidation-reduction process	Pyrroline-5-carboxylate reductase activity
BAA00886.1	Aromatic amino acid lyase	L-phenylalanine catabolic process	Catalytic activity ammonia-lyase activity
BAC81657.1	Superoxide dismutase (Cu/Zn)	Superoxide metabolic process	Metal ion binding Superoxide dismutase activity
BAD97439.1	Plant peroxidase	Response to oxidative stress	Peroxidase activity Heme binding

Pyrroline-5-carboxylate reductase (P5CR) is the protein that catalysis the final step in the proline biosynthesis from glutamate. It is also capable of converting delta-1-piperideine-6-carboxylate (P6C) into pipecolic acid (Struys et al., 2014) and its expression enhances in stress circumstances (Delauney and Verma, 1990; Hu et al., 1992; Delauney and Verma, 1993). PAL turns phenylalanine to coumaroyl-CoA the first step in the phenylpropanoid pathway (Bonawitz and Chapple, 2010; Hyun *et al.*, 2011) in the flavonoids biosynthesis and is associated with ER (Kumar et al., 2018). SOD is an essential antioxidant that protects against oxidative damage. (Foyer and Halliwell, 1976; Yahya et al., 2021). SOD is the most important O_2^- scavenger and converts via an enzymatic reaction into H_2O_2 and O_2. As H_2O_2 is toxic so CAT and POX are required to break it down into water and oxygen. (Halliwell, 1976; Ahmad et al., 2012). Plant peroxidases are monomeric glycoproteins with two calcium ions and four conserved disulfide bridges (Schuller et al., 1996).

Discussion

Plant growth and development are strongly influenced by several kinds of abiotic and biotic stresses like insects, pathogens, cold, salinity, and water-deficit stress (Suzuki et al., 2014). To assess the pea response at the genetic level against salt stress and water-deficit stress, the relative expression of four pea genes was analyzed under varying levels of salt stress treatments, *i.e.*, Control (5.4 mM), 50 mM, 75 mM, 100mM and water-deficit stress treatments, *i.e.*, water irrigated to 100%, 75%, and 50% of filed capacity. RT-qPCR analysis was carried out to find out the expression level of salt and water-deficit stress-

related genes. Genes, D1-pyrroline carboxylate reductase (*proC*), phenylalanine ammonia-lyase (*PAL1*), superoxide dismutase (*SOD*), and peroxidase (*POX*) were used for relative expression analysis in salt stress and water-deficit stress treated pea plants. In the current study, an increase in the relative expression of *proC* was detected with increasing levels of both salt stress and water-deficit stress as compared to controls. Climax showed maximum relative expression of *proC* as compared to Green grass and Meteor at 100 mM salt stress treatment and 100% water-deficit treatment. *proC* is involved in the proline biosynthesis pathway and its expression is enhanced under stress circumstances including salt and water-deficit stresses (Maghsoudi et al., 2018). It uses NADPH as a cofactor and is the primary enzyme in the proline biosynthesis pathway. It is reported that its expression is upregulated under salt stress and water-deficit stress (Deuschl *et al.*, 2004; Chen et al., 2021). Proline accumulation is a common response of plants to different stress conditions, and it acts as an osmoprotectant (compatible metabolite), a safeguarding factor for enzymes and cellular membranes. It plays a role in the scavenging of ROS once formed and provides a transient reserve of organic nitrogen to plants (Deuschl *et al.*, 2004; Nguyen et al., 2021). In the present study, no obvious change was observed in the relative expression of the *PAL1* gene at 50 mM and 75 mM salt treatments as compared to the controls in all three pea varieties. However, 100 mM salt treatment resulted in elevated levels of relative expression of *PAL1* in all the varieties. Climax showed better tolerance by maintaining maximum relative expression as compared to the other two varieties. Similar results were also observed in the water-deficit stress experiment suggesting a common response of both kinds of stresses. *PAL1* is the flavonoid biosynthesis pathway gene (Hyun *et al.*, 2011; Liu et al., 2021), which is an integral part of the plant antioxidant defense system against ROS (Kumar et al., 2018). The involvement of *PAL1* in plant defense system against abiotic stress including salt stress leads to the accumulation of phenolic compounds like flavonoids (Hou et al., 2019). During plant exposure to stress-like water-deficit conditions, flavonoids are generated as scavengers of ROS (Mahajan and Yadav, 2014; Kumar et al., 2018).

