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ORIGINAL ARTICLE

A Novel Zn Transporter Gene (*ClZIP1*) from Turmeric (*Curcuma longa* L.) and Expression Analysis in Presence of a Zn-Solubilizing Bacteria

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Abstract

Zinc (Zn) deficiency is widespread in plants and molecular mechanism of uptake and transport within organelles is unclear. A novel Zn transporter gene was identified and characterized from turmeric (*Curcuma longa* L.) and expression analyzed in presence of zinc oxide (ZnO) and *Bacillus safensis*, a Zn-solubilizing bacteria (ZSB). Initially, eleven Zn transporters were mined from rhizome-specific transcriptome of turmeric and the one with highest transcript abundance designated as *ClZIP1* was cloned and sequenced to give 1268-bp gene encoding 366 amino acids (ORF-1101 bp). In silico analysis and deduced protein designated ClZIP1 to Zn/Fe-regulated transporter (ZRT/IRT)-related protein (ZIP) family with 70.0% identity to Zn transporters from *Musa acuminata*. ClZIP1 possessed eight transmembrane (TM) domains with a variable region between TM-3 and TM-4 and conserved histidine-rich ZIP signature motif. A single 10-bp Zn deficiency-responsive element (ZDRE) was present in the promoter. *ClZIP1* expression evaluated in in vitro plantlets in presence of Zn applied as ZnO (10–100 ppm), with/without ZSB indicated that it was higher in basal portion than leaf. This is the first report on ZIP gene from turmeric, which showed higher expression in the absence of ZSB and vice versa with maximum downregulation at 100 ppm Zn (88%).

Keywords *Bacillus safensis* · *ClZIP1* · Soil Zn · ZIP gene · Zn-solubilizing bacteria · Zn fortification

Introduction

Zinc (Zn) plays a critical and essential role in key catalytic and structural processes associated with growth, development, and reproduction in crops. It is a key element in an array of enzymes and in most instances interacts with amino acids like histidine and cysteine and forms Zn fingers, whose binding is imperative for the activation of transcription factors (Lira-Morales et al. 2019). Since both quantitative and qualitative yields are strongly dependent on this micronutrient, maintaining sufficient levels of available Zn in soil, regulating Zn uptake from this labile pool, and maintaining sufficient intracellular Zn level in the plant are imperative. The mechanisms underlying Zn transport encompass uptake by root from the soil labile pool, root-to-shoot translocation, and remobilization of Zn within the plant organs (Liu et al. 2019).

While Zn-solubilizing bacteria (ZSB) can enhance the labile Zn pools in the soil by solubilizing recalcitrant Zn compounds like ZnO, $Zn_3(PO_4)_2$, and $ZnCO_3$ (Dinesh et al. 2018; Saravanan et al. 2011), the Zn ions from this available pool need to be transported into the root system and subsequently distributed all across the plant system, traversing the cellular and organelle membranes (Guerinot 2000). Since the labile Zn in soil is always very low (< 2.0 µg g^{-1}), the plants employ robust high-affinity root membrane transport mechanism regulated by the Zn transporter genes to ferry Zn between cells and take them deep into the sub-cellular niches (Bashir et al. 2012; Ishimaru et al. 2011; Kambe et al. 2006; Milner et al. 2013).

Key Messages
- A novel Zn transporter gene (*ClZIP1*) is reported for the first time in turmeric
- Zn-solubilizing bacteria (ZSB) downregulated the expression of *ClZIP1*
- Absence of ZSB led to Zn stress and upregulation of *ClZIP1*
- *ClZIP1* can be used as a biomarker for Zn availability

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During low Zn availability, plants adapt by modulating their capability for Zn uptake and thereby maintain Zn homeostasis involving a tightly regulated network of Zn status sensors and signaling, which in turn control the coordinated expression of transporters involved in Zn uptake from the soil, mobilization to plant cells, and accumulating it in cellular components (Assunção et al. 2010b). Understanding Zn homeostasis and the genes involved is essential to increase Zn availability in plants. The Zn transporter genes are housekeeping genes belonging to the ZIP (ZRT, IRT-like protein) family of metal transporters and play a significant role in the uptake, transport, and redistribution of Zn among the plant organs and upkeep of cellular Zn homeostasis. They operate at all growth stages (Gainza-Cortes et al. 2012) and function in all plant organs (Li et al. 2013). Therefore, the ZIP family is considered to be a key player in the transport of Zn from soil to plant (Olsen and Palmgren 2014) and play a vital role in ensuring synchrony among various assimilatory pathways and the physiological demands and show specific expression patterns at different Zn concentrations (Lira-Morales et al. 2019). The *cis*-element ZDRE, present in the promoter region of Zn transporter genes, are crucial molecular switches involved in transcriptional regulation of the genes. These motifs respond to Zn deficiency stress and are known to exist in ZIPs that are Zn deficiency induced (Assunção et al. 2010a). Besides, the expression of ZIP family genes is under the influence of the cations like Zn^{2+}, Fe^{2+}, Cu^{2+}, and Mn^{2+} in rice (Lee et al. 2010) and the gene expression is reported to vary between plant tissues and developmental stages (Ishimaru et al. 2011) and is also dependent on Zn transportation efficiency (Chen et al. 2008).

