International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Int. J. of Life Sciences, 2023; 11 (2): 159-164
ISSN:2320-7817(p) | 2320-964X(o)

Research Article Open Access

Cloning and Expression of a Gene Encoding Phosphomevalonate Kinase from an Endophytic *Talaromyces* species

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Manuscript details:

Received: 30.05.2023
Accepted: 28.06.2023
Published: 15.07.2023

Cite this article as:
Shenoy Priyanka N, Sneha Bhaskar, Shailasree Sekhar and Kini K Ramachandra (2023) Cloning and Expression of a Gene Encoding Phosphomevalonate Kinase from an Endophytic *Talaromyces* species, *Int. J. of Life Sciences*, 11 (2): 159-164.

Available online on http://www.ijlsci.in
ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

ABSTRACT

Terpinoids, a diverse class of important bioactive compounds with therapeutic value, are produced through Mevalonate pathway (MVA) in fungi, plants, animals and some bacteria. Phosphomevalonate kinase (PMK) is a key enzyme and catalyses one the rate limiting steps of this pathway. This enzyme is generally expressed in very low levels and strategies for metabolic engineering of this enzyme may pave way for increased production of this important pathway metabolites. In an earlier study, an endophytic *Talaromyces* sp. strain YS-1 isolated from a medicinal plant *Syzygium samarangense* was subjected to whole genome sequencing. Genome mining and bioinformatics analysis with AntiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) annotation identified 9 terpenoid gene clusters including the PMK gene which may be involved in biosynthesis of important bioactive terpenoids. In the present study this PMK gene of 1455 bp length ORF encoding 484 amino acids was cloned into pRSF expression vector and expressed in *Escherichia coli* as a His-tag fusion protein. The recombinant PMK was purified to near homogeneity using Nickel-CL agarose column. Its molecular weight was found to by 50 kDa when separated on SDS-PAGE. The expressed protein can be further characterized for its biological activities and can be metabolically engineered for production of value-added products.

Keywords: Endophytic *Talaromyces* sp., MVA pathway, Phosphomevalonate kinase, Cloning, Terpenoids, Gene annotation.

INTRODUCTION

Plants and their endophytes have extensive secondary metabolism which includebiosynthetic pathways for production of several important metabolites. One among them is Mevalonate pathway (MVA) also known as isoprenoid pathway, and responsible for production of several polyisoprenoid metabolites, carotenoids, steroids, meavalonic acid etc. This pathway which is ubiquitous in fungi, plants, animals and

some bacteria plays a critical role in multiple cellular processes as well. Some of the key enzymes involved in the pathway are HMG Co A synthase, HMG Co A reductase, Mevalonate Kinase (MK) and Phospho-mevalonate kinase (PMK). Acetyl CoA is the first precursor and DMAPP (dimethylallyl diphosphate) and IPP (Isopentenyl diphosphate isomerase) are the end products produced (Miziorko, 2011).

Terpenoids which are produced through MVA pathway are one of the diversified group of compounds derived from C5 isoprene units having important ecological functions, as defense and signaling molecules, insect attractors, antagonistic chemicals and plant growth promotors. They have high pharmacological value in therapeutic drugs like paclitaxel, camptothecin, Vinca alkaloids, and fusidic acid (Galindo-sol & Fernandez, 2022). Endophytic fungi have been reported as one of the promising sources of terpenoids (Chen *et al.*, 2021). The endophytic fungi *Aspergillus fumigatus*, isolated from *Ligusticum wallichii*, produced two new sesquiterpenes, fumagillene A and fumagillene B having anticancer activities (Li *et al.*, 2020). Another endophytic isolate of *Preussiaisomera* XL-1326 produced a pair of norsesquiterpenoidal enantiomers, preuisolactone A having moderate antibacterial activities (Xu *et al.*, 2019). Endophytic *Cerrena* sp. A593 was reported to produce two new triquinane-type sesquiterpenoids, named cerrenins D and E demonstrating moderate anticancer activities (Liu *et al.*, 2020).

Although several sources are present for the production of these terpenoid metabolites, the amount of these compounds generated under laboratory conditions are very minimal. Hence metabolic engineering approaches have to be adopted for optimizing the production of these commercially important metabolites. This requires the knowledge and ability to manipulate the key enzymes of the biosynthetic pathways and the genes encoding them. In the MVA pathway two key enzymes, viz., Mevalonate Kinase (MK) and Phosphomevalonate kinase (PMK) have been identified as major bottlenecks for optimum productions of the pathway metabolites (Garcia & Keasling, 2014). Cloning and characterization of the genes encoding these key enzymes may pave way for devising biotechnological strategies for enhanced activities of these enzymes and resultant increase in the levels of pathway products.

