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RESEARCH ARTICLE

Cloning and characterization of Patatin like Protein (PLP), a phospholipase gene involved in salinity stress induced lipid signaling

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Abstract

Sugarcane (*Saccharum* spp.) is a large perennial grass that is cultivated in tropical, semi-tropical, and subtropical regions of the world. Salinity stress is one of the important abiotic stresses limiting crop growth and productivity. It involves numerous changes in the physiological and metabolic processes, depending on severity, stage of the crop and duration of the stress. Plasma membranes are the primary sites where stress signals are perceived and transmitted for rapid gene expression/enzyme activity. Lipid signaling is one such perfect mechanism for the transmission of information between plasma membrane, cytosol, organelles, particularly nucleus and has found an integral place in stress signaling in plants. Phospholipases (PLs) constitute a family of lipid-hydrolyzing enzymes that catalyze lipid remodeling and generate rapid responses to environmental cues. In this study we have cloned and characterized Patatin like Protein (PLP) a phospholipase gene from *Erianthus.* The saline (8 ds/m) treated sugarcane hybrids such as Co 11015 and Co 12009 along with *Erianthus* was used for estimation of the copy number of PLP in various tissues. Our results showed that, the leaves of Co 11015 had high copy number of PLP followed by the leaf tissues of *Erianthus*. This study aids in understanding of PLP gene expression involved in salinity stress induced lipid signaling.

Keywords: Lipid signaling; Phospholipases; Salinity; Sugarcane

Introduction

Sugarcane being a perennial crop is exposed to numerous biotic and abiotic stresses during its entire life span. Among the various stresses the abiotic stress has a consequential effect due to varying stress levels such as high salinity, dehydration and low temperature leading to adverse growth conditions, specifically affecting the development, longevity and productivity. Abiotic stress tolerance depends on the inter connections of signal transduction pathways towards detoxification, homeostasis, and growth regulation. Lipid signaling is one of the major signaling network activated in plants as an adaptive response upon exposure to various abiotic stress.

Salinity stress affects the crop in two different phases, osmotic stress which occurs during the initial phase of the stress, followed by ionic stress due to high concentrations of Na^+. Osmotic stress decreases water absorption by the roots and causes various reductions of photosynthetic activities and the production of reactive oxygen species (ROS). Ionic stress is caused by an increased absorption of ions, primarily Na^+ and Cl^-, which results in, high concentrations of intracellular Na^+ which prevents the uptake of K^+, an essential element for several cellular processes. Plants have evolved a complex mechanism for adaptation to osmotic and ionic stresses, such as accumulation of osmo-protectants, and compartmentation of ions. Multiple signal transduction pathways are also activated at cellular level to adjust the gene expression in plants in turn triggering response pathways (Munnik and Testerink 2009). During salinity stress phospholipase (PLs) produce lipid mediators such as phosphatidic acid (PA) which are the key regulators of endomembrane organization

and thus switch on or modulate signaling cascades that lead to changes in gene activation which results in stress tolerance (Singh et al. 2012).

Patatin-like Protein belongs to the phospholipase-A family, which plays an important role in stress tolerance. (Scherer et al. 2010) identified thirteen patatin-related genes in *Arabidopsis* and classified into four subfamilies such as Patatin related Phospholipases A (pPLA) I, II (α, β, γ, δ, ε), III (α, β, γ, δ). This study confirmed that knockout of *pPLAIIIγ* produced salinity sensitive plants, while over expression of *pPLAIIIγ* increased plant tolerance to salinity and drought stress. This study proved that pPLAIIIγ acts a positive regulator of salt and osmotic stress tolerance in *Arabidopsis*. (Wei et al. 2023) has identified 33 pPLA genes using a genome-wide analysis in cotton, and through phylogeny they have classified these genes into three groups. They showed that these pPLA genes are unevenly distributed on all the 26 chromosomes and contain a few introns. They have also detected four genes were expressed at varying levels during drought and salt stresses. This study published recently has clarified the role of pPLA in the response to drought and salt tolerance. PLP gene is studied for its expression in different species such as peanut, Arabidopsis, soybean, oilseed rape, maize, rice, and potato (Gao et al. 2021).

