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Molecular Characterization of Proline Rich Regions in *Lens culinaris* under Abiotic Stress

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ABSTRACT

The present study attempt was made to isolate and characterized *Lens culinaris* full length proline rich gene. EST Sequences of proline gene (GT-622346.1) from *Lens culinaris* was retrieved from Genbank. This sequence was used as query against chickpea. Similarity sequence search has shown up to 90% similarity with EST sequence of chickpea having the Accession No. GR 398344 and up to 85% similarity with EST sequence having Accession No. GT 622346 of *Lens culinaris*. The positive results were subjected to sequencing and were further *in-silico* analysis carried out. BLASTp of the lentil sequence showed 98% similarity with *Phaseolus vulgaris* (accession number-CAJ43592), 97% with *Cicer arietinum* (accession number-XP004506458), 96% with *Medicago truncatula* (accession number-Q40375) and 91% similarity with *Pisum sativum* (accession number-CAB63486). Ten conserved repeats of 180 amino acids each in case of *Lens culinaris* having the repeat sequence "KPPVEKPPVY" was observed. The sequence was further translated into amino acid sequence and the Interproscan results for proline rich protein in *Lens culinaris* was found. These findings may help to develop more tolerant lentil crop against drought having a higher potential to satisfy demands for production by increasing the crop productivity.

KEYWORDS: Abiotic stress, biotic stress, cloning, est, lentil, proline, sequencing

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1. INTRODUCTION

Plants encounter several abiotic and biotic stresses in their natural surroundings. In lentil crop, drought stress is one of the most common environmental factors affecting growth and fertility while causing several metabolic, mechanical and oxidative changes (Kumar et al., 2015). Drought is a major environmental stress seriously limiting plant growth and crop productivity. So, it is important to improve crop yields by breeding crops with enhanced stress tolerance. Plants have evolved many mechanisms to adapt to environmental stresses via changes at physiological, morphological, and molecular levels (Todaka et al., 2012). Several studies have shown that drought stress causes an increase in proline which is an important parameter in determining the extent of tolerance to drought (Singh et al., 2016).

Under abiotic stresses, genes related with proline rich protein (PRP) exhibit an irregular pattern of transcriptional regulation depending on intensity of stress and time. Wu et al. (2014) have found differentially expressed genes (DEGs) between terminal drought and optimal irrigation treatments in two different genotypes of common bean i.e. Long 22-0579 and Naihua. Transgenic plants which over express the genes related with PRP show an improved tolerance to abiotic stresses (Gujjar et al., 2019). Drought resistant lentil varieties have been analyzed to accumulate less proline content than drought sensitive varieties (Mishra et al., 2014).

Earlier research related with plant proline/ hydroxyproline rich proteins suggested their localization on plant cell wall (Fowler et al., 1999). But in recent years, the research reports illustrate the sub-cellular localization of plant PRPs/ hybrid PRPs at plasmalemma (Qin et al., 2013; Li et al., 2014). These proteins have very important role in the development, maintenance of plant and cell wall integrity. Most well reported mechanisms of drought stress tolerance have been related to accumulation of n metabolites like proline, glycine betaine, soluble carbohydrates antioxidants etc. Which help in maintaining the vital properties of the cell (Muscolo et al., 2015). A precise localization of PRPs in various organs of plant accelerates the inconsistency in their functions. For proline accumulation in cells during the exposure to abiotic stresses, two additional pathways may be suggested besides natural synthesis of proline: 1. Downstream regulation of PRP genes during specific stages of drought stress (Gujjar et al., 2018) and 2. Degradation of PRPs during osmotic stress to get free proline (Barthakur et al., 2001). Plants under drought stress conditions often have different physiological adaptations and these adaptations are openly related to the stress resistance mechanism (Shrestha et al., 2006). The genes conferring tolerances to various abiotic stresses can offer a platform for scientific development of crop's yield under stress conditions and contribute to improvement and stabilization of crop productivity.

Various proteins present in plants develop the innate immunity system in plants during the biotic stress. Expression of gene related with Glycine rich protein (SbGPRP1) in tobacco plants let to improved tolerance towards infection of R. fascians (Halder et al., 2019). Proline accumulation has been reported during conditions of drought (Choudhary et al., 2005), high salinity**,** high light and UV irradiation**,** heavy metals**,** oxidative stress and in response to biotic stress (Fabro et al., 2004).

