OsPLB gene expressed during seed germination encodes a phospholipase in rice

Achintya Kumar Dolui^{1,2} · Mahadev Latha^{1,2} · Panneerselvam Vijayaraj^{1,2}

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Abstract

Hydrolysis of phospholipid monolayer by phospholipases is an important event in the mobilization of stored lipids for seed germination. However, the identification and functional characterization of cereal phospholipases, especially during rice germination, are limited. In the present study, we have identified and characterized a phospholipase *OsPLB* gene expressed during germination. The full-length coding region of *OsPLB* was cloned into pRSETA as well as pYES2/NTC vector. The recombinant protein was successfully expressed in both *E. coli* and *Saccharomyces cerevisiae*. The recombinant protein was purified to homogeneity by affinity chromatography, and it was further confirmed by MS/MS analysis. In vitro lipase assay and lipidome analysis using high-resolution mass spectrometry showed phosphatidylcholine (PC) specific phospholipase B activity. The results revealed that protein encoded by *OsPLB* gene prefers to hydrolyze PCs with C28, C32, and C34 containing unsaturated fatty acids. Collectively, the present study describes the identification and characterization of a phospholipase B, which hydrolyze PC, a major component of phospholipid monolayer covering storage lipid, as an initial event during rice seed germination.

Keywords Seed germination · Phospholipases · Phospholipids · Lipidome · Rice

Introduction

Plant embryo and endosperm store a significant amount of triacylglycerol (TAG) in oleosome during the final stage of seed maturation. Oleosomes consist of phospholipid monolayer associated with proteins, which prevents the vesicles from coalescing (Murphy 2001). Hydrolysis of stored TAG by lipases plays a crucial role in plant growth and development, particularly during the initial stage of

seed germination. The released free fatty acids (FFAs) are converted to acetyl-CoA by β -oxidation and is ultimately converted to sugars by the glyoxylate cycle and gluconeogenesis. It supplies the required energy to embryos for its post-germinative growth and development (Graham 2008). Before TAG breakdown, activation of phospholipases and hydrolysis of phospholipid monolayer is an essential event for the initiation of energy mobilization. During the initial period of seed germination, the expression of phospholipase and subsequent hydrolysis of phospholipid monolayer pave the way for TAG degradation (May et al. 1998). Disturbance of phospholipases activity results in severe abnormalities like delayed germination and defective vegetative growth (Tsai and Chung 2014). Reports evidence that lipase activity is rapidly enhanced during the onset of seed germination (Graham 2008). Among cereals, the maximum lipase activity was reported in rice bran, which contains 15–20% oil with a favorable fatty acids composition suitable for human consumption (Borgston and Brockman 1984). Palmiano and Juliano (1973) reported that the lipase activity was significantly higher than the esterase activity during the first week of rice germination. It shows that lipases play a significant physiological role during germination for proper plant

Achintya Kumar Dolui and Mahadev Latha contributed equally to the article.

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✉ Panneerselvam Vijayaraj
vrlipid@cetri.res.in

¹ Department of Lipid Science, CSIR-Central Food Technological Research Institute, Mysuru, Karnataka 570020, India

² Academy of Scientific and Innovative Research, Ghaziabad, Uttar Pradesh 201002, India

establishment. Lipases/esterases were identified from rice bran and seeds of other plants such as *Zea mays*, *Helianthus annuus* L. cv. Morden, cucumber (Bhardwaj et al. 2001; Chuang et al. 2011; Kim 2004; Gupta and Bhatla 2007; May et al. 1998). In Arabidopsis, TAG lipase, i.e., sugar-dependent-1 (SDP1) initiates hydrolysis of stored TAG during germination (Eastmond 2006). However, there are not enough reports on the functional characterization of phospholipases during germination. Further, in a genome-wide analysis of *Oryza sativa*, many putative lipase genes have been identified (Lin and Huan 1984; Li et al. 2007; Chepyshko et al. 2012), but their functional characterization is lacking. Hence, the identification and functional characterization of rice lipases, particularly phospholipases, are essential to understand the mobilization of stored lipids in germination and also to control the lipase activity during storage. In the present study, we identified the Os11g0655800 gene (designated as OsPLB), which is highly expressed during seed germination and functionally characterized using enzyme assay and mass spectrometric analysis. The recombinant OsPLB protein showed phospholipase activity towards phosphatidylcholine (PC) with unsaturated fatty acids.