Phosphomevalonate kinase (PMK) is the key enzyme catalyzing the ATP-dependent phosphorylation of mevalonate-5-phosphate to form mevalonate-5-diphosphate in the MVA pathway (Pilloff *et al.*, 2003). The enzyme, which belongs to GHMP kinase familyis aregulatory enzyme of the pathway and is generally expressed at relatively lower levels (Garcia & Keasling, 2014).

Fungus *Talaromyces* sp. belonging to Trichocomaceae family have been known to produce many terpenoid bioactives. In an earlier study from our laboratory an endophytic *Talaromyces* sp. Strain YS-1 isolated from a medicinal plant *Syzygium samarangense* was subjected to whole genome sequencing. Genome mining and bioinformatics analysis with AntiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) annotation identified 76 biosynthetic gene clusters (BGCs) of secondary metabolites and their biosynthetic pathways. Nine terpenoid gene clusters were detected which may be involved in biosynthesis of important bioactive terpenoids. Funannotate analysis revealed the presence of the genes encoding mevalonate kinase and Phosphomevalonate kinase of the MVA pathway. With a long-term objective of metabolic engineering of the MVA pathway, in the present study the gene encoding Phosphomevalonate kinase was isolated from *Talaromyces* strain YS-1, cloned and expressed in *E. coli* expression system.

MATERIAL AND METHODS

Growth of *Talaromyces* YS-1 strain

Talaromyces strain YS-1 isolated from *Syzygium samarangense* host medicinal plant in an earlier study was used. Pure culture of the strain was maintained by sub-culturing on potato dextrose agar medium supplemented with ampicillin (100mg/ml) and was incubated at 25 ± 2℃ with alternative dark and light cycles. The fungal mycelium grown on potato dextrose broth was subjected for RNA isolation.

Isolation ofRNA and preparation of cDNA transcripts

RNA was isolated from an endophyte *Talaromyces* YS-1 strain by RNeasy Plant Mini Kit (QIAGEN) and the isolated RNA was quantified by Nano drop 2000C spectrophotometer (Thermo fisher scientific). The obtained RNA was subjected for cDNA synthesis by High-capacity cDNA reverse transcription kit (Applied Biosystems).

Designing of primers and PCR amplification

Specific primers for PMK gene PMK F1 forward (5'-**GGATCC**ATGCCCGTCCATCCCAAC-3') and PMK R1 reverse (5'-**AAGCTT**TTATAGCCATGCTAGGTAATTGCC-3') having restriction sites BamHI and HindIII (underlined) respectively were designed for the full length PMK gene sequence obtained from the whole genome sequence of *Talaromyces* YS-1 strain deposited in the GenBank (temporary SRA submission ID-SUB13053267). The synthesized cDNA was used as a template for PCR amplification with the programing as follows: the initial denaturation at 95ºC for 3 min followed by 35 cycles of denaturation at 92 ºC for 1 min, primer annealing at 55 ºC for 1 min and extension at 72 ºC for 2 min with the final extension at 72ºC for 10min.

Cloning of PMK secondary metabolite genes and Colony PCR

Following amplification, the purified PCR product was digested with BamHI and HindIIIand cloned into pRSF vector to generate an expression construct. The recombinant vector was transformed into expression host *E.coli* BL21(DE3) strain. Then transformed cells were subjected to colony PCR to check for the presence of the inserted gene and the reading frame was confirmed by DNA sequencing.

Overexpression of recombinant PMK gene

The *E.coli* BL21 (DE3) strain containing the recombinant plasmid selected from colony PCR was further subjected toprotein expression. Luria Bertani (LB) broth medium supplemented with antibiotic Kanamycin (50µg/ml) containing recombinant cells were grown overnight at 37ºC with proper shaking. The overnight culture was transferred to fresh LB broth and incubated for 3 to 4 hours till the O.D at A_{600} reaches 0.7-1.0. The recombinant protein was induced with 1mM Isopropyl β- d-1-thiogalactopyranoside (IPTG) and further incubated for 4 hours at 37ºC. The cells were harvested by centrifugation at 5000rpm for 15 min at 4ºC and lysed in 20mM phosphate buffer (pH 7.4) by sonication. The cell lysate was centrifuged at 15,000 g for 30 min at 4ºC and the pellet and supernatant obtained were evaluated for the presence of recombinant protein.