The ZIP family and their expression have been reported in several plants, viz., *Oryza sativa* (Ramesh et al. 2003; Ishimaru et al. 2005; Lan et al. 2013; Lee et al. 2010), *Medicago sativa* (Burleigh et al. 2003), *Arabidopsis thaliana* (Nishida et al. 2011; Jain et al. 2013; Milner et al. 2013), *A. hallieri* (Lin et al. 2009; Shanmugam et al. 2013), *Phaseolus vulgaris* (Astudillo et al. 2013), *Triticum turgidum* (Durmaz et al. 2011), *Vitis vinifera* (Gainza-Cortes et al. 2012), *T. aestivum* (Evens et al. 2017), *Zea mays* (Li et al. 2013; Mondal et al. 2014; Xu et al. 2010), *Solanum lycopersicum* (Eckhardt et al. 2001; Pavithra et al. 2016), *Thlaspi japonicum* (Wu et al. 2009), *Poncirus trifoliata* (Fu et al. 2017), and *Nicotiana tabacum* (Barabasz et al. 2019). However, only very few of these ZIP genes have been characterized functionally and structurally (Ajeesh Krishna et al. 2020), though the crystal structure of ZIP protein and metal transport mechanism have been predicted (Zhang et al. 2017).

Also, studies on soil bacteria-induced expression pattern of ZIP genes in plants are scarce. Such studies will serve as a good measure of the size of the soil labile Zn pool, Zn assimilation from this pool by the roots, and remobilization to various plant organs. Ergo, unraveling the molecular basis of microbe-mediated ZIP gene expression will help in improving Zn accumulation by plants even under Zn limiting conditions. What's more, the expression pattern will also help in indirectly validating the solubilization efficiency of the promising ZSB because in most cases, both over- and underexpression of the ZIP genes are induced by the extent of Zn availability to the plant (Vert et al. 2001). However, very few attempts were made to elucidate the role of nutrient-solubilizing bacterial strains on metal transporter gene expression in crops.

The objectives of the study were to identify and characterize a novel Zn transporter gene from turmeric and analyze the gene expression during solubilization of recalcitrant ZnO by a promising ZSB isolated from the turmeric rhizosphere. We hypothesized that the expression pattern of Zn transporter genes in turmeric tissues will vary markedly with increased solubilization of Zn by the ZSB. A similar study on ZIP genes of rice is based on the transcriptome information available in the public domain (Krithika and Balachandar 2016). However, in the present study, we have utilized a novel Zn transporter gene identified from turmeric rhizomes by RNA seq approach and then analyzed its expression at varying levels of added Zn as ZnO in the presence of a promising ZSB (*B. safensis*) and characterized the gene by in silico methods.

Materials and Methods

Soil Sampling and Isolation of Bacteria

Rhizosphere soils collected from major ginger and turmeric producing states of India (Kozhikode and Wayanad districts of Kerala; Mysore and Chamarajanagar of Karnataka; Coimbatore and Erode of Tamil Nadu, Guntur and Krishna of Andhra Pradesh, and Nizamabad and Jagtial of Telangana) were used for isolation of ZSB. Isolation was done using the serial dilution technique (up to 10^{-6}). At this dilution, the suspension was pour-plated onto different media, viz., nutrient agar (NA), Jensen's, Burk's, and Pikovskaya's, and incubated at 28 ± 2.0 °C for 2–4 days. Sub-culturing of individual colonies was done in NA medium.

Identification of Promising ZSB

All the bacterial isolates were tested for Zn solubilization by using basal medium (glucose—10.0 g, $(NH_4)_2SO_4$—1.0 g, KCl—0.2 g, K_2HPO_4—0.1 g, $MgSO_4$—0.2 g, double-distilled water—1000 ml, buffered to pH 7.0; Saravanan et al. 2004) augmented with 0.5% Zn as ZnO. After autoclaving at 121 °C for 15–20 min, it was transferred to

sterilized Petri plates. One loop full (10 µL) of overnight matured cultures of bacterial isolates was inoculated onto the Petri plates and incubated at 26 ± 2.0 °C for 48 h. The zone of solubilization was determined after 7–10 days and expressed in millimeter. All the assays were done in triplicate. The isolates showing maximum zone of clearance were identified.