Lentil plant is very good to adapt itself and is one of the most significant food legumes, which is cultivated globally encounter various abiotic and biotic stress tolerance among which drought is one of the most common stress (Mostafaei, 1999). Lentil crop also contains various genes/ proteins which help this crop to conquer abiotic stresses. Identification and analysis of a number of stress inducible genes/ proteins in this crop will be of vast significance for improvement of crops susceptible to abiotic stresses.

2. MATERIALS AND METHODS

2.1. Source of explants

The plant samples of lentil were taken from Chirauri agriculture farm of Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India. The samples were brought in lab in ice box and were kept in -20 0C deep freezer.

2.2. Extraction of genomic DNA by CTAB method

DNA was extracted from the leaves by using method proposed by (Doyle and Doyle, 1990). Modified at the NCSU Forest Biotechnology Laboratory.

2.3. PCR (Polymerase chain reaction) amplification, dna sequencing and bioinformatics analysis of the query sequence

2.3.1. Primer designing and validation of Prp2 gene

The Primers used for the PCR analysis were specific to proline rich gene was designed by primer-3 bioinformatics software. Primers were design by EST sequence that is G.T-622346.1, by using the optimum parameters during the primer designing such as GC content (40–60%), Tm value (55°C), Length of product (750bp) etc. Total DNA isolation was done from fresh plant tissue by using genomic DNA extraction kit supplied by Qiagen gmbH, Germany from *Lens culinaris* by following the manufactures's instructions. After DNS isolation, DNA was stored in the TE buffer at -20°C. The DNA PCR amplification was performed by using a Thermocycler with 50 µl reaction having 1X of PCR buffer, 10µM of each dNTPs, 25mM MgCl2, 337.6 µg and 309.1 µg of PF/PR primers and 1Unit/µl of Taq polymerase enzyme (MBI, Fermentas). During PCR amplification each cycle consisted of denaturation step at

94°C for 10 min, primer annealing step at 55°C for 45 sec, and extension step at temperature 72°C for one minute and final extension step for ten minutes at 72°C. After DNA amplification, DNA was excised and eluted from the gel by using Q1A quik gel extraction kit (Qiagen). In the present investigation, PCR profile for Gene Amplification of *Lens culinaris* was standardized. The primer used for the gene amplification was custom synthesized commercially by BioServe Biotechnologies (India) Pvt. Ltd., Hyderabad and acquired sequence of DNA was established for the occurrence of PRP2 gene by performing BLASTn and tBLASTn. The sequence data obtained after sequencing was then validated by performing BLAST [www.ncbi.nih.gov/BLAST] analysis to ensure that the sequences are true. The sequence obtained from forward primer was then reverse complemented and aligned with sequence obtained from forward primer so as to get the sequence in *Lens culinaris*.

The sequence which was obtained after sequencing deposited in to Genbank database and respective accession numbers were obtained-AccesionNo-KF310527. The sequence found similar to the query sequence in NCBI database were retrieved, aligned and used for the construction Phylogenetic tree construction. The sequence was further translated into amino acid sequence and the Interproscan results for proline rich protein in *Lens culinaris*.

3. RESULTS AND DISCUSSION

3.1. Genomic DNA Isolation from lentil

In an approach towards our reach to the Proline gene in lentil (*Lens culinaris*), Sample (plant) were obtain from Chirauri form Sardar Vallabhbhai Patel University of Agriculture and Technology Meerut during the month of February 2012. The genomic DNA was extracted and purified from Lentil leaves using C-TAB method of DNA isolation protocol. The isolated DNA was used for further analysis. The integrity of the DNA isolated was verified by visualization on agarose gel (0.8%) with DNA standards. Presence of single, sharp band was indicative of high molecular weight DNA. The DNA after purification was quantified by spectrophotometer.