Affinity purification

The His tagged recombinant PMK protein was purified using His-Tag Fusion protein purification Kit (Genei, Bangalore) according to manufacturer's instructions. The Nickel-CL agarose column was equilibrated with the equilibration buffer and the cell lysate loaded onto the column. The bound His-tagged fusion PMK protein was eluted with elution buffer and the purified fraction was analysed by SDS-PAGE.

Protein analysis

Proteins extracted inpellet and supernatant of cell lysate were subjected to SDS-PAGE on 10% acrylamide gel. Staining was carried with coomassie brilliant blue and protein expression was observed.

RESULTS AND DISSCUSSION

cDNA amplification, Cloning of PMK secondary metabolite gene and Colony PCR

Whole genome sequencing of *Talaromyces* YS-1 strain and annotation using Funannonate indicated that the PMK gene of this fungal endophyte is 1455bp in length. PCR amplification of this gene from cDNA using the designed specific primers PMK-F1 and PMK-R1 followed by agarose gel electrophoresis resulted in a band of ~1450bp **(Fig 1)**. The amplicon and the expression vector pRSF were digested with restriction enzymes with BamHI and HindIII and ligated to generate a recombinant vector construct. This was transformed into *E.coli* BL-21(DE3) expression host cells. The recombinant cells were identified by colony PCR **(Fig 2)**.

Heterologous Expression of recombinant proteins

The expression of recombinant pRSF containing PMK cDNA insert was induced by 1mM IPTG in *E.coli* BL-21 for 4. The recombinant protein will be expressed as a fusion protein with an N-terminal 6-His tag derived from the pRSF expression vector. The control sample was also maintained simultaneously without any induction. The obtained protein samples from pellet and supernatant of induced and uninduced samples were analysed through SDS-PAGE. The recombinant protein was observed as an overexpressed band of ~50 kDa in the induced pellet and supernatant fractions of the cell lysate **(Fig.3)**.

Purification of the recombinant protein

The recombinant PMK with His tag was purified using Nickel-CL agarose column affinity chromatography. The SDS-PAGE of the purified fraction revealed a ~50kDa protein with near homogeneity (**Fig. 4).** The molecular mass of the protein corresponded with the theoretically predicted molecular mass.

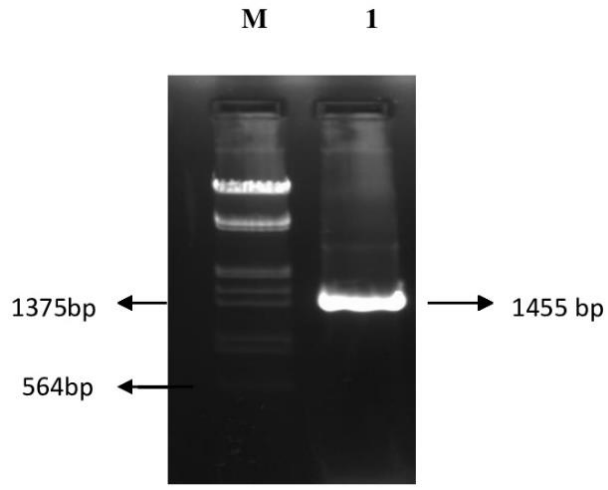

Fig. 1. Agarose gel electrophoresis of PMK gene from *Talaromyces* sp strain YS-1. Lane M-Lambda DNA/EcoRI+HindIII Marker, 1- Amplified PMK cDNA transcript

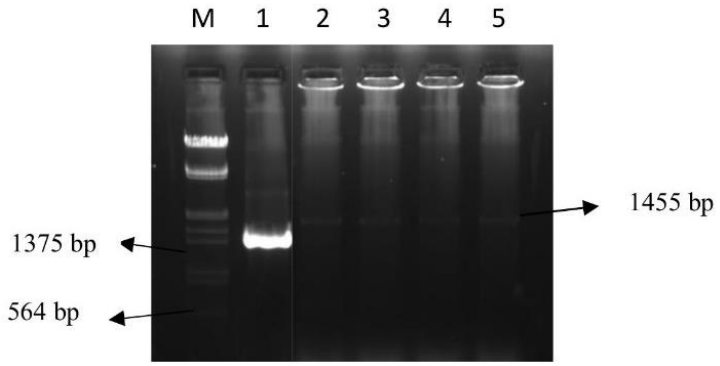

Fig. 2 Agarose gel electrophoresis of colony PCR products of cloned PMK gene.
M- Lambda DNA/EcoRI + HindIII Marker, 1- positive control PMK cDNA amplified product and 2 to 5- positive colonies containing the cloned PMK gene