Identification of Shortlisted Isolates Using 16S rRNA Gene Sequencing

Briefly, the genomic DNA from the shortlisted bacteria was isolated (Sambrook and David 2000) and the 16S rRNA gene was amplified using the 27F-1492R primer pair, i.e., 27F (*5′*-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (*5′*-GGTTACCTTGTTACGACTT-3′), in 25.0 µL of reaction mixture containing 1 × buffer with $MgCl_2$ (2.5 µL), dNTPs mix (2.0 µL), Taq polymerase (0.3 µL), 0.5 µM of each primer, and template DNA (2.0 µL) and nuclease-free water (17.2 µL). The PCR conditions involved initial denaturation for 2 min at 94 °C followed by 30 cycles of denaturation (1.0 min at 94 °C), annealing (1.0 min at 52 °C), and extension (1.0 min at 72 °C) followed by final extension (10 min at 72 °C). The amplified products were analyzed on agarose gel (1.5%) in presence of ethidium bromide along with a 1.0-kb ladder as size standard. The bands were visualized under UV and photographed using Gene Sys software. The PCR products were sequenced at AgriGenome, Kochi, Kerala, India. Following BLAST, the sequences were compared with registered sequences in the GenBank database using NCBI Blast server (http://www.ncbi.nlm.nih.gov). The analysis indicated 99–100% similarity with 16S rRNA gene sequences of plant growth-promoting rhizobacteria (PGPR) strains already available in NCBI. The 16S rDNA sequence of the PGPR strain was deposited in the GenBank database of NCBI.

Turmeric In Vitro Culture and ZSB Inoculation

Uniform healthy in vitro raised plantlets of a high curcumin turmeric variety (IISR Prathibha) were transferred to Murashige and Skoog (1962) liquid medium (without Zn) supplemented with 0, 10, 25, 50, and 100 ppm ZnO along with a similar set of treatment with ZSB. For this, pure culture of *B. safensis* at a final concentration of 10^{11} colony-forming units (cfu) mL^{-1} was pelletized and centrifuged at 5000 g for 20 min at 5.0 °C and cell pellets were resuspended in 10 mM $MgSO_4$ and centrifuged twice and resuspended in 10 mM $MgSO_4$. The bacterial titer was adjusted to the OD_{600} of 0.05 (10^8 cfu mL^{-1}) and 20 µL of bacterial suspension was added to the media (Krithika and Balachandar 2016). All plantlets were maintained at 25 ± 1 °C under cool white fluorescent light at an intensity of 16-h light (at 11.7 µmol m^{-2} s^{-1})/8-h dark cycles per day for 7 days. These plantlets were used for gene expression analysis and estimation of Zn.

Estimation of Zn

The Zn concentration in the medium was measured using an atomic absorption spectrophotometer (Varian AA 240FS). For estimation of Zn concentration in plant tissue, the basal portion was digested using a tri-acid extract (nitric:sulfuric:perchloric in the ratio of 9:2:1), made up to 50 mL, and Zn content in the extract was determined using AAS.

Transcriptome Analysis

Plant Material

Freshly harvested rhizomes from 4-month-old field-grown plants (11.2994° N, 75.8407° N), of a high curcumin variety, *viz.*, IISR Prathibha, released by Indian Institute of Spices Research (ICAR-IISR, Kozhikode, Kerala) was used for transcriptome sequencing. The plants were grown under normal management conditions following standard package of practices (Srinivasan et al. 2016).

RNA Preparation, cDNA Library Synthesis, and Illumina Sequencing

Total RNA was extracted from rhizomes as per the protocols standardized in our lab (Deepa et al. 2014). Total RNA (1.0 µg) was treated with 1U of RNase-free DNase I (Thermo Scientific) to remove residual genomic DNA. The purity of total RNA was analyzed with an Agilent 2100 Bioanalyzer and the RNA integrity number (RIN) value was found to be 7.8. cDNA was synthesized using a Thermo Scientific Revert Aid First Strand cDNA synthesis kit (#k1622) following the supplier's instruction. The RNA-seq library preparation and sequencing was carried out at AgriGenome Labs, Pvt Ltd., Kochi. Transcriptome library was constructed using an Illumina's TrueSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions and sequenced in Illumina HiSeq 2500 platform by paired-end sequencing.