3.2. PCR based amplification of proline gene isolated from genomic DNA of lens culinaris

The quantity of DNA estimated showed a higher yield of DNA sample. The two sets of primers "Lens F and Lens R" were used for amplifying gene of interest. The Tm temperature was set for Proline gene specific primers designed through Primer-3 bioinformatics software. The primers were compatible to amplify the 750bp of band nearby spanning the section between the gene respectively (Figure 1a) Taq DNA polymerase was used since Pfu. DNA polymerase was not found compatible to get the desired results and resulted in wavy bands. The band obtained was eluted using gel elution kit (company: Qiagen, Germany), Quantified in agarose gel (Figure 1b), was sent for sequencing at BioServe Biotechnologies (India) Pvt. Ltd. Hyderabad.

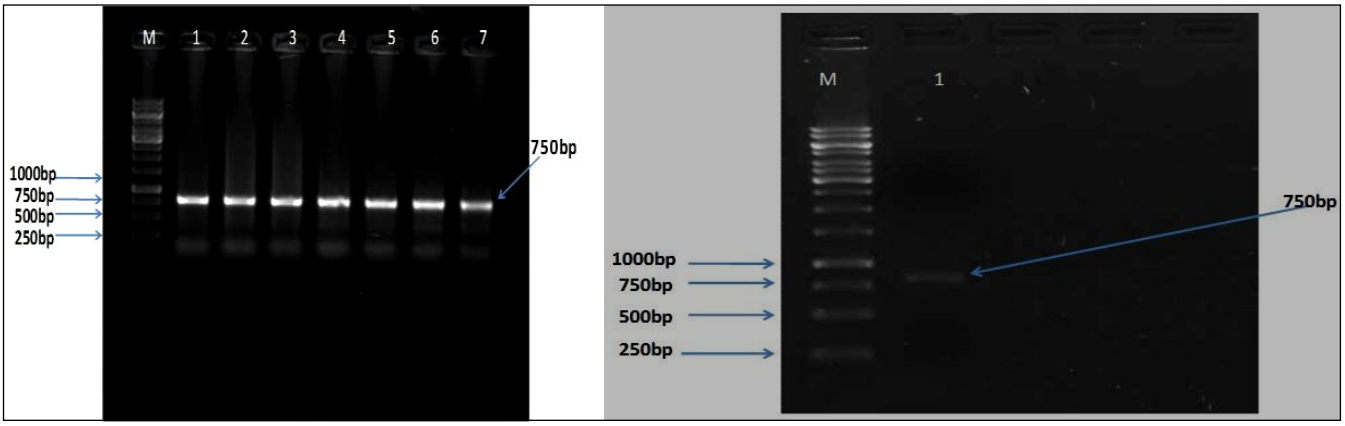

Figure 1: Amplification (PCR based) of Proline rich region in Lens culinaris: a) M- Marker (1kb), 1-7 lanes (Amplified PCR product); b) M- Marker (1kb), 1 lanes (Gel eluted product of~750bp)

3.3. In-silico analysis of sequences

For sequencing, the PCR amplified product was sent to BioServe Biotechnologies (India) Pvt. Ltd. Hyderabad. After getting the DNA sequence, these sequences were confirmed for the occurrence of PRP2 gene by doing BLASTn and tBLASTn (http://www.ncbi.nlm.nih.gov/BLAST.html). This sequence was also matched with expasy tool Interproscan (Table 1). After the DNA sequence was submitted to Gene Bank with the accession no.: KF310527.

3.4. PRP2 gene identification and analysis in lens culinaris EST database

The EST sequence of gene associated with proline (GT-622346) from *Lens culinaris* was retrieved from Genbank. This identified sequence was used as query against EST

Table 1: Translated sequence obtained from Expasy with their respective positive and negative strand