Materials and methods

Materials

The lipid substrates and SPLASH LIPIDOMIX MS-Standard were procured from Avanti Polar Lipids (Alabaster, AL). cDNA Synthesis Kit and other enzymes were procured from Thermo Scientific. pRSETA, pYES2/NTC vectors, and *E. coli* strains were purchased from Invitrogen. Triolein, monoolein and all other chemicals were procured from Sigma-Aldrich.

In silico analysis of OsPLB protein sequence

Sequence analysis, secondary structure prediction, domain analysis, and three-dimensional structure prediction were performed using PHYRE2 Protein Fold Recognition Server. The presence of the lipase motif was further analyzed using the PROSITE database.

Plant material, RNA isolation, and cDNA synthesis

Freshly harvested rice (*O. sativa*, IR64) seeds were surface sterilized using 0.1% mercury chloride. Afterward, seeds were thoroughly washed with sterile water. Seeds were allowed to germinate up to 192 h and sampling was done at 0, 48, 96, and 192 h. From each sample total RNA was isolated following protocol as described by Vicent and Delseny (1999). cDNA was synthesized from 1 µg RNA

using kit as per manufacturer instructions (Cat No. K1671, Thermo Scientific) and used for amplification of the *OsPLB* and qPCR analysis.

Profile of *OsPLB* expression

The mRNA expressional status of *OsPLB* during different stages of germination [48 h, 96 h, 192 h (two-leaf stage) with 0 h used as control] was studied using gene-specific primer by qPCR analysis. Actin was used as an internal control. The primers are listed in Supplementary Table 1. The experiments were performed in triplicates with 10 µl reaction mixture using the cDNAs, gene-specific primers and iTaq Universal SYBR Green Supermix (Bio-Rad) in CFX Connect™ Real-Time PCR Detection System.

Cloning and heterologous expression of *OsPLB* in *E. coli* and *Saccharomyces cerevisiae*

The full-length coding sequence of the *OsPLB* gene was amplified from cDNA using gene-specific primers (Supplementary Table 1). For bacterial expression, the gene was cloned into the pRSET-A vector with *EcoRI* and *HindIII* restriction sites, whereas it was cloned into the pYES2/NTC vector for yeast expression using *BamHI* and *EcoRI* restriction sites. Positive clones harboring the gene were confirmed by both restriction digestion and by nucleotide sequencing. The *OsPLB* gene construct and empty vector were transformed into Rosetta (DE3) pLys. Cells were induced with various concentrations of isopropylthio-β-galactoside (IPTG) for 4 h at 37 °C to enable gene expression. Cells were harvested by centrifugation at 2400 × g. Pellets were washed with sterile water and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, and 300 mM NaCl, 2 mM MgCl₂, 10% glycerol (v/v), and 1 mM PMSF). The cell lysates were centrifuged at 2400 × g at 4 °C for 5 min, and the cell-free extract was further centrifuged at 13,000 × g at 4 °C for 30 min. The membrane fraction was solubilized, and the recombinant protein was purified using Ni²⁺-NTA affinity chromatography. OsPLB expression was confirmed by immunoblot analysis using an anti-His₆ tag monoclonal antibody (Cat no. H1029, Sigma).

In yeast, the *OsPLB* construct and pYES2/NTC empty vector were transformed into yeast lipase mutant strain, *yju3Δ* using the lithium acetate, and transformants were selected by uracil depletion media (SM-U). The resulting transformants were grown to the late log phase in SM-U containing 2% dextrose. For induction of OsPLB protein, the cells were harvested and washed with cold water by centrifugation and induced at 0.4 OD (*A*₆₀₀) with 2% (w/v) galactose and 1% (w/v) raffinose in SM-U media for 24 h. Cells were harvested at 2400 × g at 4 °C for 5 min and suspended in lysis buffer as mentioned earlier. Proteins were extracted

after cell lysis using sterile glass beads at 4 °C using glass beads. The expression of recombinant protein was confirmed by immunoblot using an anti-His₆ tag monoclonal antibody.

