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RESEARCH ARTICLE

Functional Characterisation of a Calmodulin-Binding Receptor-Like Cytoplasmic Kinase (*GmCBRLCK1*) in *Glycine max* (L.) Merr. using Bioinformatic Tools

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ABSTRACT

An understanding of the function of signaling genes/proteins in soybean is vital for comprehending plant growth and development. The objective of this study was to functionally characterize a calmodulin-binding receptor-like cytoplasmic kinase gene (Glyma.13G161700) from *Glycine max*. Bioinformatic analyses were performed for the characterization. Expression profile of *GmCBRLCK1* gene in soybean tissue was assessed using Genevisible. Functional genomic analysis for gene expression regulation and co-expression analysis was evaluated using micro array data from Affymetrix Soybean Genome Array platform in GENEVESTIGATOR v3. Gene ontology functional predictions were determined through FFPred 2.0. The results showed that the calmodulin-binding receptor-like cytoplasmic kinase gene is predominantly expressed in the pericycle and syncytium in root seedlings and in the palisade cells of the legume. The gene was shown to be highly upregulated in response to root exposure to *Phytophthora sojae*, *Heterodera glycines* and aluminium stress. Co-expressed genes during the legume development showed Pearson's correlation co-efficient of 1 to Glyma.13G161700. Gene ontology predictions confirmed the signaling and metabolic functions of the kinase gene and its primary locations are the membrane and endomembrane system of *G. max*. The study therefore suggests that *Glycine max* calmodulin-binding receptor-like cytoplasmic kinase (*GmCBRLCK1*) is involved in receptor signaling pathways to enhance seedling tolerance to root infection by *P. sojae*, *H. glycines*, and to aluminium stress. The kinase gene is also involved in regulation of metabolic processes that aid in growth and development of soybean seedling.

Keywords: Calmodulin-binding receptor-like cytoplasmic kinase, Gene, *Glycine max*, Kinase, Protein, Signaling, Soybean.

1 Introduction

Signal perception and transduction during growth, development and response to adverse environmental stimuli can be facilitated by specific calmodulin-binding receptor like cytoplasmic kinases in plants. Calmodulin (CaM) is a small acidic protein and one of the many calcium binding proteins in plants. Most receptor like kinase (RLK) family proteins possess a receptor configuration that have an extracellular domain, a transmembrane domain and a kinase domain and these constitute approximately 75% while the remaining 25% is cytoplasmic containing a kinase domain only hence the name receptor like cytoplasmic kinases (RLCKs) [1]. Most studies have focused on RLK and it is only recently that few plant RLCKs have been characterised such as Pto, PBS1, CRCK1 (At5G58330) and CRCK3 (At2G11520). Pto and PBS1 have been observed to be responsible in conferring resistance to the pathogen *Pseudomonas syringae* in *Solanum lycopersicum* [2] and in Arabidopsis [3], respectively. CRCK1 plays a

major role in stress signal transduction during cold and salt stress [1] whereas CRCK3 is important in constitutive defense responses of *mekk1*, *mkk1* and *mpk4* which are an integral part of the MAP kinase signaling [4].

Work on RLK kinase in genus *Glycine* have been performed in *G. max* [5] and *G. soja* [6]. In *G. max* a CaM-binding receptor-like kinase from the legumes' nodule GmCaMK1, was characterised, which is a homolog of Arabidopsis calcium/ calmodulin-regulated receptor-like kinase 1 (CRLK1), At5G54590 [5]. In *G. soja* a calcium dependent CaM-binding receptor-like kinase (GsCBRLK) was functionally characterised and reported to be involved in regulating transgenic plant tolerance to salt stress [6]. However, there has been no evidence of published literature on calmodulin-binding receptor-like cytoplasmic kinases in *G. max*.

Glycine max is an important cash crop that is grown widely for its high oil and protein content, phytochemicals and production of biodiesel and is reportedly affected by biotic and abiotic factors during its development. The sequencing of *G. max* genome was completed in 2010 [7] and has therefore provided a platform to study gene function in the legume. Genome sequencing has made it possible to use a variety of computational methods for predictions of gene functions and this is now a well-established field [8-10]. Most authors have successfully used data mining tools to predict function of genes in species such as *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae* and *Escherichia coli* [11, 12], and most of these gene predictions have been confirmed [13]. In this paper we used bioinformatic tools to functionally characterize a calmodulin-binding receptor-like cytoplasmic kinase from *G. max* Glyma.13G161700 an ortholog of *Arabidopsis* CRCK3. The protein sequence of the gene is available in UniprotKb (https://www.uniprot.org/). Functional characterisation of the calmodulin-binding receptor-like cytoplasmic kinase gene in *G. max* using bioinformatic tools is crucial in understanding of its role as a signaling molecule in plant responses during its growth development, therefore providing a platform for further biochemical characterisation.