In the current study, the *SOD* gene showed a decline in the relative expression under all salt and water-deficit treatments as compared to the control in all three varieties. A considerable effect of 100 mM salt treatment and 50% of water-deficit treatment was observed on the relative expression of *SOD* in all three varieties. The relative expression of the *POX* gene showed a rise under higher levels of both salt and water-deficit stress as compared to the control treatments in all three varieties. Maximum relative expression was observed in Meteor at 100 mM salt treatment and 50% of water-deficit treatment. *SOD* and *POX* constitute the plant's antioxidant defense system to strengthen plant tolerance to salt stress (Ahmad et al., 2012; Dixit, 2022). *SOD* is the key player in the first line of plant defense against ROS activity and is considered a stable marker for tolerance to several forms of abiotic stress (Berwal and Ram, 2014). The decline in the relative expression of *SOD* in current analyses can be attributed to *SOD* isoforms. Since it is well established that the majority of plant species have many *SOD* isoforms that differ in their active site (Berwal and Ram, 2014). In a recent study, it was revealed that the expression of *POX* increased under salt stress in soybean, and overexpression of *POX i*nduced salt tolerance (Jin et al., 2019). *POX* plays a crucial role to eliminate ROS and H_2O_2 in rice (Cui et al., 2015).

Phylogenetics is the study concerning the evolutionary genetic relationship among various organisms (Potter, 2008; Newman et al., 2016). Here, we have examined the utility and effectiveness of conventional alignment-based phylogenies for reconstructing the evolutionary relationships among the genes of salt and water-deficit stress in peas. Currently, DNA, RNA, or protein sequences are being used for phylogenetic analysis (Zhang et al., 2018). Phylogenetic trees visually represent evolutionary relationships among different species by way of a common ancestor, while each node with descendants constitutes the ultimate recent common ancestor of the descendants (Bichindaritz and Potter, 2004; Theobald, 2008). Multiple sequence alignment (MSA) is a technique used to generate sequence alignment of more than two biological sequences including DNA, RNA, or protein to determine regions of similarity. Alignments help to reflect an evolutionary modification between sequences of organisms being descendants from a common predecessor (Felsenstein and Felenstein, 2004; Dwivedi, 2009). There exist various methods to construct phylogenetic trees based on aligned genetic sequences and the most popular among these methods is the CLUSTAL W alignment algorithm (Chatzou et al., 2016). Overall results of the current phylogenetic analysis based on DNA sequence data reflected that the important secondary metabolite biosynthesis pathway genes of *P. sativum* including *proC* and *PAL1* that express under salt stress and water-deficit are comparatively more closely related to *C. arietinum* and *T. pratense* showing common ancestry. *SOD* showed common ancestry with *V. radiate* and *POX* showed the same ancestry with *M. truncatula*. *P. sativum* genes have a close resemblance to *M. truncatula*, *C. arietinum*, *T. pretense*, *V. radiata* and belong to legumes (Fabaceae family). The results of the genes studied for phylogenetic analysis in *P. sativum* suggested the common origin of these genes belonging to the Fabaceae family.

The NCBI's Conserved Domain Database (CDD) is a database of protein family and domain models. A protein domain is a small, discrete unit of three-dimensional structure that ranges in size from 50 to 200 amino acids. It is considered a unit of molecular evolution, which can be used to define evolutionary classifications (Yang et al., 2020). It is generally linked with a discrete feature of a function of protein, like, enzymatic activity, nucleic-acid binding, or membrane transport (Yang et al., 2020). *In silico* analysis provides an excellent way for phylogenetic analysis of genes and conserved domain analysis in proteins (Zhu, 2002). The current *in silico* analysis was focused on identifying the phylogenetic relationship of *P. sativum* salt stress and water-deficit stress-related genes with genes pool of important plant species and identification of conserved domains of respective proteins of these genes. The proteins coded by these genes also contain important conserved domains belonging to important protein superfamilies. PLNO 2688, PLN02457, Cu-Zn superoxide dismutase, and secretory peroxidase conserved domains were identified in the current analysis. Protein family classification showed that the oxidation-reduction process was the most common family biological process involved in these stresses given to peas, which validated the relationship of these proteins. These results indicated that the genes and their respective proteins are known to play a significant role in stress tolerance by expression of different metabolic pathways and are largely conserved among plant species. It means these stresses will also show the same effects in the other species. It will allow us to made an effective study about the cross relation between these plant species.