Erianthus arundinaceus, a wild genus of sugarcane was chosen for this study, as an important source of resistance genes towards salinity stress. In this present study we have cloned and characterized Patatin-like Protein involved in salinity stress tolerance from *Erianthus* IK-76-48. The study (Boulter et al. 2016) shows that the tissue-specific, temporal and spatial patterns of gene expression play pivotal roles in understanding the functionality of a biological system and it could be analyzed by real-time quantitative polymerase chain reaction (qPCR) technique recognize the single gene expressions. Hence, the PLP gene copy number has also been quantified from the cDNA of different tissues (leaf, stem and root) of various clones such as IK76-48 (*Erianthus),* Co 11015 and Co 12009 after salinity treatment. The results of our study might be exploited to develop climate resilient varieties along with the enhanced sugarcane production yield.

Materials and Methods

Sample collection

The *Erianthus* clone (IK 76-48), Co 11015 and Co 12009 were collected from ICAR-Sugarcane Breeding Institute, Coimbatore.

Genomic DNA isolation and Primer designing

The total genomic DNA was isolated from the leaves tissues of 10 month old *Erianthus* clone IK 76-48 using CTAB method (Aboul-Maaty and Oraby 2019). The isolated Genomic DNA was diluted and used as template for PCR amplification. The partial sequence of patatin-like protein genes from sorghum, maize, rice, and switch grass species was retrieved from Genbank NCBI. The sequences were aligned using multiple sequence alignment-CLUSTALW2. The conserved domain was identified and Primer 3 plus software was used to design the patatin-like protein gene specific primer. The primer sequences are GDPLP-forward- 5'-GCTCATCACTAGTCAC ACGTACAC-3', GDPLP-reverse- 5' TACATTTTGTTAGAATTA GTTTATGTTTT TCTTTC-3'.

PCR reaction setup and Cloning of PLP gene

To amplify PLP gene, with the genomic DNA of *Erianthus* (IK 76-48) of 2 µl (60 ng/ µl), LA-Taq polymerase of 0.3 µl, 2 X GC buffers 12.5µl, dNTPs 4µl (0.2 mM), GD_PLP gene specific primers 1 µl (10 pico moles/µl) and the PCR reaction was setup for 34 cycles of 94°C for 3 min for initial denaturation, 94°C for 1 min for

denaturation, 56°C for 45 sec for primer annealing, 72°C for 1.15 min extension and final extension for 72°C in 10 min. The products were visualized on 1% agarose gel and the required fragment was eluted using QIA-quickgel elution kit (Qiagen, USA). The amplified fragment was T/A cloned using InsTA cloning kit (Thermofisher, USA). Then the ligation mixture was incubated at 37°C for 2 hrs and plated with x-gal and IPTG on LB agar plates. The transformed colonies were picked by initial screening. The cloned fragment was confirmed through PCR with plasmid DNA isolated from the white colonies and sequenced at University of Delhi South Campus, New Delhi.

Bioinformatics analysis

Nucleotide and amino acid sequences were analyzed using NCBI and EXPASY tools. Alignment of amino acid sequences were made with the Clustal X program, while BLAST searches were carried out using the National Center for Biotechnology Information Website (http://www.ncbi.nlm.nih.gov). The phylogeny analysis was performed using MEGA 11 software. The motif identification for this gene was detected using MEME (https://meme-suite.org/meme/tools/meme) molecular evolutionary genetics analysis using UPGMA, Test of Phylogeny: Bootstrap method with 1000 Bootstrap Replications.

Expression profiling after salinity treatment

The Co 11015 and Co 12009 are high yielding and high quality clones under varied agro-climatic conditions, which were chosen for performing molecular expression profiling of PLP gene along with *Erianthus* clone IK 76-48. Pot culture experiment was conducted using the above-mentioned clones for expression studies of PLP genes under salinity stress. The salt mixture composed of sodium sulphate (1.58 g/l), sodium chloride (2.252 g/l) and calcium chloride (2.052 g/l) in the ratio of 2:2:1 was used with an electrical conductivity of 8 dS/m for this experiment (Vasantha et al. 2010). The *Erianthus* clone, Co 11015 and Co 12009 were planted and grown for 100 days where the plant's tillering stage takes place, the plants retaliate to the molecular response and produce salinity tolerance transcripts (Kasirajan et al. 2022). To begin the stress treatment experiment, the pots were irrigated with the salt mixture for seven days and the morphological changes such as withered leaves and leaf tip drying were observed from the 7^{th} day. Furthermore, the samples from various tissues (leaf, stem and root) were collected on 9^{th} day. Controls were maintained by irrigating with normal.