2 Materials and Methods

2.1 *GmCBRLCK1* Expression within Soybean

A review of the expression profiles for the gene in *G. max* tissues were obtained from Genevisible Affymetrix Soybean Genome Array at https://genevisibe.com/search/ server. The expression of the gene in soybean tissues were tested by GENEVESTIGATOR across 54 samples. The expression profile was expressed as expression level on a log2 scale. The Genevisible interface was able to query and verify expression of *GmCBRLCK1* gene in soybean tissues and is powered by GENEVESTIGATOR v3. GENEVESTIGATOR v3 is a database and web-browser datamining interface for Affymetrix GeneChip data.

2.2 Functional Genomic Analysis

The Compendium Wide Analysis perturbation tool available in GENEVESTIGATOR v3 (http://www.genevestigator.ethz.ch.) [14] was employed to explore gene expression responses of *GmCBRLCK1* in response to *Phytophthora sojae*, *Phakopsora pachyrhizi*, *Heterodera glycines*, aluminium stress and GmMPK4 silencing. The tool uses expression profiling through microarrays from the [Soybean] Affymetrix Soybean Genome Array platform. The platform is ideal for functional genomics since the possibility to correlate genes to conditions that regulate them is possible and this is fundamental in understanding the function of the gene under investigation [14]. A fold change of 1.5 and a p-value of 0.05 were used to set the selection for the array data for perturbations and the expression level of the gene was presented as expression values on a log2 scale.

2.3 Co-expression Analysis

A similarity search tool, Co-expression available in the Compendium Wide Analysis in GENEVESTIGATOR v3 was used to assess soybean genes that are functionally co-expressed with Glyma.13G161700. The co-expression was used to find genes that responded to the same perturbations as *GmCBRLCK1* and to identify genes with similar profile during development.

Pearson correlation coefficient (*r*) was used as a measure of similarity of the co-expressed genes against the *GmCBRLCK1* gene.

2.4 Gene Ontology

Gene ontology (GO) was predicted for biological processes and cellular component predictions through FFPred v2.0 which was accessed at the interface of the PSIPRED Protein Analysis Workbench http://bioinf.cs.ucl.ac/psipred/ [15]. The FFpred 2.0 is a tool useful for general eukaryotic protein function prediction. The tool uses support vector regression models for the prediction of GO terms of proteins by providing accurate functional assignments to their role in regulatory, signaling, developmental and metabolic processes [15].

3 Results and Discussion

Glyma.13G161700 a calmodulin-binding receptor-like cytoplasmic kinase is largely expressed in the primary growth tissues of the soybean plants presented as expression levels on a log2 scale in the pericycle and syncytium as shown in Figure 1. Considerable expression levels are also present in primary cell, paraveinal mesophyll, mesophyll, parenchyma and palisade parenchyma. The pericycle and syncytium are components of the root system while the later are largely part of the plant leaf. The pericycle is a primary tissue of plant root that consists of a cylinder of mostly parenchyma cells and lies just interior to the endodermis. It is largely responsible for the initiation of lateral roots [16]. The syncytium is associated with soybean root regions and is a multicellular feeding site for *Heterodera gylcines* thus, holding the only source of nutrients for the cyst nematode [17]. It has been reported that roots of *G. max* are susceptible to the soybean cyst nematode *H. glycines*. This parasite is believed to penetrate roots and migrate towards vascular tissue before selecting a pericycle or endodermis cell to initiate the formation of a syncytium, which serves as a feeding site [18, 19].