Conclusions

Salt and water-deficit stress effects in peas can be managed by the production of suitable plant varieties using molecular breeding and molecular biology by taking evolutionary information from the phylogenetic analysis in the future. This study provides valuable insights into the expression patterns and evolutionary relationships of stress-related genes in pea plants under salt stress and water-deficit conditions. The findings highlight the importance of proline biosynthesis, flavonoid biosynthesis, antioxidant activity, and peroxidase activity in stress tolerance mechanisms. These findings contribute to our understanding of plant adaptation to stress and can aid in the development of strategies to enhance stress resilience in agricultural crops. As Climax showed better tolerance under salt stress and drought stress, it would be a good candidate for further study. Future studies are suggested to unravel the activation of different isoforms of antioxidant enzymes by considering the complete profile of antioxidant enzymes isoforms under salt stress and drought stress. It would be useful to study the plant root extract for antioxidant activities under salt stress and drought stress to compare the activation of different isoforms.

Declarations

Compliance with Ethical Standards

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Conflict of Interest

The authors declare that they have no financial and competing interest.

Data Availability Statement

Data supporting the findings of this study are available from the corresponding author on request.

Author Contributions

All authors contributed to the study conception and design. Muhammad Farooq performed the investigation by carrying out experiments. Rafiq Ahmad and Muhmmad Shahzad helped in the designing of experiments and data analysis. Saad Ur Rehman and Yasar Sajjad performed the statistical analysis and also proofread the article. Amjad Hassan and Mohammad Maroof Shah helped in writing, review and editing and also supported in research facilities. Amber Afroz helped in the research experiments in the lab. Sabaz Ali Khan conceptualized the project, got funding together with Muhammad Farooq.