Transcript Assembly, Annotation, and Retrieval of Zn Transporter Genes

Raw RNA seq data in fastq format was trimmed and low-quality bases were removed. The reads possessing average quality score less than 20 in any of the paired end and those possessing Illumina adapter sequences were filtered out. The trimmed reads were then assembled individually employing SOAPdenovo31mer algorithm opting default parameters. Only those transcripts with length ≥ 150 bp were used for gene expression analysis using Bowtie2 parser program and only transcripts having FPKM (fragments per kilobase

million) ≥ 1 were annotated. The assembled transcripts were annotated using in-house pipeline programs for de novo transcriptome assembly and compared using BLASTX program with NCBI non-redundant protein database. Matches with similarity score ≥ 40% and *e*-value ≤ 10^{-5} were further annotated. As the present study is concerned with Zn transporter genes, we had utilized the transcriptome data to mine only those homologous transcripts of Zn transporter genes within the text of annotated genes, and the sequences were confirmed using BLASTn (https://blast.ncbi.nlm.nih.gov/).

Expression Analysis and Validation of Zn Transporter Gene

The in vitro plantlets were removed from the media and third leaf as well as the basal portion along with attached roots was cut carefully with a sharp scalpel and washed with sterilized water to remove adhering media and dried on tissue paper towels. RNA was prepared and converted to cDNA as per Deepa et al. (2014, 2017). For relative gene expression analysis, quantitative real-time PCR (qRT-PCR) was carried out. The Zn transporter gene-specific primers were designed using Primer Quest tool, an online primer designing software (https://eu.idtdna.com/PrimerQuest), and was synthesized by IDT, USA. The primers c42415_g1_i1 qfp 5′AGGCTTCTT CCTCTCAAGTG3 ′ and c42415_g1_i1 qrp 5′GGTGGA AAGAGAGGGCTAAA3 ′ were used for amplification. The reaction was done in Rotor Gene Q (Qiagen) with a 36-well rotor using QuantiFast SYBR Green PCR kit (Qiagen). The reaction mixture comprised of 10 µL of 2 × SYBR green, 10 pmol each of forward and reverse primers, and 0.5 µL cDNA and made up to a final volume of 20 µL with sterile nuclease-free water. PCR amplification was carried out with initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 10 s, and 60 °C for 30 s. For all real-time RT-PCR analyses, three biological and three technical replicates were used. Elongation factor 1-alpha (*EF1α*) gene was used as a reference gene in order to normalize expression values (Deepa et al. 2017). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The size and intensity of amplified fragments were also confirmed by gel electrophoresis.

Full-Length Gene Cloning and Sequence Analysis

The contig c42415_g1_i1 with highest FPKM and length 1448 bp was selected from the set of Zn transporter contigs (Table 1.) and open reading frame (ORF) was predicted by ORF Finder (Rombel et al. 2002). Primers were designed based on flanking sequences of the ORF using Primer 3 plus tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) for cloning of the full-length gene. A 20-µL PCR was performed using 10 µL EmeraldAmp® GT PCR Master Mix (Takara, http://clontech.com) and 10 pmol each of forward and reverse primers and made up to 20 µL using nuclease-free water. One hundred nanograms of cDNA was added to the mixture and amplified using automated Mastercycler. The PCR program consisted of initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (59 °C for 1 min), and extension (72 °C for 2 min); final extension was done at 72 °C for 10 min. The PCR product was then loaded to the 1% agarose gel with EtBr staining and run at 90 V for 45 min. The PCR product was then transformed into a plasmid vector (Thermo Scientific InsTA cloning kit#K123, #K1214), recombinant clones were analyzed by colony PCR using M13 primers, and the insert carrying plasmid was lyophilized and sent for sequencing at AgriGenome Labs Pvt Ltd., Kochi, Kerala.

Analyses of Zn Transporter Genes/Proteins and Phylogenetic Relationships

NCBI BLAST was used for all sequence similarity searches (Altschul et al. 1990). The query sequence of Zn transporter gene identified from the transcriptome was subjected to BLASTn analysis to find closely related sequences. The nucleotide sequence of isolated Zn transporter from turmeric was also analyzed using BLASTn. All the probable ORFs for the Zn transporter were picked out by ORF finder (Rombel et al. 2002). The deduced amino acid sequence from the longest ORF was used as query for searching homologous sequences using BLASTp program. About 25 significantly similar sequences were used to construct phylogenetic tree by neighbor joining method using MEGA7 software. The bootstrap consensus tree was deduced from 1000 replicates and used to find the evolutionary relationship of the sequences analyzed.