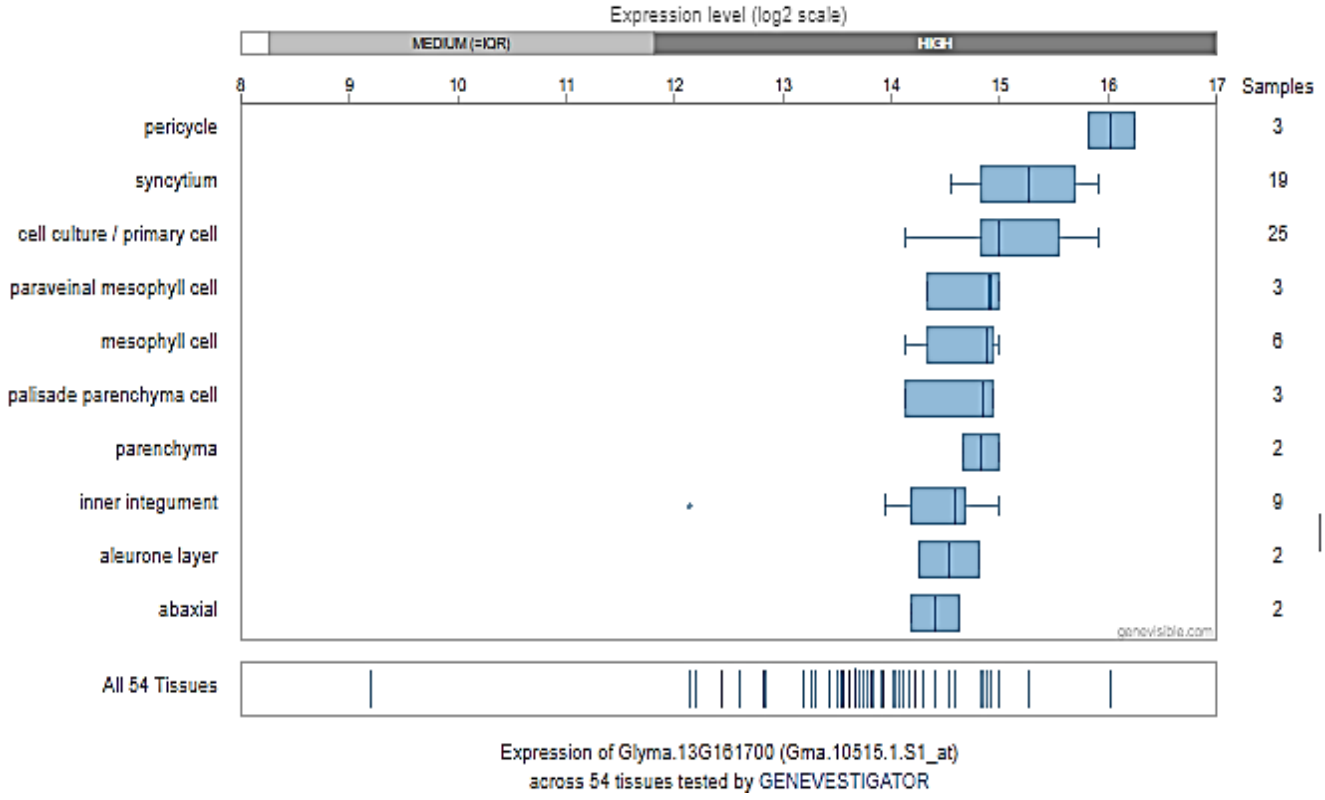

Figure 1: *Expression profile of GmCBRLCK1 Glyma.13G161700 gene in soybean tissue as tested by Genevisible in GENEVESTIGATOR v3.*

Our results show that the *GmCBRLCK1* gene expression in the syncytium is initially downregulated during 48hour exposure (Figure 2). However, prolonged exposure of 120 hours results in upregulation of the gene. The initial downregulation to the expression of *GmCBRLCK1* gene could be because once the soybean root has been penetrated by the pathogen, the parasite induces many changes in the pericycle cell or endodermal cell it selects by causing cell hypertrophy and dissolving the cell wall. It is believed that by 42 hours, the parasite would have caused cell wall perforations [18], causing the cytoplasm of the pericycle and endodermis to merge forming the syncytium. Once the feeding site has been formed, expression of Glyma.13G161700 is upregulated in the syncytium. Changes in host gene expression during nematode infection of roots has been demonstrated especially within the developing syncytium [20, 21].