5'3' Frame 1	L P Stop P S Stop C S F F L L L S F L R G L A T Met R N L P S T S H L Stop R N L P S I S H L L R N L L F T N H R Stop R N H L C T N H Q L K S L R F T N H L L K N L R S T N H Q L K N H L F T S H Q Stop R S L R C T S L Q L K S L Q F T S H Q Stop R N L R C T S L Q L K S L P F T S H Q Stop R N L P C T S L Q L K S L P F T N H Q Stop R N L P Y T S L Q L K S H Q S T N H Q Stop R N L Q Y T S H Q L R N L R F I S H R W G I S H Q F T R L H S R S H K T N Q V P Y D G T C V Stop K L L L R R Y A K Stop G L Q S Met G Y I F L Stop S S F Q S F V Y N V I A K T R Met Stop V Stop N Stop E R K L C F C S P H
5'3' Frame 2	F H N L P S V P S S C S C H S S G V W Q L Stop E T S H L Q A T C R E T S R L Stop A T C Stop E T S C L Q T T G R E T T C V Q T T S Stop K A S G L Q T T C Stop K T S G L Q T T S Stop K T T C L Q A T S R E A S G V Q A S S Stop K A S S L Q A T S R E T S G V Q A S S Stop K A S R L Q A T S R E T S R V Q A S S Stop K A S R L Q T T S R E T S R I Q A S S Stop K A T S L Q T T S R E T S S I Q A T S Stop E T S G L Stop A T G G V S A T S L H A S I L E A T K P T R Y P Met Met V L V S R N C Y Stop G G Met L N K V Y K A W D I Y S C N L H F N P L F T Met L L R K Q E C E C K T E K E N C A F V L H I
5'3' Frame 3	S I T F L V F L L L A L V I P Q G F G N Y E K P P I Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V E K P P V Y K P P V G Y Q P P V Y T P P F Stop K P Q N Q P G T L Stop W Y L C L E I A I K E V C Stop I R F T K H G I Y I L V I F I S I L C L Q C Y C E N K N V S V K L R K K I V L L F S T
3'5' Frame 1	N V E N K S T I F F L S F T L T F L F S Q Stop H C K Q R I E Met K I T R I Y I P C F V N L I Stop H T S L I A I S R H K Y H H R V P G W F C G F Stop N G G V Stop T G G Stop Y P T G G L Stop T G G F S T G G L Y T G G F S T G G L Stop T G G F S T G G L Y T G G F S T G G L Stop T G G F S T G G L Y T G G F S T G G L Stop T G G F S T G G L Y T G G F S T G G L Stop T G G F S T G G L Y T G G F S T G G L Stop T G G F S T G G L Stop T G G F S T G G L Stop T G G F S T G G L Y T G G F S T G G L Stop T G G F S T G G L Stop T G G F S T G G L Stop T G G F S T G G L Stop Met G G F S Stop L P N P Stop G Met T R A R R R N T R K V Met E 3'5' Frame 2 Met W R T K A Q F S F S V L H S H S C F R N N I V N K G L K Stop R L Q E Y I S H A L Stop T L F S I P P Stop Stop Q F L D T S T I I G Y L V G F V A S R Met E A C K L V A D T P P V A Y K P E V S Q L V A C I L E V S L L V V C R L V A F Q L E A C I R E V S L L V V C K R E A F Q L E A C T R E V S L L V A C K R E A F Q L E A C T P E V S L L V A C K L E A F Q L E A C T P E A S L L V A C K Q V V F Q L V V C R P E V F Q Q V V C K P E A F Q L V V C T Q V V S L P V V C K Q E V S Q Q V A Y R R E V S L Q V A C R W E V S H S C Q T P E E Stop Q E Q E E G T L G R L W K
3'5' Frame 3	C G E Q K H N F L S Q F Y T H I L V F A I T L Stop T K D Stop N E D Y K N I Y P Met L C K P Y L A Y L L N S N F Stop T Q V P S Stop G T W L V L W L L E W R R V N W W L I P H R W L I N R R F L N W W L V Y W R F L Y W W F V D W W L F N W R L V Y G R F L Y W W F V N G R L F N W R L V H G R F L Y W W L V N G R L F N W R L V H R R F L Y W W L V N W R L F N W R L V H R R L L Y W W L V N R W F F N W W F V D R R F F N R W F V N R R L F N W W F V H R W F L Y R W F V N R R F L N R W L I D G R F L Y R W L V D G R F L I V A K P L R N D K S K K K E H Stop E G Y G

database of chickpea and *Lens culinaris* in tblastn search. Sequences having maximum similarity were downloaded from genbank and clustering was completed by using bioedit tool. The Expasy tool, RADAR (Rapid Automatic Detection and Alignment of Repeats) was used to identify repeats motifs in the amino acid sequence of PRP2 gene.