Physicochemical parameters of the deduced protein such as number of amino acids, molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and Grand Average Hydropathy (GRAVY) were predicted using ExPASy's Protparam server (http://web.expasy.org/protparam/). InterProScan was used for functional analysis of the protein and ExPASy's ScanProsite was employed to find signature sequences and possible post translational modifications. The transmembrane proteins and their orientations were predicted using the TMpred program (Hofmann 1993) and the positions of transmembrane domains identified using TOPCONS server (Tsirigos et al. 2015) (http://topcons.net/). Multiple sequence alignment of the protein with the closely related Zn transporters was performed using ClustalW (Thompson et al. 1994) and conserved protein motifs were characterized by Mondal et al. (2014).

Table 1 Zinc transporter unigenes identified from the *Curcuma* transcriptome

No	Contig ID	Sequence length	FPKM	Sequence description	Blast per. identity	Blast top hit description	GO ID and definition	KEGG Orthology and definition
1	c42415_g1_i1	1448	9.83	PREDICTED: zinc transporter 7-like (*Musa acuminata* subsp. *malaccensis*)	77.20%	*Musa acuminata* subsp. *malaccensis* zinc transporter 10 (LOC103976440), transcript variant X2, mRNA (XM_009391637.2)	0,005,385 zinc ion transmembrane transporter activity	K14709 solute carrier family 39 (zinc transporter), member 1/2/3
2	c68557_g1_i1	319	1.17	PREDICTED: probable zinc transporter protein DDB_G0282067 (*Musa acuminata* subsp. *malaccensis*)	83%	PREDICTED: *Glycine soja* probable zinc transporter protein DDB_G0291141 (LOC114421600), mRNA (XM_028387611.1)	-	No entries annotated
3	c32208_g2_i1	427	2.20	PREDICTED: zinc transporter 10-like (*Musa acuminata* subsp. *malaccensis*)	72%	*Musa acuminata* subsp. *malaccensis* zinc transporter 10 (LOC103976440), transcript variant X2, mRNA (XM_009391637.2)	0,005,385 zinc ion transmembrane transporter activity	K14709 solute carrier family 39 (zinc transporter), member 1/2/3
4	c98111_g1_i1	303	1.70	PREDICTED: zinc transporter 7-like (*Musa acuminata* subsp. *malaccensis*)	85.80%	*Musa acuminata* subsp. *malaccensis* zinc transporter 7-like (LOC103986617), mRNA (XM_009404654.2)	-	No entries annotated
5	c32208_g3_i1	420	2.46	putative zinc transporter (*Triticum aestivum*)	80%	*Musa acuminata* subsp. *malaccensis* zinc transporter 10 (LOC103976440), transcript variant X2, mRNA (XM_009391637.2)		No entries annotated
6	c41847_g1_i3	928	2.07	PREDICTED: zinc transporter ZTP29 (*Musa acuminata* subsp. *malaccensis*)	81%	*Musa acuminata* subsp. *malaccensis* zinc transporter ZTP29 (LOC103981337), transcript variant X2, mRNA (XM_009398038.2)	-	K07238 zinc transporter, ZIP family
7	c41847_g1_i1	906	3.68	PREDICTED: zinc transporter ZTP29 (*Musa acuminata* subsp. *malaccensis*)	83%	*Musa acuminata* subsp. *malaccensis* zinc transporter ZTP29 (LOC103981337), transcript variant X2, mRNA (XM_009398038.2)	-	K07238 zinc transporter, ZIP family
8	c28885_g1_i1	493	8.58	PREDICTED: zinc transporter 3-like (*Musa acuminata* subsp. *malaccensis*)	82%	*Musa acuminata* subsp. *malaccensis* zinc transporter 4-like (LOC103993643), mRNA (XM_009413786.2)		No entries annotated
9	c33357_g1_i1	782	7.03	PREDICTED: zinc transporter 4-like (*Musa acuminata* subsp. *malaccensis*)	78%	*Musa acuminata* subsp. *malaccensis* zinc transporter 4-like (LOC103979002), mRNA (XM_009394988.2)	0,005,385 zinc ion transmembrane transporter activity	K14709 solute carrier family 39 (zinc transporter), member 1/2/3

Table 1 (continued)

No	Contig ID	Sequence length	FPKM	Sequence description	Blast per. identity	Blast top hit description	GO ID and definition	KEGG Orthology and definition
10	c33538_g1_i1	1226	6.24	PREDICTED: zinc transporter 2 (*Musa acuminata* subsp. *malaccensis*)	77%	*Musa acuminata* subsp. *malaccensis* zinc transporter 2 (LOC103996197), mRNA (XM_009417057.2)	0,005,385 zinc ion transmembrane transporter activity	K14709 solute carrier family 39 (zinc transporter), member 1/2/3
11	c44858_g1_i2	1861	8.28	PREDICTED: putative zinc transporter At3g08650 (*Musa acuminata* subsp. *malaccensis*)	85%	*Musa acuminata* subsp. *malaccensis* putative zinc transporter At3g08650 (LOC104000020), transcript variant X3, mRNA (XM_018819839.1)	-	No entries annotated