Apart from the roots, the *GmCBRLCK1* gene is largely expressed in the leaf which is the main photosynthetic organ of all plants where the paraveinal mesophyll, the mesophyll and the palisade parenchyma are found. The photoassimilate is used to provide metabolic energy to sinks such as roots, leaf primordia and storage organs such as seeds. Expression of the *GmCBRLCK1* gene in palisade parenchyma entails its likely location within chloroplasts which consists of the thylakoid and stroma. The paraveinal mesophyll is a single layer of branched cells that extend between the vascular bundles in soybean trifoliate leaves [22]. They function as an intermediary between the mesophyll cells and the vascular bundles in transporting photoassimilate and nitrogenous compounds [22, 23], therefore provides an ideal nutrient distribution system for developing leaf tissues. This entails that *GmCBRLCK1* is largely involved in regulation of metabolic processes that ensure adequate supply of nutrients for the developing *G. max* seedlings.

Gene expression responses of *GmCBRLCK1* in response to *Phytophthora sojae* (A) *Phakopsora pachyrhizi* (B), *Heterodera glycines* (C) aluminium stress (D) and GmMPK4 silencing (F) showed a general upregulation of the gene under the different stimuli as shown in Figure 2. The expression level of *GmCBRLCK1* are shown on a log2 scale. The silencing of GmMPK4 resulted in upregulation of the *GmCBRLCK1* after 480 hours. GmMPK4 is a negative regulator of defense responses.

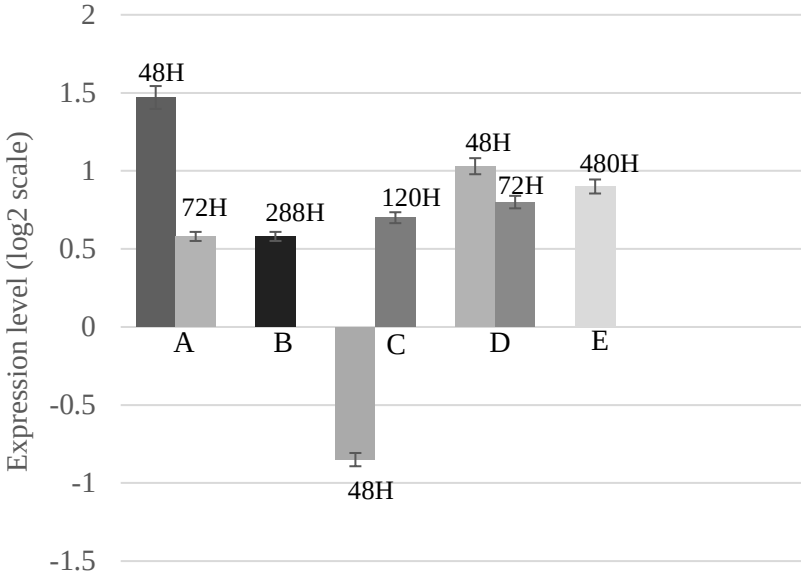

Figure 2: *Gene expression responses of GmCBRLCK1 Glyma.13G161700 to perturbations obtained from array experiments available on the GENEVESTIGATOR v3 Affymetrix Soybean Genome Array platform. The different stimuli are presented as A to E. (A) Gene expression level in response to root/hypocotyl exposure to Phytophthora sojae for 48 and 72 hours (B) Gene expression response to trifoliate leaf exposure to Phakopsora pachyrhizi for 288 hours (C) Gene expression response to syncytium exposure to Heterodera glycines for 48 and 120 hours (D) Gene expression responses to primary root exposure to aluminium stress (excess Al) (E) Gene expression response to GmMPK4 silencing after 20 days.*

The results showed an upregulation of the kinase gene to tissue exposure to the pathogens *Phytophthora sojae*, and *Phakopsora pachyrhizi*. This implies the involvement of *GmCBRLCK1* gene in defense response to pathogen attack and most importantly *P. sojae* which peaked expression level to 1.47 on a log2 scale within 48 hours exposure of soybean root/hypocotyl to the pathogen. *Phytophthora sojae* is a pathogen that causes root and stem rot in the legume accounting for major crop losses worldwide. Exposure of soybean primary root to aluminium stress resulted in upregulation of *GmCBRLCK1* (expression level

1.03 log2 scale) within 48hours exposure implying an active role played by the kinase gene in maintaining aluminium stress tolerance in soybean seedlings. Aluminium toxicity has been shown to lead to inhibition of root elongation and modification of membrane properties in soybean seedlings [24]. The inhibition of root elongation and modification of membranes affects plant growth and development.