3.5. pBLAST analysis

After sequence analysis 750bp amplicons were observed in *Lens culinaris* (Figure 1A and 1B). DNA sequences were aligned using CLUSTALW and aligned sequences were also compared with BioEdit Sequence Alignment Editor (Hall, 1999) (Figure 2). pBLAST results of the

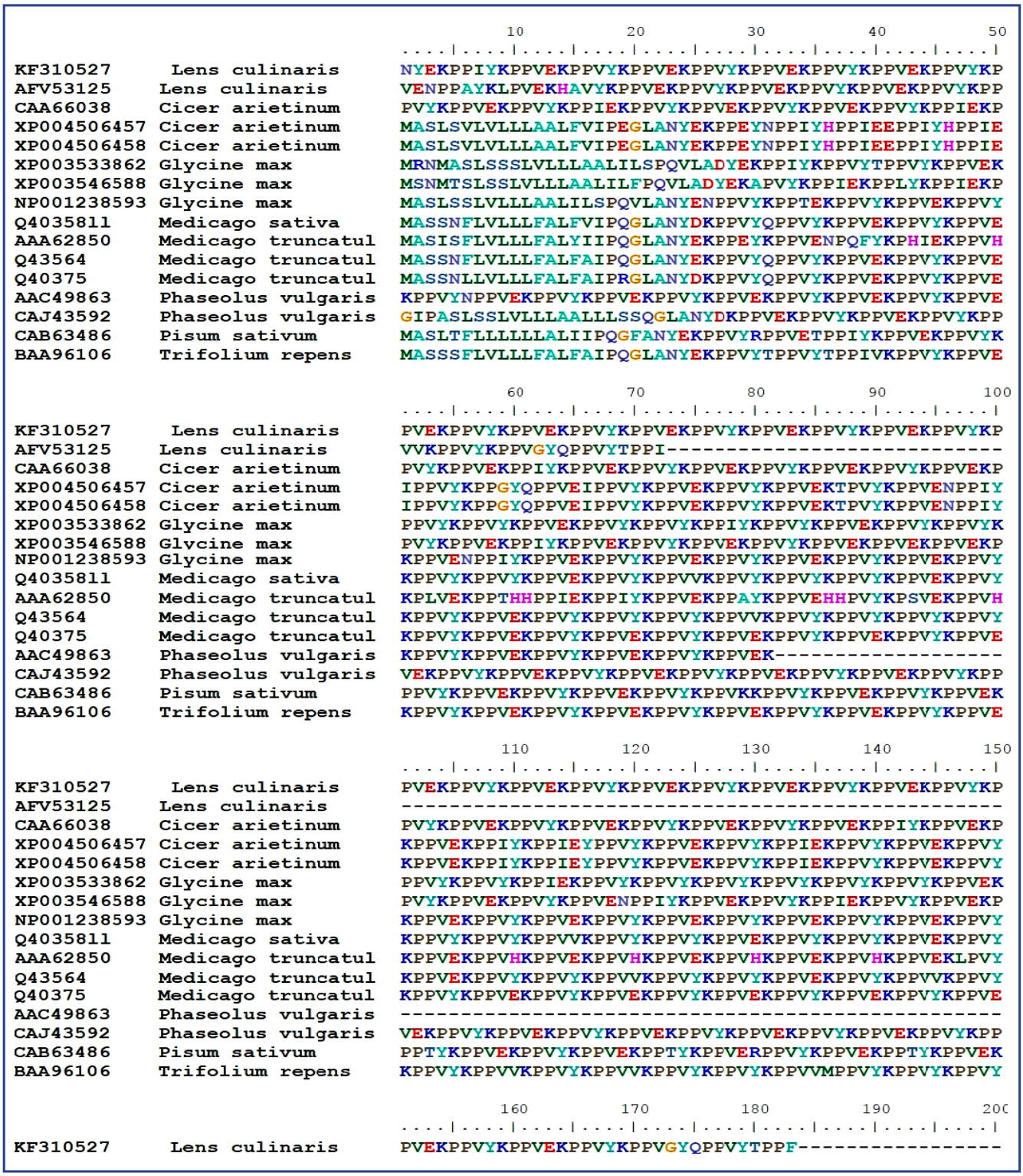

Figure 2: Sequence (amino acid) alignment from various related species Lens culinaris, *Medicago truncatula*, *Glycine max*, *Phaseolus vulgaris Pisum sativum* and *C. arietium* using Bioedit tool