cDNA for absolute quantification

Tissue samples from stem, leaves, and root were collected from the clones IK76-48, Co 11015 and Co 12009 after salinity treatment and the samples were frozen in liquid nitrogen. Total RNA was isolated from all the tissues using Trizol method (Liu et al. 2018). The isolated RNA was converted into cDNA using thermofisher cDNA Synthesis Kit (Thermofisher scientific, USA) and it was normalized using a house keeping gene 25s rRNA. Subsequently, the PLP gene specific primers PLP_RT_Forward 5' CTCGCGGACTACTT CGACTAC 3' and PLP_RT_ Reverse 5' ATGTC CTTGGCAGCGTAGAG 3' was used in the expression study.

Construction of standard curves for copy number determination

A 15-fold serial dilution of plasmid DNA harboring the PLP gene was performed so that, the copy number ranges from 1×10^{5} to 1×10^{9}copies/µl, which was used to construct the standard graph. The concentration of the plasmid was measured using a fluorometer and the corresponding copy number was calculated using the following equation 1 (Mahboudi et al. 2018).

Equation 1

$$\text{DNA (copy)} = (6.02\times10^{23}) \left(\frac{copy}{mol}\right) \times$$

$$\text{DNA amount (bp)} \times 660 \left(\frac{g}{mol}/dp\right)$$

CT values in each dilution ($10^{-1}$to 10^{-15}) were measured in duplicate using a real-time qPCR to generate the standard graph respectively. The CT values were plotted against the logarithm of their initial template copy numbers. Each standard graph was generated by a linear regression of the plotted points. From the slope of standard curve, PCR amplification efficiency (E) was calculated according to the equation 2(Ruijter et al. 2009).

Equation 2

$$E = 10^{(-\frac{1}{slope})}$$

Results and Discussion

activity. Phospholipases (PLs) on the plasma membrane constitute a family of lipid-hydrolyzing enzymes that catalyze lipid remodeling, and generate rapid responses of plants to environmental cues. Phospholipase are classified into phospholipases A (A1, A2), C and D according to the site of phospholipid cleavage. There are many phospholipase families responsible for stress tolerance mechanism (Wang et al. 2019). These genes produce the plant protecting enzymes and protect the plant from both biotic and abiotic stresses.

Cloning of PLP gene and sequence analysis

Genomic DNA was isolated from *Erianthus* clone IK 76-48 and it was treated with RNAse (Fig.1a). The targeted PLP gene was amplified using gene specific primer and with an amplicon size of 2.2 kb (Fig.1b).

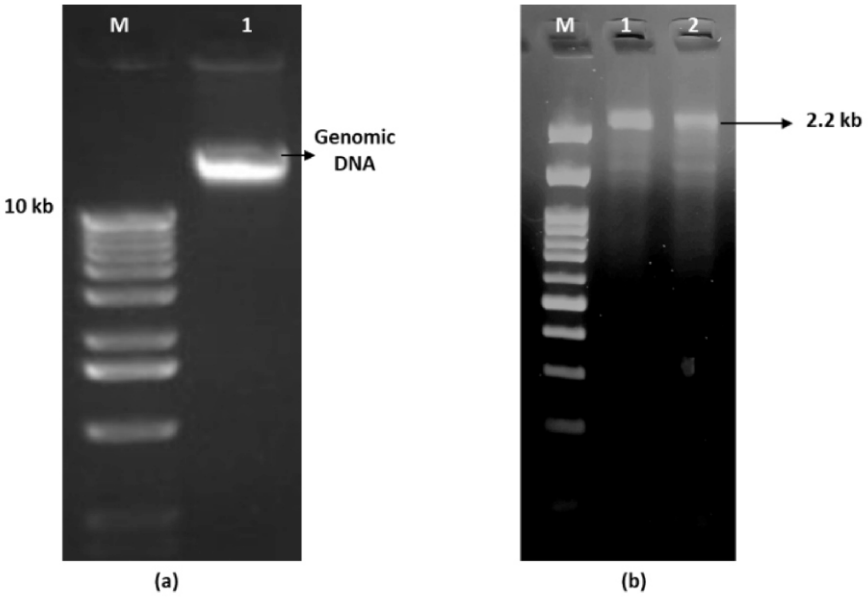


Figure 1a. Genomic DNA isolated from the IK 76-48 clone. b) PLP gene amplification using PCR. M: 100 bp DNA ladder, Lane 1& 2: amplification of fragment at ~2.2kb.