Micro array data available from Affymetrix Soybean Genome Array platform showed that genes co-expressed during perturbations had the highest *r* of 0.78 at $p \leq 0.05$ (Table 1). Co-expressed genes during development of soybean are highly correlated with the *GmCBRLCK1* gene ($r = 1$ $p \leq 0.05$) as shown in Table 2.

Table 1: *Co- expressed genes during perturbations ($r \geq 0.65$).*

Gene	*R*	Description
Glyma.18G275700	0.78	Protein kinase superfamily
Glyma.11G051400	0.72	Eukaryotic aspartyl protease family protein
Glyma.13G243600	0.71	SNF2 domain-containing protein/helicase domain containing protein/ zinc finger protein-related
Glyma.07G237900	0.70	RNA binding abscisic acid binding
Glyma.13G335700	0.69	U-box domain containing protein kinase family protein
Glyma.07G253900	0.69	Protein phosphatase 2C family
Glyma.20G001300	0.69	Myosin 1
Glyma.04G195700	0.68	HCP-like superfamily protein with MYND-type zinc finger
Glyma.15G092200	0.68	RNI-like superfamily protein
Glyma.15G232000	0.68	TGACG motif-binding factor 6
Glyma.11G138400	0.66	SCARWCROW-like 14
Glyma.03G136000	0.66	ATP-binding cassette A1
Glyma.07G231800	0.65	Telomerase activating protein Est 1
Glyma.15G070100	0.65	SNF2 domain-containing protein/helicase domain containing protein/ zinc finger protein-related
Glyma.07G128500	0.65	Mitochondrial substrate carrier family protein

Co-expression analysis revealed a protein kinase superfamily protein Glyma.18G275700 to be highly co-expressed with the *GmCBRLCK1* gene. Normally genes that serve the same function are co-expressed, and the kinase superfamily proteins have been shown to be involved in the regulation of key aspects of cellular function such as cell division, metabolism and response to external signals [25]. Protein kinases are enzymes that use the γ-phosphate of ATP to phosphorylate serine, threonine or tyrosine residues in proteins [26]. Plant protein kinases have been reported to be components of signaling networks that function in cell cycle regulation, developmental processes, modulating vesicle transport and regulating cellular metabolism [27 -30]. The gene Gyma.11G051400 a eukaryotic aspartyl protease family protein shares a similar function with Glyma.13G161700. The eukaryotic aspartyl protease family protein was studied in *Arabidopsis thaliana* and was observed to be involved in disease resistance [31]. Breitenbach et al. [32], were able to associate aspartyl protease activity in the model plant with feedback regulation during plant innate immunity while to screening for systematic acquired resistance regulators. In *Oryza sativa* the enzymes were reported to be responsible for triggering programmed cell death (PCD) during development [33]. The involvement of *GmCBRLCK1* in response to plant pathogen attack maybe a feedback regulation of plant innate immunity.

RNA binding proteins have been reported to play important roles in post-transcriptional regulation [34]. A report by Lorkovic [35], provided insight into the basic roles of RNA-binding proteins as important governors of diverse developmental processes and are involved in adaptation of plants to various environmental stresses through modulating expression of specific transcripts. The RNA-binding proteins are plant specific hence perform plant specific functions. In *G. max* RNA-binding abscisic acid binding protein Glyma.07G237900 has been shown to be co-expressed with *GmCBRLCK1*. The RNA-binding ABA-binding protein is likely to be involved in regulating stress responses that are related to water availability, hence play vital roles in regulating growth and development of the

juvenile legume plant. In *Arabidopsis*, these proteins have been shown to participate in ABA signaling during germination and drought tolerance. Work by Raab et al. [36] showed the localization of such proteins in chloroplasts thus suggesting the role of *GmCBRLCK1* in regulating chloroplast gene expression through changes in RNA metabolism. Not only is the chloroplast involved in photosynthesis, but also represent sites for important for metabolism of starch, fatty acid and amino acids. All these processes are essential for plant growth and development. The RNA-binding ABA binding protein have been shown to play vital roles in germination, freezing tolerance, and light regulation of protein supply to the chloroplast [36]. Co-expression gene analysis during development showed that highly correlated genes are produced during development of the legume. Over 100 genes in soybean have a Pearson correlation co-efficient of 1 to *GmCBRLCK1* Glyma.13G161700. The top 50 genes are presented in Table 2.