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Figures

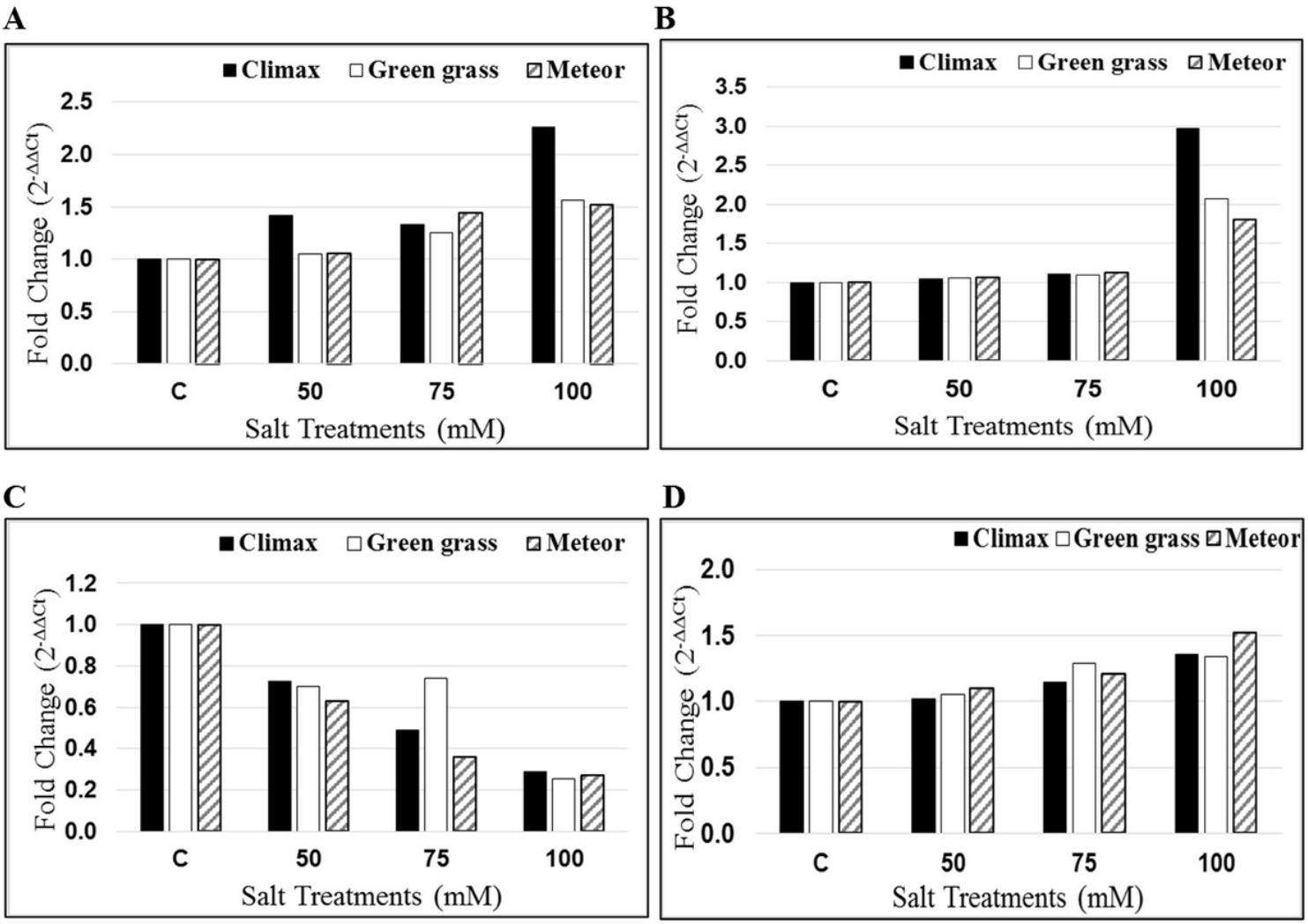

Figure 1

Relative expression of *P5CR* (**A**) *PAL1* (**B**) *SOD*(**C**) *POX* (**D**) normalized with housekeeping gene *Actin* using quantitative real-time PCR under different salt treatments (50 mM, 75 mM, 100 mM) and without salt stress (control; C = 5.4 mM).