Lentil sequence have 98% with *Phaseolus vulgaris* (accession number-CAJ43592), 97% with *Cicer arietinum* (accession number-XP004506458), 96% with *Medicago truncatula* (accession number-Q40375) and 91% with *Pisum sativum* (accession number-CAB63486). Ten conserved repeats of 180 amino acids each in case of *Lens culinaris* having the repeat sequence "KPPVEKPPVY" (Figure 2) were observed. Results of Interproscan have also revealed the similarity with Proline rich protein (PRP) (IPR003883) in the database of Pfam (Figure 3).

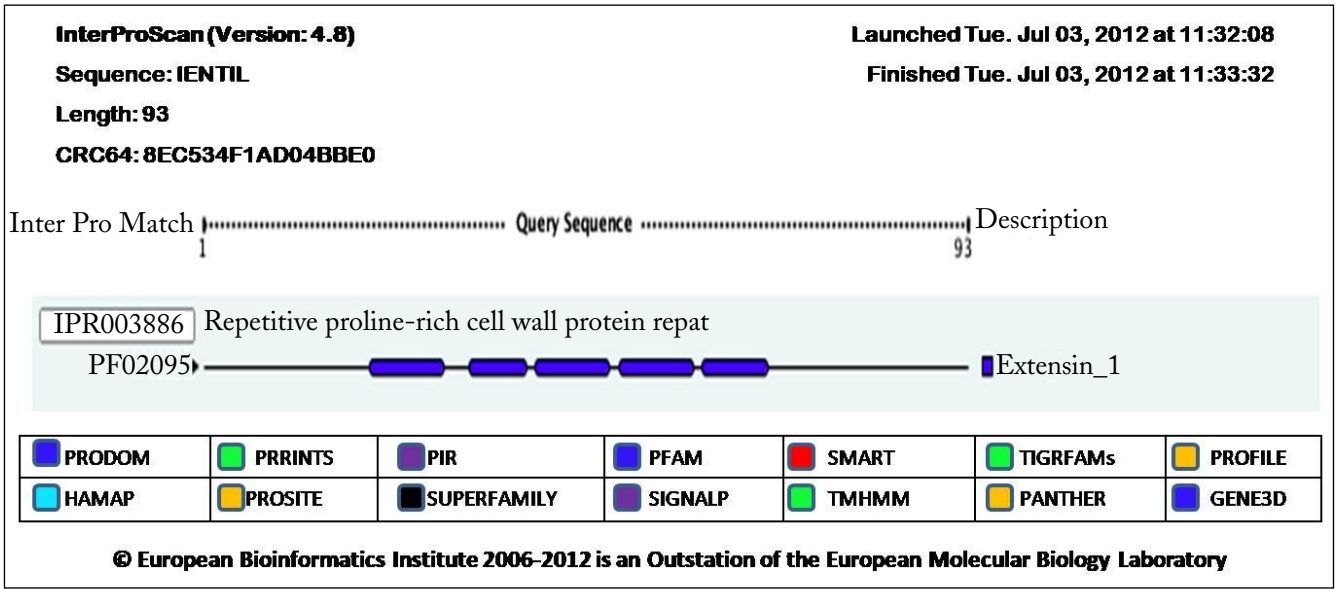

Figure 3: Results of Interproscan for PRP in *Lens culinaris*

In either way, PRPs use themselves as valuable proteins under all environmental stress conditions. It has been validate by their over expression and silencing in genetically modified plants where over expression of PRP genes enhanced tolerance to various abiotic stresses (Liu et al., 2015; Mellacheruvu et al., 2016). Recently, promoter analysis of PRP genes in *Nicotiana tabaccum* and *Glycine soja* verified the existence of stress inducible elements, viz., GATA box (binding site for helix-loop-helix), W boxes (binding sites for WRKY), and MYBs on it (Chen et al., 2014; Liu et al., 2015).