Table 2: *Top 50 co-expressed genes during development (r = 1)*

Gene	Description
Glyma.06G172700	Glyceraldehyde-3-phosphate dehydrogenase C
Glyma.07G119500	Vacuolar protein sorting 11
Glyma.16G070400	Sucrase/ferredoxin-like family protein
Glyma.18G039000	DegP protease 7
Glyma.08G282800	Copper amine oxidase family
Glyma.08G025800	Carbon-nitrogen hydrolase family protein
Glyma.08G072100	Calmodulin-binding transcription activator protein with CG-1 and Ankyrin
Glyma.01G126000	Tautomerase/MIF superfamily protein
Glyma.13G059400	Chorismite mutase 1
Glyma.08G007500	Acyl-CoA oxidase
Glyma.02G123700	Phosphoinositide 4-kinase gamma 7
Glyma.13G245500	Protein of unknown function (DUF300)
Glyma.18G128200	Protein of unknown function (DUF569)
Glyma.12G211700	Protein of unknown function (DUF1637)
Glyma.14G007300	Protein of unknown function (DUF1423)
Glyma.17G261600	Ubiquitin-like superfamily protein
Glyma.13G162800	Inorganic H pyrophosphate family protein
Glyma.04G001400	Protein of unknown function (DUF300)
Glyma.04G217200	Like COV 2
Glyma.06G173700	Zinc finger C-x8-C-x5-C-x3-H type family protein
Glyma.14G179900	Sequence-specific DNA binding transcription factors; transcription regulators
Glyma.03G128800	SEC 14 cytosolic factor family protein/
Glyma.02G134600	CDPK-related kinase
Glyma.12G112900	Lumazine-binding methylase 1
Glyma.11G031800	Ubiquitin-like superfamily
Glyma.08G119100	BTB/POZ domain-containing protein
Glyma.09G285300	P-loop containing nucleoside triphosphate hydrolase superfamily
Glyma.16G082400	Sec23/Sec24 protein transport family protein
Glyma.08G187100	XAP5 family protein
Glyma.04G030600	pfkB-like carbohydrate kinase family protein
Glyma.06G036300	Serine protease inhibitor (SERPIN) family protein
Glyma.18G028600	Arginine-tRNA protein transferase 1
Glyma.13G131700	Actin depolymerising factor 4
Glyma.07G270100	snRNA activating complex family protein
Glyma.07G183600	Aldehyde dehydrogenase 6B2
Glyma.03G252100	WHIRLY 2
Glyma.19G017300	Leucine-rich repeat kinase family
Glyma.18G028600	Arginine-tRNA protein transferase 1
Glyma.13G131700	Actin depolymerizing factor 4
Glyma.07G270100	snRNA activating complex family protein
Glyma.07G183600	Aldehyde dehydrogenase 6B2
Glyma.03G252100	WHIRLY 2
Glyma.19G017300	Leucine-rich repeat protein kinase family protein
Glyma.11G227700	RP non-ATPase subunit 8A
Glyma.18G056100	Protein kinase superfamily protein
Glyma.02G160100	Enhancer of polycomb-like transcription factor
Glyma.20G116300	Regulator of Vps4 activity in the MBV pathway
Glyma.08G302500	Basic-leucine zipper (bZIP) transcription factor family
Glyma.03G169700	Protein of unknown function (DUF3741)
Glyma.01G170400	RTE-homolog