Plants constantly face challenging environment but they cope with them through their metabolism and physiological adjustments. Lipid signaling is an important mechanism which has gained attention recently due to their regulatory effect on cellular

The cloning was confirmed through colony PCR and the plasmid DNA was isolated from the colony PCR positive white colonies. It was reconfirmed through PCR using the plasmid DNA as template, which resulted in 2.2 kb of band size shown in (Fig. 2).

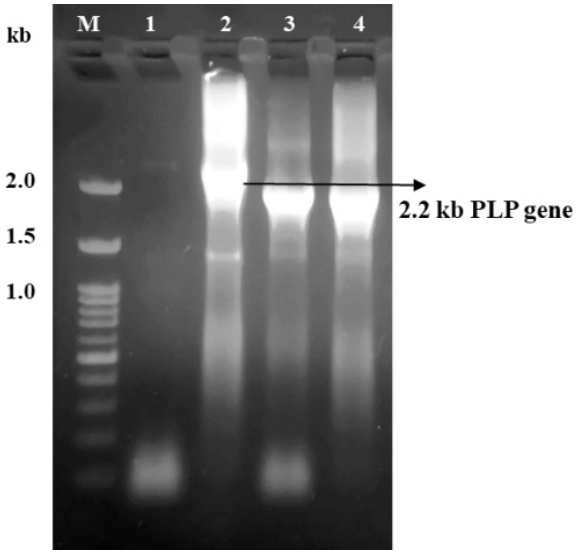


Figure 2. Plasmid PCR from positive colonies and negative colonies, M: 100bp ladder, Lane: 2,3,4: positive colony with the amplification of 2.2 kb, Lane: 1 Negative colonies.

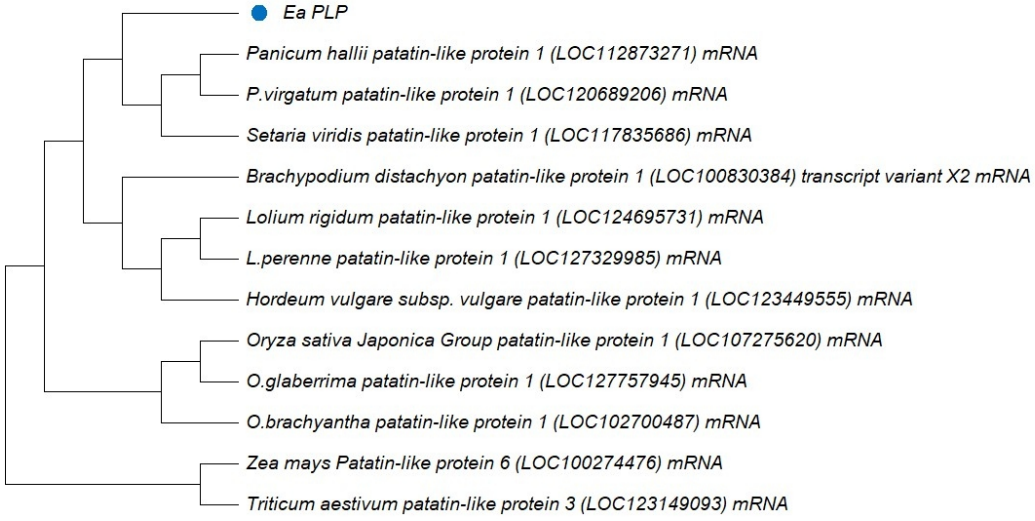


Figure 3. Phylogeny analysis of PLP gene showing the clustering of EaPLP with *Panicum* species (Switch grass)

The positive plasmid was sequenced and the sequencing results produced a fragment of 1.664 kb coding for PLP gene. The sequence analysis showed 88.5 and 88.3 % sequence similarity with patatin like protein of *Setaria viridis* and *Panicum virgatum* respectively. The phylogeny analysis also showed that Ea_PLP gene clustered with *Panicum virgatum*, *Panicum hallii* and *Setaria viridis.*

A set of 7 protein sequences, between 330 and 437 in length were analyzed through MEME software which showed that it had a conserved serine hydrolase/lipase motif (GXSXG) and the phosphate-binding element DGGGXR (Fig. 4a & b). Our results are similar to (Holk et al. 2002) in which they identified four patatin-related PLA genes (*At*PLA I, *At*PLA IIA, *At*PLA IVA, and *At*PLA IVC) from Arabidopsis (ecotype Columbia). They also identified patatin- specific motifs which may function in plant signal transduction.