Confirmation of recombinant OsPLB protein by in-gel trypsin digestion

In-gel trypsin digestion of purified recombinant protein was performed following protocol, as described by Shevchenko et al. (2007). MS–MS ions search were performed using MASCOT search engine using Mascot Generic File (MGF) against NCBI nr with the following set parameters: enzyme specificity of trypsin, Taxonomy filter of *O. sativa*. Missed cleavage of 1 was allowed. Static modification of carbamidomethyl, variable modification of oxidation of methionine was also allowed. Mass tolerance was set to ± 1.5 Da for precursor mass and ± 0.5 Da for product ion masses.

In vitro enzyme assay

The acyl hydrolase activity of OsPLB was monitored using various individual polar and neutral lipid substrates. The assay was performed for 30 min at 37 °C in the presence of 20 µg recombinant OsPLB protein or protein from vector background as enzyme source. The assay mixture consisted of 50 mM Tris–HCl (pH 8.0), 1 mM MgCl₂, 1 mM KCl, 10% Glycerol in the presence of 50 µM natural substrates or 0.1 µM fluorescent substrate. For phospholipases activity, 10 mM CaCl₂ was incorporated into the assay buffer. After the incubation period, the reaction was stopped by the addition of 2:1 (v/v) CHCl₃:CH₃OH. The enzymatic product formation was monitored by silica-TLC using the following solvent system and quantified. For neutral lipid, petroleum ether:diethyl ether:acetic acid (70:30:1) as solvent system whereas chloroform:methanol:ammonia (65:25:5) for phospholipid. The individual lipid moieties were identified based on the *R_f* value of the respective standards. The assay was further validated with NBD-fluorescent fatty acid-labeled lipids substrates, and fluorescent fatty acid release was identified on a TLC by scanning at 488 nm using GE typhoon FLA 9500.

In vivo lipid profiling of yeast cell overexpressing OsPLB

The gene and pYES2/NTC empty vector were transformed in yeast and were grown as mentioned above. After 24 h induction, equal OD (*A*₆₀₀ = 30) of cells were harvested, and total lipids were extracted (Bligh and Dyer, 1959). Subsequently, neutral lipids were separated using the solvent system mentioned above. Phospholipids were separated by two-dimensional TLC using chloroform: methanol: ammonia (65:25:5, v/v) as the first dimension followed by chloroform:

methanol: acetone: acetic acid: water (50:10:20:15:5, v/v) as the second dimension solvent systems. Finally, phospholipids were visualized by MnCl₂ charring of TLC plate at 120 °C. The individual lipid moieties were identified based on the *R_f* value of the respective standards and quantified by densitometry analysis.

Lipidome analysis of yju3Δ cell overexpression OsPLB by HRMS

After 24 h induction, total lipids were extracted from equal OD (*A*₆₀₀ = 30) cells, concentrated using a vacuum concentrator and redissolved in 500 µl of solvent (chloroform: methanol (1:2) containing 7.5 mM ammonium acetate). All samples were analyzed by direct infusion using MS/MS^{ALL} on an ABSciex TripleTOF™ 5600 System. The samples were loaded at a flow rate of 7 µl/min. For identification of molecular species of PC, the positive mode was used. For the MS/MS^{ALL} technique, TOF MS experiment was set to scan *m/z* from 100–1200, which was followed by product ion analyses (mass range 100–1500 in MS/MS experiments). The following parameters were included for the TOF MS experiment. Ion source gas 1 (GS1) at 15 psi, ion source gas 2 (GS2) at 20 psi, curtain gas (CUR) at 25 psi, the temperature of 200 °C, ion spray voltage floating at +5100. A declustering potential (DP) of ± 80 , collision energy (CE) of ± 50 eV and collision energy spread (CES) of 30 were used. A washing step was mandatorily performed between samples to wash away any carryover from the previous sample. For identification of PC molecular species, the precursor ion (*m/z*) of 184 was set in peak view software (AB Sciex). Data were further processed by LipidomeDB Data Calculation Environment (DCE) (Fruehan et al. 2018).

Statistical analysis

Data were expressed as mean $\pm$ standard deviation of three independent experiments. The significance of difference at *p* < 0.05 was determined by one-way ANOVA with Tukey's post hoc test using IBM SPSS Statistics software.