Our analysis of co-expressed genes during development revealed over 100 genes with Pearson correlation co-efficient value of 1. A Pearson correlation co-efficient of 1 shows that the *GmCBRLCK1* gene Glyma.13G161700 is highly involved in the primary growth of *G. max* as evidenced by its expression in the legume primary tissues root pericycle, leaf palisade parenchyma, paraveinal mesophyll cell and mesophyll cell. The functions of co-expressed genes during perturbations showed that the genes are involved in regulating processes directly associated with the legume development. The top co-expressed gene Glyma.06G172700 is a glyceraldehyde-3-phosphate dehydrogenase C protein, which is a cytosol enzyme. The enzyme is vital in catalyzing the conversion of glyceraldeyde-3-phosphate to 1,3-biphosphoglycerate through reduction of NAD^+ to NADH [37]. Glyma.06G172700 may play a central role in plant primary metabolism. In Arabidopsis, a study by Bertomeu et al. [37] has revealed that the enzyme plays a vital role in plant development by affecting the supply of Ser to roots and a mutant of the enzyme coding genes resulted in impairment in the growth and development of roots. This suggests involvement of *GmCBRLCK1* in root development as seen by its high expression level within the pericycle. The vacuole is a multifunctional organelle that performs crucial physical and metabolic functions including cellular responses to environmental and biotic factors [38 - 39]. A vacuolar protein sorting gene Glyma.07G119500 is co-expressed with the *GmCBRLCK1* gene. This co-expression suggests the *GmCBRLCK1* gene is involvement in protein sorting in *G. max*. The vacuole is part of the endomembrane system which is involved in biosynthesis and sorting [40] though the chloroplast does not belong to the endomembrane system [41]. Studies have shown that the endomembrane system is involved in abiotic and biotic stress responses ([42]. Thus, *GmCBRLCK1* is likely to be involved in the sorting of proteins related to *P. sojae*, *P. pachyrhizi*, *H. glycines* and aluminium stress tolerance. A chloroplast related protein DegP protease [43] in the form of DegP protease 7 is co-expressed with *GmCBRLCK1* gene. The co-expression suggests that the Glyma.13G161700 is expressed within the photosynthetic machinery particularly the thylakoid membrane since DegP is strongly associated with the inner side of the thylakoid membrane [43]. In bacteria DegP has been described as a heat shock protein indicating that its expression is stimulated by heat. This allows the bacteria to survive at elevated temperatures [44]. In pea plants it was shown that DegP may be involved in chloroplast response to transient elevated temperatures. Elevated DegP levels were seen to be associated with elevated temperature 25°C to 40°C [43]. Therefore, heat stress is likely an abiotic stress factor which results in the expression *GmCBRLCK1* gene in chloroplast.

Only GO terms with higher (H) support vector machine (SVM) reliability values are shown in Table 3. for both biological processes and cellular component processes. The GO terms indicate the involvement of the *GmCBRLCK1* gene in signaling pathways, metabolic processes and phosphorylation. The gene is primarily an integral component of membrane and endomembrane system in *G. max*.

Table 3: *Cellular component and Biological processes predictions for GO terms*

GO term	Name	SVM Reliability
Biological Process Prediction		
GO: 0007166	Cell surface receptor signaling pathway	H
GO:0006796	Phosphate-containing compound metabolic process	H
GO:0016310	Phosphorylation	H
GO:0009059	Macromolecule biosynthetic process	H
GO:0019222	Regulation of metabolic process	H
GO:0006468	Protein phosphorylation	H
GO:0007167	Enzyme linked receptor protein signaling pathway	H

Cellular Components Predictions		
GO:0016020	Membrane	H
GO:0016021	Integral component of membrane	H
GO:0005886	Plasma membrane	H
GO:0031224	Intrinsic component of membrane	H
GO:0071944	Cell periphery	H
GO:0005576	Extracellular region	H
GO:0005887	Integral component of plasma membrane	H
GO:0031226	Integral component of plasma membrane	H
GO:0012505	Endomembrane system	H
GO:0005615	Extracellular space	H
GO:0031982	Vesicle	H
GO:0070062	Extracellular vesicular exosome	H
GO:0031988	Membrane—bound vesicle	H
GO:0005783	Endoplasmic reticulum	H
GO:0031090	Organelle membrane	H
GO:0098588	Bounding membrane of organelle	H

4 Conclusions

Our bioinformatic functional characterisation has provided an initial insight into the role of Glyma.13G161700 a calmodulin-binding receptor-like cytoplasmic kinase in *G. max*. The outcome of the analysis show that *GmCBRLCK1* gene is involved in receptor signaling pathways that are related to the primary development of soybean seedling through regulating transcription of proteins related to metabolic processes and pathogen and abiotic stress tolerance. The bioinformatic characterisation will however need to be complemented with the biochemical characterisation of the gene to further provide concrete evidence on its physiological functions in plants. We believe with further biochemical analysis overexpression/or expression analysis of this gene can be investigated in transgenic plants. Such studies may lead to improved crop tolerance to environmental factors and increase crop productivity.

5 Declarations

5.1 Competing Interest

Authors declared no potential conflict of interest exists.

How to Cite this Article:

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