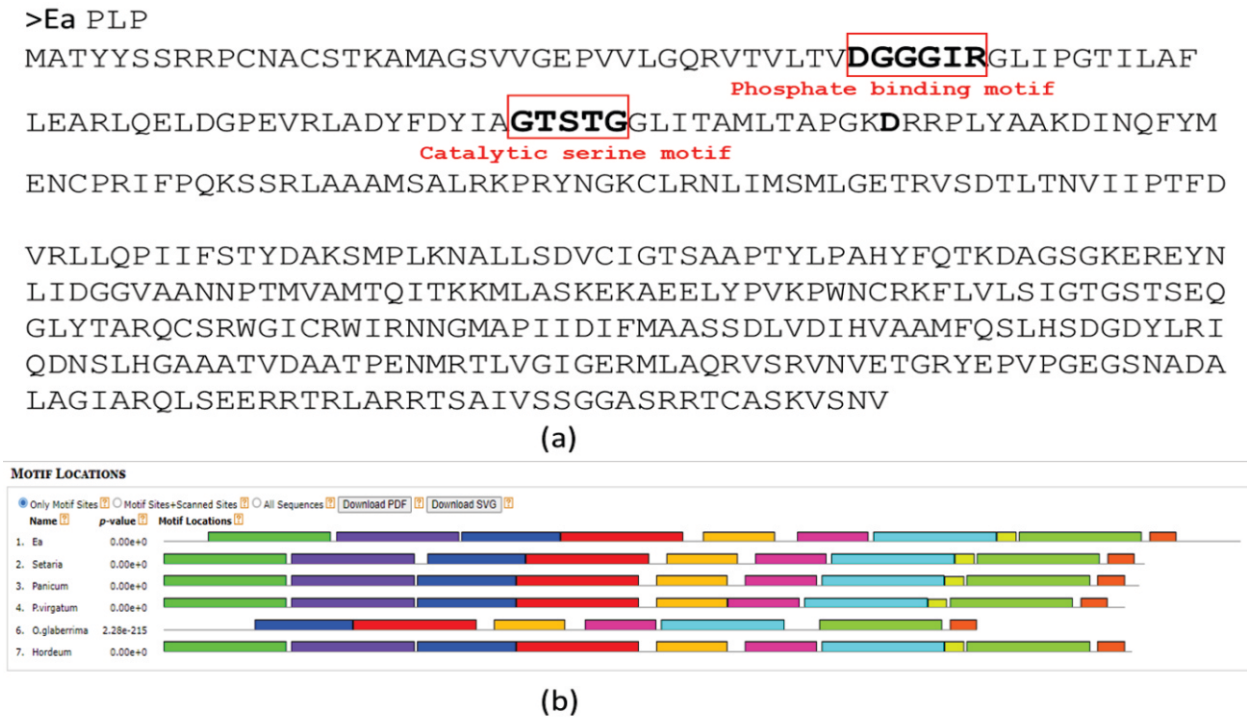


Figure 4. Amino-acid sequence of PLP gene and Motif analysis of the EaPLP along with related species

Expression profiling of PLP gene after salinity stress

After, the saline treatment the root, stem and leaves samples were collected from sugarcane varieties (Co 11015 and Co 12009) and *Erianthus* (IK 76-48). Earlier study has reported that Co 12009 has a wide adaptability to varied environments, resistant to red rot and a promising donor for drought tolerance (Alarmelu et al. 2022). Co 11015 variety is also reported to be a consistent high yielder under varied agro-climatic conditions across Tamil Nadu. The developer has identified this clone as a drought tolerant, early maturing clone which has made three crops in two years (Hemaprabha et al. 2019). Henceforth, these clones along with *Erianthus* has been chosen and studied for their salinity tolerance limits. The total RNA was isolated from the respective clones and tissues and converted into cDNA, normalized by 25s rRNA shown in (Fig. 5) for further study.