Results and discussion

Identification and functional characterization of lipase, particularly phospholipase genes are essential to get an insight into the physiological significance of lipid mobilization in seed germination. There are several putative lipase genes that have been reported in *O. sativa* (Li et al. 2007; Chepyshko et al. 2012). However, their biochemical characterization is yet to be confirmed.

***OsPLB* gene highly expressed during germination**

In the present study, we retracted the *OsPLB* gene from the rice genome database using in silico analysis. *OsPLB* gene contains 1101 base pair which encodes 367 amino acid protein. The domain and motif analysis reveal that it contains an α/β hydrolase fold with signature lipase motif (Fig. 1a). The classical lipase GX SXG motif is present inside the consensus sequence LIV-KG-LIVFY-LIVMST-G-HYWV-S-YAG-G-GSTAC, and it shows *OsPLB* could be a lipase. The lipase motif is spanned between 189 and 198 amino acids with active site serine at 195. Secondary structure analysis reveals that *OsPLB* encodes a protein that is predicted to have 36% alpha-helix, 16% beta-strand, 16% disordered, and 4% TM helix. Amino acid residues 24–326 of *OsPLB* aligned with 82% sequence coverage to *Rhizomucor miehei* lipase with 32% identity (Fig. 1b). The best fit 3D model of the protein was achieved with 92% residues of the sequence at > 90% confidence (Fig. 1c). To understand the expression of *OsPLB* during germination, the mRNA expression levels were analyzed at different time point 0, 48, 96, and 192 h, using quantitative real-time PCR. Actin was used as an internal reference gene to normalize gene expression (Tsai and Chung 2014). The amplified product (147 bp) was verified by agarose gel electrophoresis. Real-time qPCR analysis reveals a time-dependent increase in expression of *OsPLB*, and it was significantly higher in 192 h. It was 1.6-fold and 2.5-fold higher than 48 and 96 h, respectively (Fig. 1d). There was no further increase after the two-leaf stage. A similar result was observed during seedling development of sunflower cotyledons (Gupta and Bhatla 2007) and cucumber (May et al. 1998). It indicates that the seeds entered into photosynthesis by this period to generate energy for further growth and development. Our results suggest that the *OsPLB* gene expressed during seed germination may have a significant physiological role in storage lipid mobilization.

Heterologous overexpression of *OsPLB* in *E. coli*

For the functional characterization, total RNA was isolated from *O. sativa* seeds and then converted into cDNA followed by cloning in pRSET-A vector. A positive *OsPLB* construct and empty vector were transformed into various bacterial expression strains. Protein expression was induced by different concentrations (up to 0.6 mM) of IPTG. However, we were able to observe the recombinant protein expression only with Rosetta (DE3) pLys strains at 0.4 and 0.6 mM IPTG (Supplementary Figure 1). This strain compensates for several rare codons in a prokaryotic host and is suitable for heterologous protein expression in *E. coli* system. The expressed recombinant protein was separated on 12% SDS-PAGE (Fig. 2a) followed by immunoblot confirmation using anti-His⁶ antibody (Fig. 2b). The protein expression was

observed in the membrane fraction. Similarly, the recombinant seed coat lipases activity was reported in the insoluble fractions (Kim 2004). The expressed protein was solubilized with 8 M urea and subsequently purified by Ni-NTA affinity chromatography. Protein purity was monitored by SDS-PAGE analysis, which showed a single homogeneous band around ~43 kDa (Fig. 2c). The polypeptide mass of the expressed protein is closer to the predicted molecular mass of 41 kDa (40,791.1 Da).

Confirmation of *OsPLB* by in-gel trypsin digestion and MS/MS analysis

To confirm the identity of the expressed *OsPLB* protein, the purified protein was further validated by in-gel trypsin digestion followed by mass spectrometry analysis. Unequivocally, protein identification relies on the analysis of peptides generated by proteolysis digestion. Mascot search showed five unique peptides, and they were exactly matched to *Oryza sativa* (XP_015615080.1) with sequence coverage of 18% (Fig. 2d) and annotated as a lipase. This accession number is synonymous with the unique OS ID of Os11g0655800. It showed that the expressed protein was in a proper frame with full-length protein. Further to study the lipase activity, we cloned the *OsPLB* gene into a yeast expression vector, pYES2/NTC. Positive clone and its empty vector were transformed into yeast lipase mutant strain, yju3 Δ to rule out the background activity. YJU3 encodes one of the major lipases, and it may contribute to lipase activity (Heier et al. 2010). The SDS-PAGE, followed by immunoblot with anti-His₆ tag monoclonal antibody, confirmed the expression of *OsPLB* (Fig. 3a).