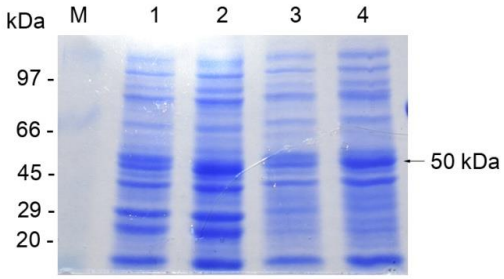

Fig 3. SDS –PAGE analysis of recombinant protein. Lane M- Protein Molecular marker, 1- Uninduced pellet, 2-Induced pellet, 3-Uninduced supernatant and 4-Induced supernatant.

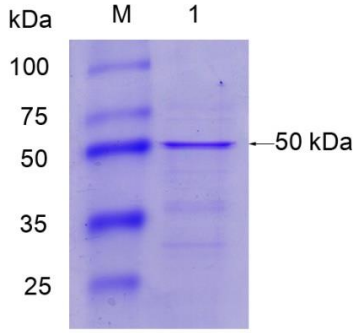

Fig. 4. SDS-PAGE profile of the recombinant PMK protein fraction purified by Nickel-CL agarose column.

Cloning and expression of genes encoding key enzymes of important secondary metabolite pathways is an important step towards developing metabolic engineering strategies for enhanced production of commercially important bioactive compounds from these pathways. Several studies have targeted endophytes for the production of terpenoid metabolites and the genes involved in the pathway(Chen et al., 2021). High value terpenoids such as vincristine and vinblastine have been produced from endophytic *Talaromyces radicus* isolated from *Catharanthus roseus* (Palem et al., 2015). Since MVA pathway is an important for the production of several terpinoid bioactive metabolites, the present study was undertaken to isolate and clone the PMK gene which encoded a key regulatory enzyme of this pathway from the endophytic fungus *Talaromyces* YS-1 strain. The gene sequence isolated in the present study had high homology with the sequence of PMK genes from other fungal species such as *Talaromyces marenffei*, *T. amestolkiae*, *Aspergillus* sp etc. There are few earlier reports of cloning and expression of this gene from different organisms. The gene SaPMK from *Santalum album* which was reported to have an ORF of 1527 bp was cloned and characterized. It was expressed at higher levels in roots and leaves and involved in the biosynthesis of santalol, a major constituent of sandalwood oil (Niu et al., 2021). The gene (SbPMK) was also cloned and characterized from a macrofungus *Sanghuangporus baumii*. This gene had a ORF of 1488 bp with 489 amino acids and predicted molecular weight of 52.47 kDa which is very similar to the PMK gene cloned in the present study (Wang et al., 2021).

Although several fungal isolates are shown to produce commercially important terpenoids the yield is very low due to several factors including weakened biosynthetic pathway due to repeated subcultivation (Chen et al., 2021). Cloning and expression of key genes of the pathway such as PMK can lead to multiple fold increase of terpenoid synthesis. The cloned PMK gene can be further taken for metabolic and protein engineering strategies like codon optimization, rational designing etc. for the overexpression of commercially important compounds.

CONCLUSION

The gene encoding PMK a key enzyme from terpenoid secondary metabolite pathway was identified through genome mining and sequence analysis of *Talaromyces* YS-1 strain isolated from *Syzygium samarangense*. Specific primers were designed for PCR amplification of this gene and it was cloned into a *E coli* expression vector pRSF. The overexpression of the recombinant protein using IPTG induction revealed the expected ~50kDa protein in the cell lysate. The protein was purified by Nickle-CL agarose column. The expressed protein can be further characterized for its biological activities and can be metabolically engineered for production of value-added products.

Acknowledgment

The authors are grateful to the Institution of Excellence (IOE), University of Mysore, for infrastructural facilities and financial assistance to PSN.

Author Contributions

KRK and SS conceptualized and designed the study. PSN and SB carried out the experiments and collected the data. PSN, SB, SS and KRK analysed the data and finalized the manuscript. All authors have read and approved the final manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical statement: This article does not contain any studies with human participants or animals performed by any of the authors.

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