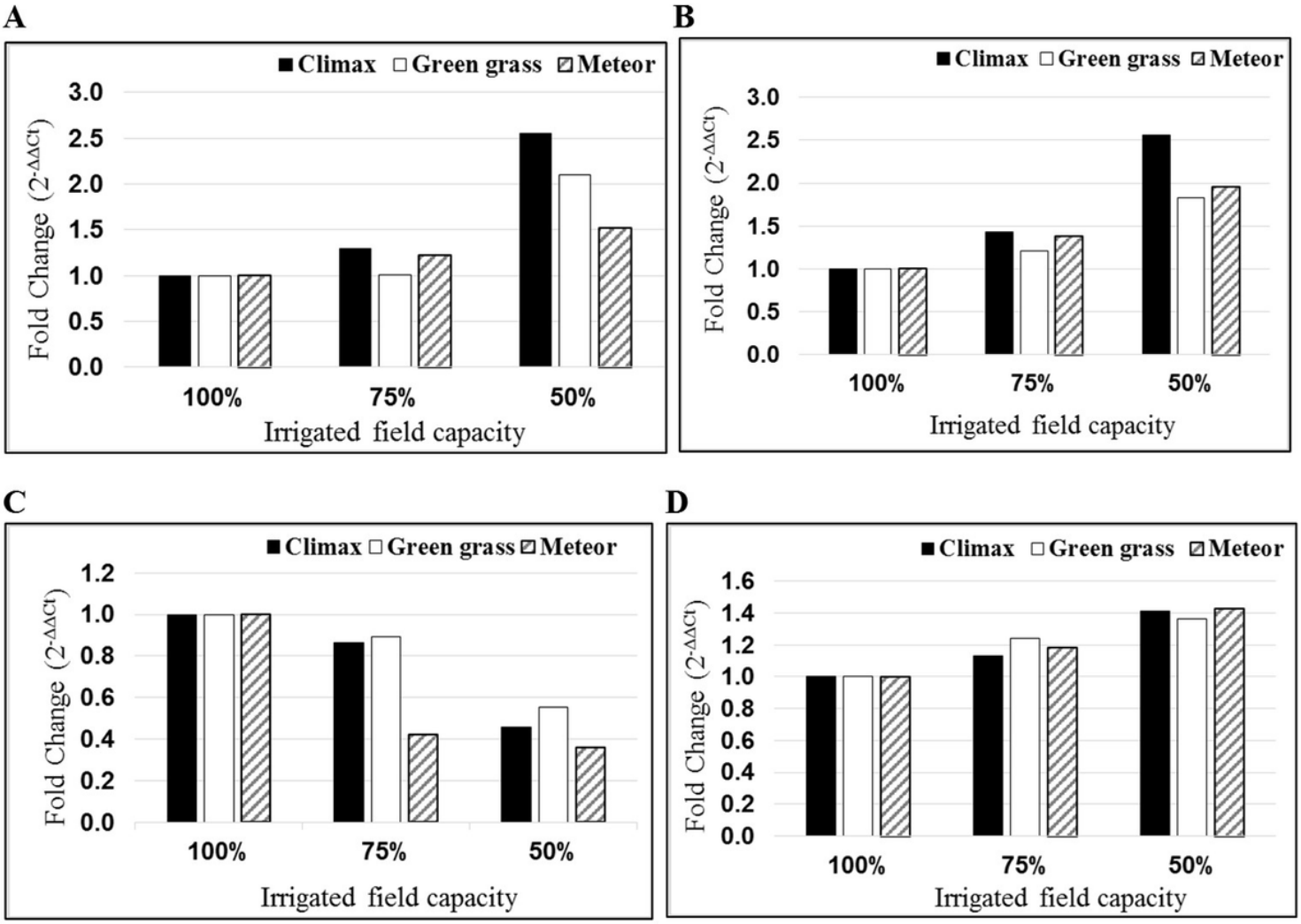

Figure 2

Relative expression of *P5CR* (**A**) *PAL1* (**B**) *SOD*(**C**) *POX* (**D**) normalized with housekeeping gene *Actin* using quantitative real-time PCR under different water-deficit treatments (water irrigation to 50% of FC, 75% of FC) and without water-deficit stress (water irrigation to 100 % of FC).

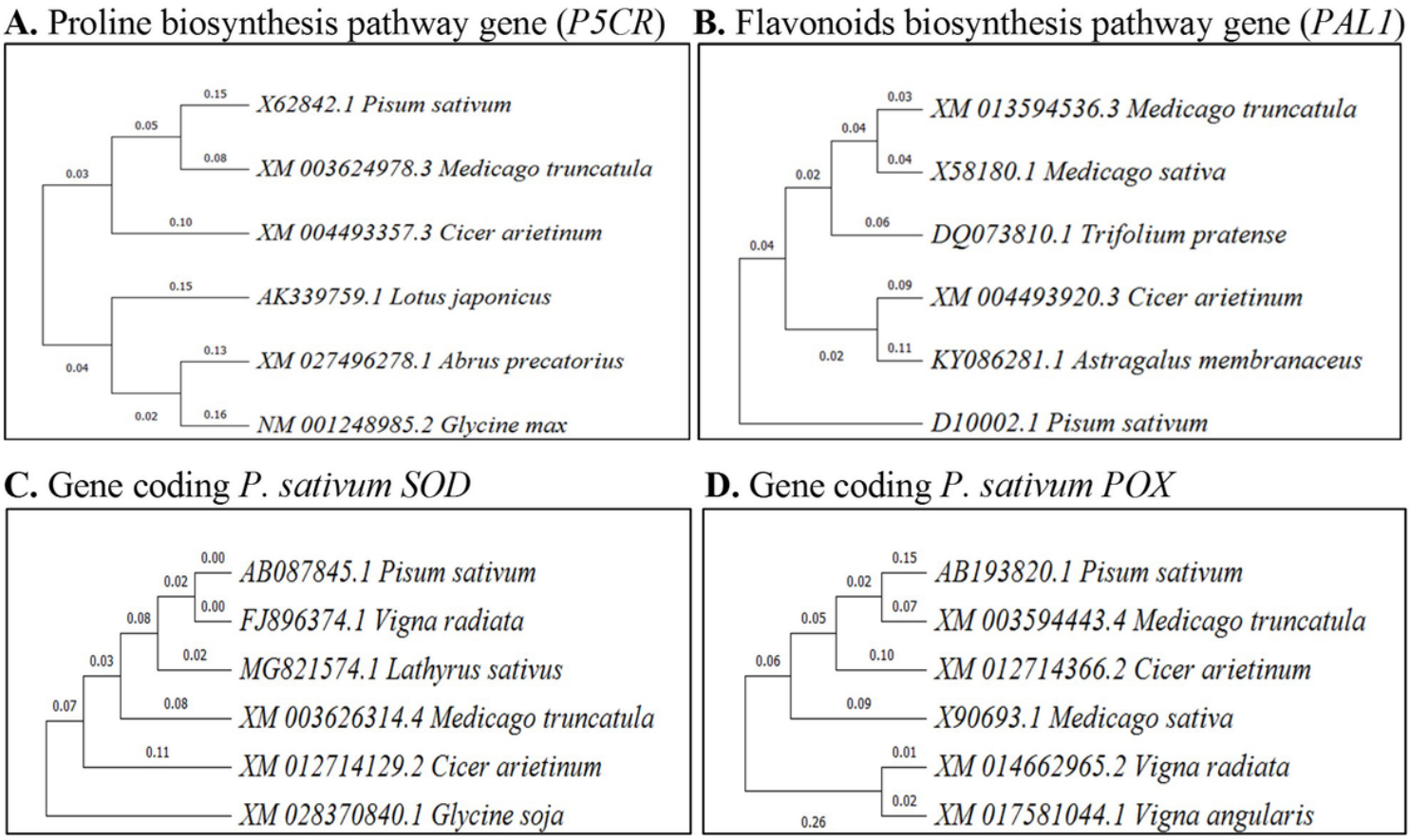

Figure 3

Phylogenetic tree of pea genes based on DNA sequence data.