Overexpression of *OsPLB* reduces the phosphatidylcholine content

Germination is accompanied by changes in structural and storage lipids, reflecting modifications in the fatty acid profile. Plant membranes composed of glycerolipids which include PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylglycerol (PG) and galactolipids (Denev et al. 2009). The impact of *OsPLB* overexpression in yeast on neutral and phospholipids was monitored. Interestingly, the FFA content was increased significantly as compared with vector control (Fig. 3b). There were no significant changes observed with other neutral lipids such as TAG, diacylglycerol (DAG), and MAG (Supplementary Figure 2). It shows that the observed increase in FFA content could be due to the hydrolysis of phospholipids. Further to confirm the source of released FFA, the phospholipids were separated by two-dimensional TLC. A significant (~20%) reduction of PC was observed in yeast expressing *OsPLB* as

Fig. 1 The domain and structural analysis of rice OsPLB. **a** Schematic representation of domains retrieved from the conserved domain database at the NCBI. **b** Secondary structure analysis of OsPLB. The red color box indicates the lipase motif. **c** Predicted 3D model

of the OsPLB protein. **d** Relative mRNA expression of *OsPLB* during germination. Value are mean $\pm$ SD of three biological replicates (* $p < 0.05$)

Fig. 2 Heterologous overexpression and confirmation of *OsPLB* in *E. coli*. **a** SDS-PAGE analysis of *OsPLB* overexpression. **b** The recombinant protein expression and the purification was confirmed by immunoblot analysis. **c** SDS-PAGE profile of enriched and the puri-

fied protein. **d** Confirmation of the expressed protein by in-gel trypsin digestion. MS/MS fragmentation pattern of the unique peptides are highlighted

compared to vector (Fig. 3c). However, there was not much increase in lysophosphatidylcholine (LPC), and it could be due to subsequent hydrolysis of LPC. It was evidenced by the increased (~37%) FFA content (Fig. 3b), which might be from hydrolysis of both PC and LPC. Besides, there were marked increases in other phospholipids, mainly PE and PA. Collectively, the overexpression of *OsPLB* significantly increases the FFA content by hydrolysis of PC. In cereals like wheat, germination proceeds with an overall reduction of membrane phospholipids except for PA, which

shows the opposite trend during germination (Minasbekyan et al. 2004). Among the phospholipids, PC, PI, and PE are predominant in rice (Glushenkova et al. 1998), and *OsPLB* may have an affinity towards endogenous PC.

OsPLB encodes PC specific phospholipases activity

Further, to confirm the phospholipase activity, we performed in vitro lipases assay using recombinant protein as an enzyme source, and its vector control was used for

Fig. 3 The rice *OsPLB* encodes phospholipase. *OsPLB* and its corresponding vector were transformed into yeast *yju3Δ*. **a** Overexpression of *OsPLB* was confirmed by western-blot analysis using anti-His monoclonal antibody. The corresponding SDS-PAGE profile is shown in the lower panel. **b** The level of FFA in yeast cells overexpressing *OsPLB*. The lower panel represents the neutral lipids profile. **c** Sepa-

ration, and quantification of phospholipids by two-dimensional TLC. 0, Origin; 1, LPC; 2, PI; 3, PS; 5, PC; 6, PE; 7, PA. **d** Phospholipase activity. The assay was performed using fluorescent PC, and the product formation was quantified. Insert represents the reduction of PC substrate. Values are mean $\pm$ SD of three independent experiments (* $p < 0.05$)