The PLP gene copy number was quantified in different sugarcane variety (Co 11015 and

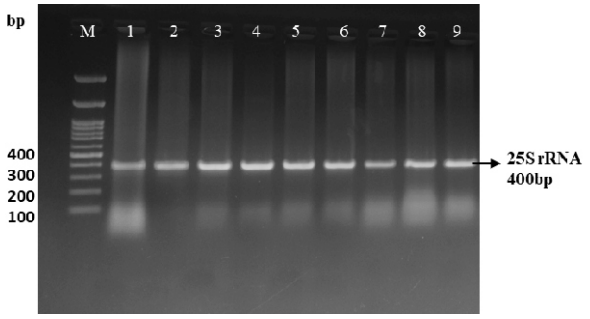


Figure 5. PCR amplification of cDNA for normalization of total RNA with 25s rRNA specific primers.

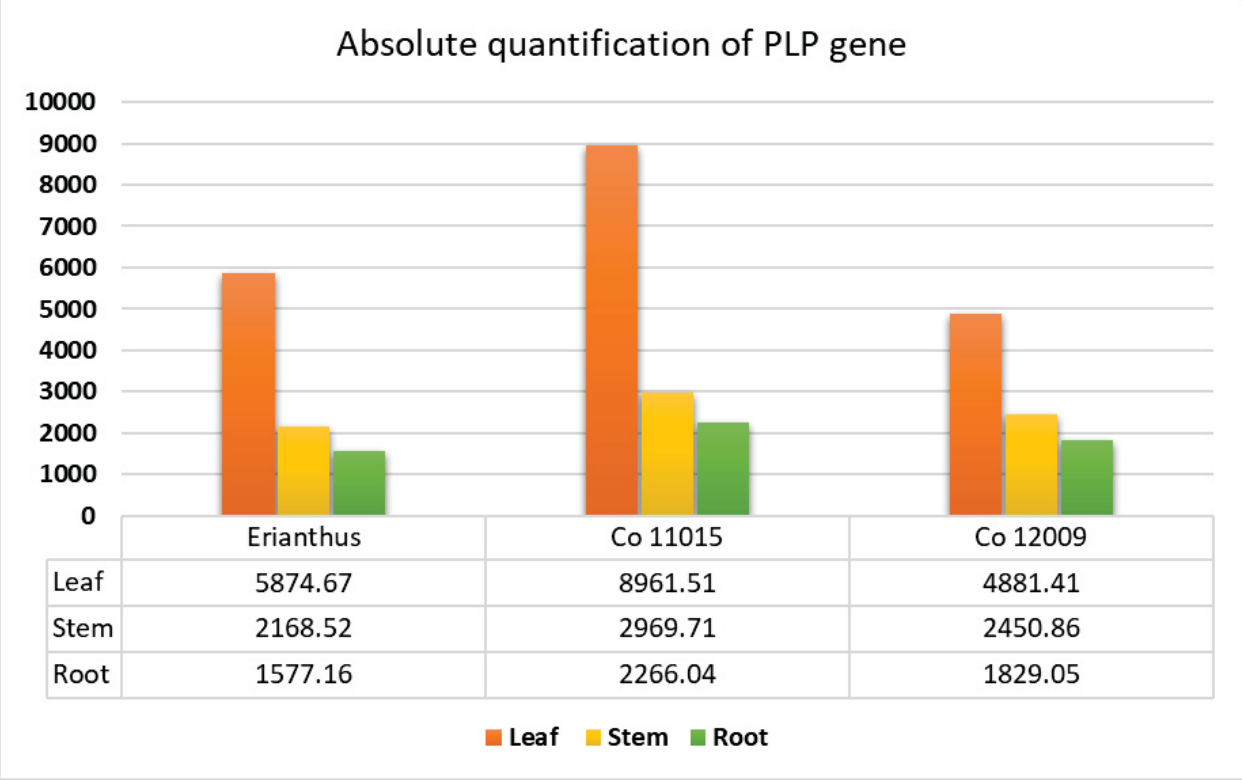
Co 12009) and *Erianthus* (IK 76-48) using the absolute quantification technique. The plasmid reference curve was determined by employing the series of serially diluted plasmid from the range of 10^{-1} to 10^{-15} with respect to the known concentration of it and the corresponding Ct values of the serial diluted plasmid DNA has been used to create the slope- intercept form. The reference curves were linear in the range tested with (R^2>0.9817) by the reactions (Sharma and Dean-Nystrom 2003). From the slopes value of -3.108 which falls under the range of - 3.1 ≤ slope ≤ - 3.6 (Mahboudi et al.