Structural Analysis of ZIP Protein Homologs

The secondary structure of ZIP proteins was predicted using self-optimized prediction method from alignment (NPS@:SOPMA secondary structure prediction (ibcp.fr)) and PSI-blast based secondary structure PREDiction (PSIPRED) (PSIPRED Workbench (ucl.ac.uk)). Three-dimensional structure predictions were done using SWISS-MODEL software (http://swissmodel.expasy.org/). The model was validated using MolProbity (http://molprobity.manchester.ac.uk.) and the quality was determined using SAVES v6.0 server (SAVESv6.0—Structure Validation Server (ucla.edu). The three-dimensional protein structure was analyzed by RAMPAGE (http://mordred.bioc.cam.ac.uk/rapper/rampage.php) to verify the accuracy of the model.

Organization of ClZIP1 and Promoter Mining

Promoter regions were amplified using forward primer, ZnP_1 5′-ATCGGCGATGATCATGGGAA-3′ (Lin et al. 2016), and reverse primer, ZnP_2 5′-ACCAACTTCAGCCAAAGT GC-3′, designed based on the 5′ regions of *ClZIP1* + 60 bp downstream of the start codon. The PCR product was cloned and sequenced at AgriGenome Labs Pvt Ltd., Kochi, Kerala, India. The promoter site was predicted by the Promoter 2.0 prediction server (Promoter 2.0 Prediction Server (dtu.dk)) (Knudsen 1999). The organization of exon and intron was analyzed by gene structure display server (http://gsds.gao-lab.org/) tool. Organization of coding sequence and the position of start site, TATA box, and splice sites were predicted using PlantCARE tool (Lescot et al. 2002) and online server TSS Plant (http://www.softberry.com/berry.phtml?topic=tssplant&group=programs&subgroup=promoter) (Table S2 and S3).

Statistics

Significance of treatments with respect to Zn content in the growth medium and in plant tissues (basal portion and leaf) was done by one-way ANOVA using the SPSS software. Least significance (LSD) test was employed for post hoc comparisons of means with significant *F* values.

Results

Selection of the Most Promising Zn-Solubilizing Bacteria

Among the 100 bacterial isolates obtained from the plant rhizosphere, three were positive with regard to Zn solubilization as evidenced by the formation of clear halo zone in vitro (Table S1). Using 16S rRNA gene sequencing, these

three were identified as *Bacillus cereus* (NCBI-IISR-GB2 MT192803), *Pseudomonas aeruginosa* (NCBI-IISR-TB5 MZ540872), and *B. safensis* (NCBI-IISR-TB4 MT192800). *B. cereus* and *P. aeruginosa* followed by *B. safensis* showed the maximum zone of clearance on the 10^{th} day of incubation (29, 27, and 25 mm, respectively) (Fig. S1). Since some strains of *B. cereus* and *P. aeruginosa* have been implicated in several human ailments, we selected *B. safensis* (NCBI-IISR-TB4 MT192800) for further studies related to ZIP gene expression.

Retrieval of Zn Transporter-Like Genes

A total of ~ 11.31 million reads including 56,527,980 number of paired-end reads with 50.97% GC were generated from *Curcuma* transcriptome using the Illumina Hi seq 2500 platform. Average base quality was above Q30 for 90% of bases and average GC content of the reads followed a normal distribution. After trimming, 3.41-Gb bases with 52.26% GC were obtained. The number of transcripts with FPKM ≥ 1 was found to be 86,949. More than 95% of the annotated contigs showed similarity to *Musa auminata*, a member of family Zingiberaceae. The raw reads generated from Illumina sequencing were deposited in National Centre for Biotechnology Information (NCBI), Sequence Read Archive database (SRA) under the Bioproject ID PRJNA698442 (http://www.ncbi.nlm.nih.gov/sra) (unpublished data; SRA accession no. SRR13594120). Eleven Zn transporter unigenes were identified from the transcriptome based on similarity searches. Among those, 10 unigenes showed > 70% identity with *M. acuminata* subsp. *malaccensis* (NCBI) with three contigs above 1000 bp in length. Contig id c42415_g1_i1 showed maximum FPKM value of 9.83 and BLAST identity of 77.2% with accession XM_009391637.2 of *Musa acuminata*. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology analyses identifiers assigned the gene to Zn transporter family (Table 1).