background activity. Phospholipases activity was measured using individual substrates such as PC, PE, PS, PI, PA, and CL in the presence of 10 mM CaCl_2 . Interestingly, prominent lipase activity was observed with PC, and the substrate reduction was correlated with the increased FFA and LPC formation (Fig. 3d). The FFA formation was found to be higher than the LPC, which could be due to the phospholipase B activity at both sn-1 and sn-2 position. Further, no measurable lipase activity was observed with other phospholipid substrates (Supplementary Figure 3). Results clearly show that *OsPLB* is expressed during germination and exhibit the phospholipase B activity towards PC. Our finding is consistent with reported phospholipase activity during seed germination (May et al. 1998; Graham 2008; Lin et al. 2019). A transient, time-dependent, and oelosome-specific expression of phospholipase (PC-specific) have been reported during seed germination in cucumber (May

et al. 1998) and sunflower seed (Gupta and Bhatla, 2007). Bhardwaj et al. (2001) reported a PLA_2 activity in rice bran with an affinity towards PC. Overexpression of phospholipase A1 enhances seed germination in *Arabidopsis thaliana* (Seo et al. 2011). In addition, PC-specific PLA_2 activity was involved in the mobilization of plant lipids in *Arachis hypogea* (Parthibane et al. 2011). Further, no measurable lipase activity was observed with TAG, DAG, and MAG in our experimental conditions (Supplementary Figure 3).

High resolution mass spectrometric analysis of PC molecular species

To understand the substrate preference of the recombinant enzyme, we analyzed the level of total PC and its molecular species by HRMS. Welti and Wang (2004) demonstrated the identification of the lipid species for the

substrate and product profiling of specific enzymes using the MS-based method. PC molecular species are described as total carbon number with unsaturation in their two acyl chains. The observed signals were normalized, and the level of PC and its molecular species were calculated. The reduction of PC was $\sim 22\%$ as compared to vector control (Fig. 4b), and the result was consistent with the lipid profiling data in two dimensional TLC. Fauconnier et al. (2003) reported the HRMS based identification of compositional changes in lipid classes that are associated with plant development. Result reveals that the overexpression of *OsPLB* significantly reduces PC32:2, PC32:1, and C28:1 as compared to vector control (Fig. 4c). However, there was a marked difference with PC34:2 and PC34:1, and no changes were observed with other species. These results suggest that *OsPLB* appears to have substrate preference of PCs with C28 and C32 containing unsaturated

fatty acids. In addition, analysis of PCs fatty acyl chain revealed a significant decrease associated with palmitoleic and oleic acid-containing PCs (Fig. 4c). May et al. (1998) demonstrated that palmitoyl-PC and linoleoyl-PC are the preferred substrate for phospholipase during germination. Further, there are no significant changes observed in the distribution of PC acyl chain length (Fig. 4d). Oleic, linoleic, and palmitic acids are the predominant fatty acids present in rice lipids (Denev et al. 2009). PC specific phospholipase activity with the preference to oleic acid, linoleic acid, and palmitic acid was reported in *Arabidopsis* and castor-seed (Rietz et al. 2010; Bayon et al. 2015). However, shorter chain fatty acids are reported to be preferred and released faster than long-chain fatty acids by rice lipases (Aizono et al. 1973; Kim 2004). Collectively, our results showed *OsPLB* gene expressed during germination encodes phospholipase B with the preference of PC.

Fig. 4 Overexpression of *OsPLB* reduces the molecular species of PC. **a** Mass spectra of PC scanned over an m/z range of 100–1500 to detect PC. The presence of fragment ion 184.0 which corresponds to the phosphocholine head group. C28, C30, C32, and C36 refer to the number of carbons in the *sn*-1 and *sn*-2 fatty acyl chains of PCs.

b Amount of total PC in yeast cells overexpressing *OsPLB* or empty vector. **c** Relative amount of each PC molecular species. **d** Relative quantification of each PC fatty acyl chain length from the total fatty acyl chains (%). Data are mean $\pm$ SD of three biological replicates (* $p < 0.05$)

Conclusion

In the present study, we functionally characterized the rice *OsPLB* gene, which encodes a phospholipase expressed during germination. It may be involved in the initial seed germination process by hydrolyzing phospholipid monolayer and accelerate the lipid mobilization process. Further study is required to understand the physiological significance of the *OsPLB* gene in energy mobilization during seed germination.

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Author contributions PV conceived the original research plans and supervised the experiments. AKD and ML carried out all the experiments. PV and AKD analyzed and discussed the data. PV and AKD wrote the manuscript.

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest to declare.

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