2018), with higher amplification efficiency of 109.77 (2.09) calculated from the above-mentioned equation 2.

The reference curve of PLP plasmid makes a linear relationship between Ct and initial amounts of total cDNA samples of root, stem, and leaf of all genotypes permitting the determination of the concentration of unknown's samples. The PLP gene molecules were high in leaf tissues of Co 11015 (8961.5) followed by *Erianthus* (5874.67) and Co 12009 (4881.4). Similarly, the PLP molecules were lesser in stem and root tissues of *Erianthus* as well as the sugarcane varieties (Fig. 6). The PLP gene copy was abundant in the leaves and shrunken expression in the stem and root tissue sample. The Arabidopsis AtPLA IIA promoter (PIIA) was analyzed and the expression was high in leaves followed by roots and stipules and not in the flowers (Holk et al. 2002). They also showed that the transcription was elevated by several hormones which was related to the plant's wounding, pathogen signaling and by phosphate and iron deficiency. The study report on Arabidopsis, Rice, Camelina and Canola plants shows that the patatin like protein were developed in response to hormones and nutritional starvation. Further the overexpression of the PLP gene leads to decreased cell elongation in vegetative and reproductive growth of rice, which resulted in reduced cellulose content, anisotropic growth, and plant height (Yang et al. 2012; Dong et al. 2014; Liu et al. 2015). Large number of studies have proven the accumulation of phosphatidic acid (PA) is induced during salinity stress in plants (Munnik et al. 2000; Bargmann et al. 2009; Darwish et al. 2009).

Conclusion

Phospholipids, which constitute the bio-membranes separates organelles and cytoplasm, and act as signaling molecules during salt stress



	Erianthus	Co 11015	Co 12009
Leaf	5874.67	8961.51	4881.41
Stem	2168.52	2969.71	2450.86
Root	1577.16	2266.04	1829.05

Figure 6. Comparison of PLP gene copy number in the following clones: Co 11015, Co 12009 and *Erianthus* using absolute quantification of gene expression on stem, root, and leaf tissues.

response. Recently phospholipids-based lipid signaling is the key focus of research due to their important role in membrane rearrangement, cytoskeletal dynamics, and abiotic and biotic stress tolerance. In-spite of the vast potential role of phospholipase very little/ no information is available about the gene sequences and their genetic control in sugarcane. Hence in this study we have cloned and characterized a patatin like protein a phospholipase gene and quantified its expression in various tissues of *Erianthus* and sugarcane hybrids during salinity stress. Patatin-like proteins has an important role in lipid signaling pathway activated during salinity stress. In the present study, we have shown patatin-like genes are induced by salinity at higher concentration in the leaf tissues which might also play a role associated to senescence.

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References

Aboul-Maaty NA-F, Oraby HA-S. 2019. Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. Bulletin of the National Research Centre 43(1):1–10.

Alarmelu S, Hemaprabha G, Shanthi RM. 2022. Co 12009: Midlate sugarcane variety for tropical India. Electron Journal of Plant Breeding 13(3):1012–1018.

Bargmann BOR, Laxalt AM, Riet B Ter, Van Schooten B, Merquiol E, Testerink C, Haring MA, Bartels D, Munnik T. 2009. Multiple PLDs required for high salinity and water deficit tolerance in plants. Plant Cell Physiology 50(1):78–89.

Boulter N, Suarez FG, Schibeci S, Sunderland T, Tolhurst O, Hunter T, Hodge G, Handelsman D, Simanainen U, Hendriks E, et al. 2016. A simple, accurate and universal method for quantification of PCR. BMC Biotechnology 16(1):27.

Darwish E, Testerink C, Khalil M, El-Shihy O, Munnik T. 2009. Phospholipid Signaling Responses in Salt-Stressed Rice Leaves. Plant Cell Physiology 50(5):986–997.

Dong Y, Li M, Zhang P, Wang X, Fan C, Zhou Y. 2014. Patatin-related phospholipase pPLAIIIδ influences auxin-responsive cell morphology and organ size in *Arabidopsis* and *Brassica napus*. BMC Plant Biology 14:1–20.