Conserved Motifs, Sequence Identities, and Phylogenetic Relationships

Complete ORF of the Zn transporter was confirmed by cloning based on primers designed against the contig id c42415_g1_i1 (SRA accession no. SRR13594120)**.** The PCR product of size ~ 1500 bp yielded a result of 1268 bp on sequencing. Sequence analysis indicated that ORF was 1101 bp in size. This gene was designated as *ClZIP1* (*C. longa* ZIP1) and deposited in NCBI (accession no.: MZ818774). On BLASTn analysis, the gene showed 76.74% identity with transcript variants X1 (XM_009391635.2) and X2 (XM_009391637.2) of transporters from *M. acuminata* subsp. *malaccensis*. The ORF of the gene encoded a predicted protein of 366 amino acids. BLASTp analysis of ClZIP1 showed highest identity with Zn transporters from *M. acuminata* subsp. *malaccensis*, viz., XP_009389912.1 and XP_009389910.2 (73.58%) followed by XP_009385792.1 (67.98%). The other plants showing similarity to ClZIP1 were *Phoenix dactylifera* (66.84%), *Cocos nucifera* (66.06%), *Phalaenopsis equestris* (65.68%), *Elaeis guineensis* (65.18%), *Nelumbo nucifera* (64.40%), *Nymphaea colorata* (63.85%), *Arabidopsis thaliana* (62.09%), *Tripterygium wilfordii* (61.56%), and *Hevea brasiliensis* (61.21%). From the dendrogram analysis, ClZIP1 was found to be clustered together with Zn transporters belonging to *Musa acuminata* (Fig. 1). Multiple sequence alignment performed with the protein sequences of the transporter from the different taxa that clustered with ClZIP1 revealed that several amino acid residues are conserved. Dark gray color represented those that are highly conserved whereas light gray color indicated those conserved among *Musaceae* (Fig. 2).

Analyses of TM Helix and Signature Sequences

The ClZIP1 protein had an estimated molecular weight of 38.47 kDa and theoretical pI of 6.51. The protein had more negatively charged residues (27) than positively charged ones (23). The instability index was predicted as 47.29 and aliphatic index was 98.17. A positive GRAVY value (0.443) was obtained for the protein. The ClZIP1 protein had transmembrane helix length between 17 and 33 residues with 8 inside to outside helix and 8 outside to inside helix and N-terminus inside 8 strong transmembrane (TM) helices. On ScanProsite analysis, a histidine-rich region with amino acid sequence HIVGMRAHAAAHRHSHAHVKGACDSHGLPVHSHAHGH was identified between TM-3 and TM-4. The predicted protein showed 12 sites for N-myristoylation. The InterProScan analysis assigned the protein to ZIP super family.

Analysis of Promoter Region

On analysis of the 685-bp promoter sequence (Fig. S2), the transcription start site was predicted to be located at 546 bp and TATA box with maximum score (6.258) at 513 bp. About 25 cis regulatory elements were predicted using PlantCARE Database from both strands of *ClZIP1*, which involves MYC and MYB binding sites and other response elements such as TCT-motif, TGACG-motif, G box, and ABA responsive element (ABRE) (Table S2 and S3). A single 10-bp imperfect palindromic ZDRE motif sequence ATGTCGAACT was identified in the upstream region at −517 bp.

Molecular Modeling and Structure Prediction

SOPMA analysis of the protein predicted 42.35% residues as α helix, 40.98% random coils, 12.02% extended

strands, and 4.64% β turn. The ClZIP1 protein did not display the presence of 3_{10} helix, Pi helix, β bridge, bend region, and ambiguous state in structure. PSIPRED results showed high confidence of helix, coils, and strands (Fig. 3). The 3D structure of the protein (Fig. 4a) predicted through the SWISS-MODEL workspace, which is an integrated web-based modeling environment, showed 16.93% similarity with the already reported crystal structure of ZIP metal transporter from *Bordetella bronchiseptica* in the protein data bank (PDB). Two-dimensional graphic representation of protein structures was analyzed by Ramachandran plot (Fig. 4b). The 3D model had an overall quality factor of 77.70. The MolProbity score obtained for the protein was 2.52. The results showed that 87.13% of the residues are in favored region and 4.68% are in outlier region.