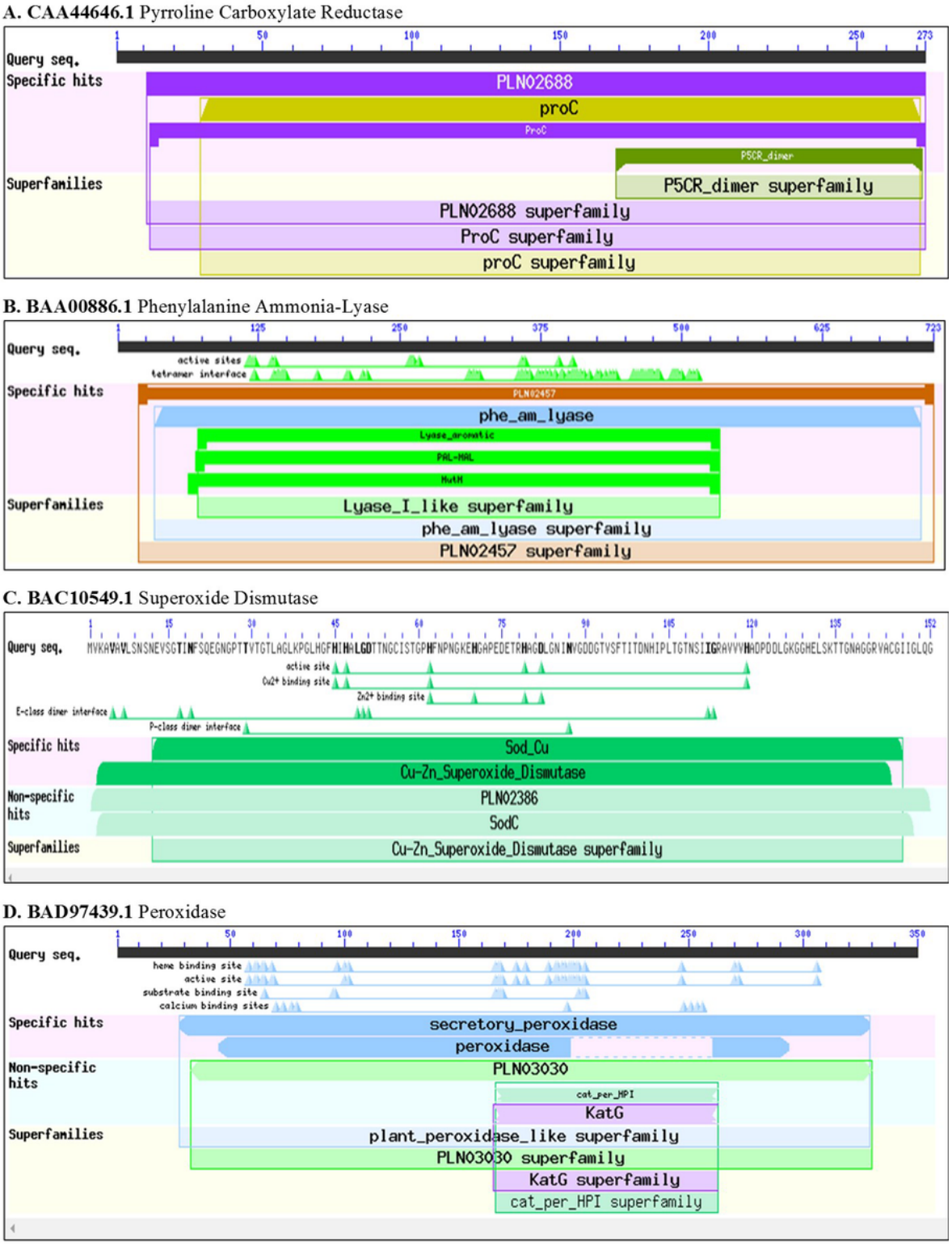

Figure 4

Schematic diagram of relative positions of conserved domains and superfamily of *Pisum sativum* salt stress and water-deficit stress related proteins **(A)** ProC **(B)** PAL1 **(C)**SOD **(D)** POX.