Gao G, Zhang X, Zhao Kunkun, Zhao Kai, Cao D, Ma Q, Zhu S, Qu C, Ma Y, Gong F. 2021. Genome wide identification and expression analysis of patatin-like protein family members in peanut (*Arachis hypogaea* L.). Reproduction and Breeding 1(1):48–54.

Hemaprabha G, Appunu C, Mohanraj K, Annadurai A, Alarmelu S, Sreenivasa V, Karuppaiyan R, Shanthi RM, Govindaraj P, Mahadevaiah C, et al. 2019. Co 11015 (Atulya) a Recently Notified Sugarcane Variety for Tamil Nadu. Journal of Sugarcane Research 9(2):193.

Holk A, Rietz S, Zahn M, Quader H, Scherer GFE. 2002. Molecular identification of cytosolic, patatin-related phospholipases A from Arabidopsis with potential functions in plant signal transduction. Plant Physiology 130(1):90–101.

Kasirajan L, Valiyaparambth R, Kamaraj K, Sebastiar S, Hoang N V, Athiappan S. 2022. Deep sequencing of suppression subtractive library identifies differentially expressed transcripts of *Saccharum spontaneum* exposed to salinity stress. Physiologia plantarum 174:1–12.

Liu G, Zhang K, Ai J, Deng X, Hong Y, Wang X. 2015. Patatin-related phospholipase A,

pPLAIIIα, modulates the longitudinal growth of vegetative tissues and seeds in rice. Journal of Experimental Botany 66(21):6945–6955.

Liu L, Han R, Yu N, Zhang W, Xing L, Xie D, Peng D. 2018. A method for extracting high-quality total RNA from plant rich in polysaccharides and polyphenols using Dendrobium huoshanense. PLoS One. 13(5):1–9.

Mahboudi H, Heidari NM, Rashidabadi ZI, Anbarestani AH, Karimi S, Darestani KD. 2018. Prospect and Competence of Quantitative Methods via Real-time PCR in a Comparative Manner: An Experimental Review of Current Methods. The Open Bioinformatics Jornal 11(1):1–11.

Munnik T, Meijer HJG, Riet B Ter, Hirt H, Frank W, Bartels D, Musgrave A. 2000. Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. Plant Journal 22(2):147–154.

Munnik T, Testerink C. 2009. Plant phospholipid signaling: "In a nutshell." Journal of Lipid Research 50 (SUPPL.).

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den hoff MJB, Moorman AFM. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research 37(6):2–12.

Scherer GFE, Ryu SB, Wang X, Matos AR, Heitz T. 2010. Patatin-related phospholipase A: Nomenclature, subfamilies and functions in plants. Trends Plant Sciences 15(12): 693–700.

Sharma VK, Dean-Nystrom EA. 2003. Detection of enterohemorrhagic Escherichia coli O157:H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. Veterinary Microbiology 93(3): 247–260.

Singh A, Baranwal V, Shankar A, Kanwar P, Ranjan R, Yadav S, Pandey A, Kapoor S, Pandey GK. 2012. Rice phospholipase A superfamily: Organization, phylogenetic and expression analysis during abiotic stresses and development. PLoS One. 7(2):30947.

Vasantha S, Venkataramana S, Gururaja Rao PN, Gomathi R. 2010. Long term salinity effect on growth, photosynthesis and osmotic characteristics in sugarcane. Sugar Tech. 12(1):5–8.

Wang P, Shen L, Guo J, Jing W, Qu Y, Li W, Bi R, Xuan W, Zhang Q, Zhang W. 2019. Phosphatidic acid directly regulates PINOID-dependent phosphorylation and activation of the PIN-FORMED2 auxin efflux transporter in response to salt stress. Plant Cell. 31(1):250–271.

Wei Y, Chong Z, Lu C, Li K, Liang C, Meng Z, Wang Y, Guo S, He L, Zhang R. 2023. Genome-wide identification and expression analysis of the cotton patatin-related phospholipase A genes and response to stress tolerance. Planta. 257(3).

Yang W-Y, Zheng Y, Bahn SC, Pan X-Q, Li M-Y, Vu HS, Roth MR, Scheu B, Welti R, Hong Y-Y. 2012. The patatin-containing phospholipase A pPLAIIα modulates oxylipin formation and water loss in *Arabidopsis thaliana*. Molecular Plant 5(2):452–460.