In lentil the identified gene *prp2* has revealed similarity with *Glycine max prp2* gene which belongs to first group containing multiple copies of "KPPVEKPPVY". Many external factors are responsible for the expression of plant PRPs (Harrak et al., 2001), had recognized a gene which encode a Pro, Thr and Gly rich protein (PTGRP) in case of Lycopersicon *chilense* which was negatively regulated under drought stress.

Reverse transcription quantitative PCR analysis explained the down regulation of a S1PRP gene under drought condition in every tomato plant: root (1314 fold), stem (11 fold), leaf (40 fold), and in flower (2 fold) (Gujjar et al., 2014; Gujjar et al., 2018). Similar results were reported earlier in *Poncirus trifoliate* (Peng et al., 2015), where osmotic stress condition lead to slow decrease in the expression of PRP genes. While complementary annotations in favor of up regulation of PRP genes under different abiotic stress conditions were also observed in cotton (Qin et al., 2013) and Glycine soja (Liu et al., 2015).

Proline rich protein MtPPRD1 in *Medicago truncatula* perform as a lipid transfer protein in the membrane biogenesis and regulate the intracellular fatty acid pool by binding and transferring the phospholipids and fatty acids between membranes. Proline is a admirable osmolyte which can protect subcellular organs and macromolecules under osmotic stress conditions (Meringer et al., 2016). Some of the major role of proline during stress conditions comprise reactive oxygen species scavenging action under oxidative stress condition.

The present research enabled in elucidating the conserved region and the relationship of *Prp-2* gene with other related PRP genes sharing the similar characteristic feature. Over all it can be concluded that the gene may be helpful in understanding the roles and distribution of proline gene in lentil. Cloning of proline rich protein in expression vector & its genetic engineering in crop plants would be of great importance.

3.6. Phylogenetic tree

Sequences of complete proline gene were obtained from the Eluted DNA products of *Lens culinaris* was aligned with similar sequences of other plant group by using MEGA 5.2. Phylogenetic tree was constructed according to the original data set by neighbour joining method. It shows resemblance of *Lens culinaris* to other related species (Figure 4).

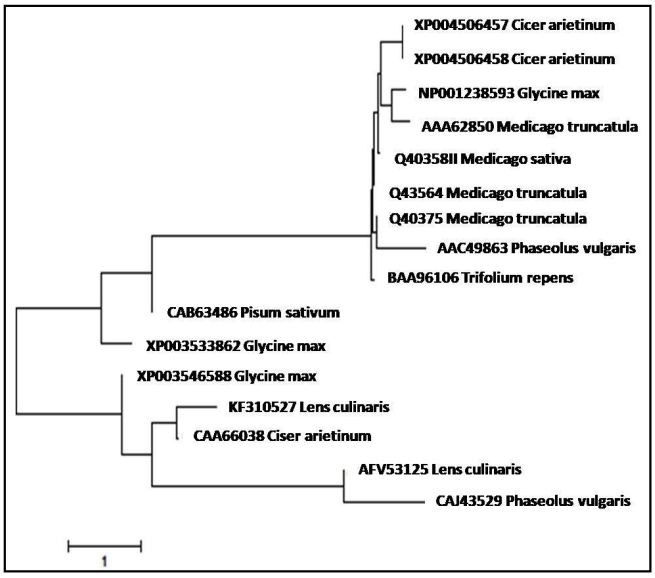

Figure 4: Phylogenetic analysis of prp2 gene from *Lens culinaris*, *Cicer arietinum* and *Glycine max*

4. CONCLUSION

The phylogenetic analysis indicated the similarity of Lens culinaris (Prp-2 gene) was closely related to Phaseolus vulgaris and Cicer arietinum with 98% followed by 97% similarity. The gene might be helpful in understanding the roles and distribution of proline gene in lentil. These results may be helpful in genetic engineering of proline rich gene for improving drought tolerance in crops plants and achieving goal of developing tolerance ability in lentil crops to increase the yield under stress.

5. ACKNOWLEDGEMENT

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