Expression Pattern of *ClZIP1* and Zn Concentration in Basal Portion and Medium

The expression pattern of *ClZIP1* was analyzed under Zn-replete and Zn-deficient conditions in both leaf and basal portions of turmeric, in presence and absence of ZSB. The expression of *ClZIP1* varied with concentration of Zn in the medium as well as presence and absence of ZSB (Fig. 5). The expression at 0 ppm Zn was taken as control and was normalized with respect to *EF1α* transcript levels. In the absence of ZSB, expression of *ClZIP1* was higher and on par in the basal portion irrespective of the Zn applied to the medium (0–100 ppm Zn). While in presence of ZSB, the expression was markedly downregulated in all treatments with the maximum at 100 ppm (88%). The gene expression was also analyzed in the leaf tissues and did not correlate with the Zn levels.

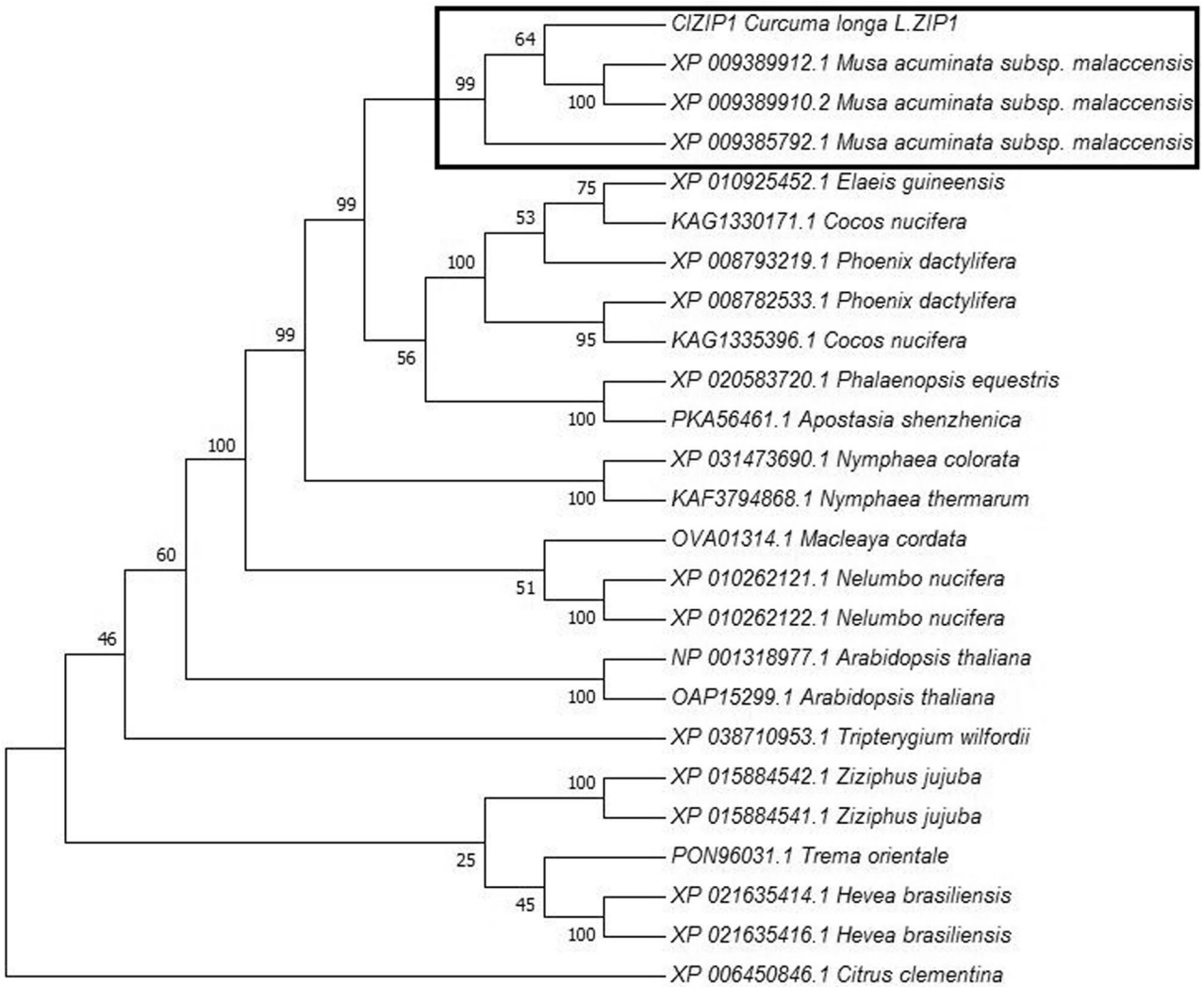

Fig. 1 Phylogenetic tree based on amino acid sequence of *C. longa* Zn transporter gene (*ClZIP1*) and closely related species

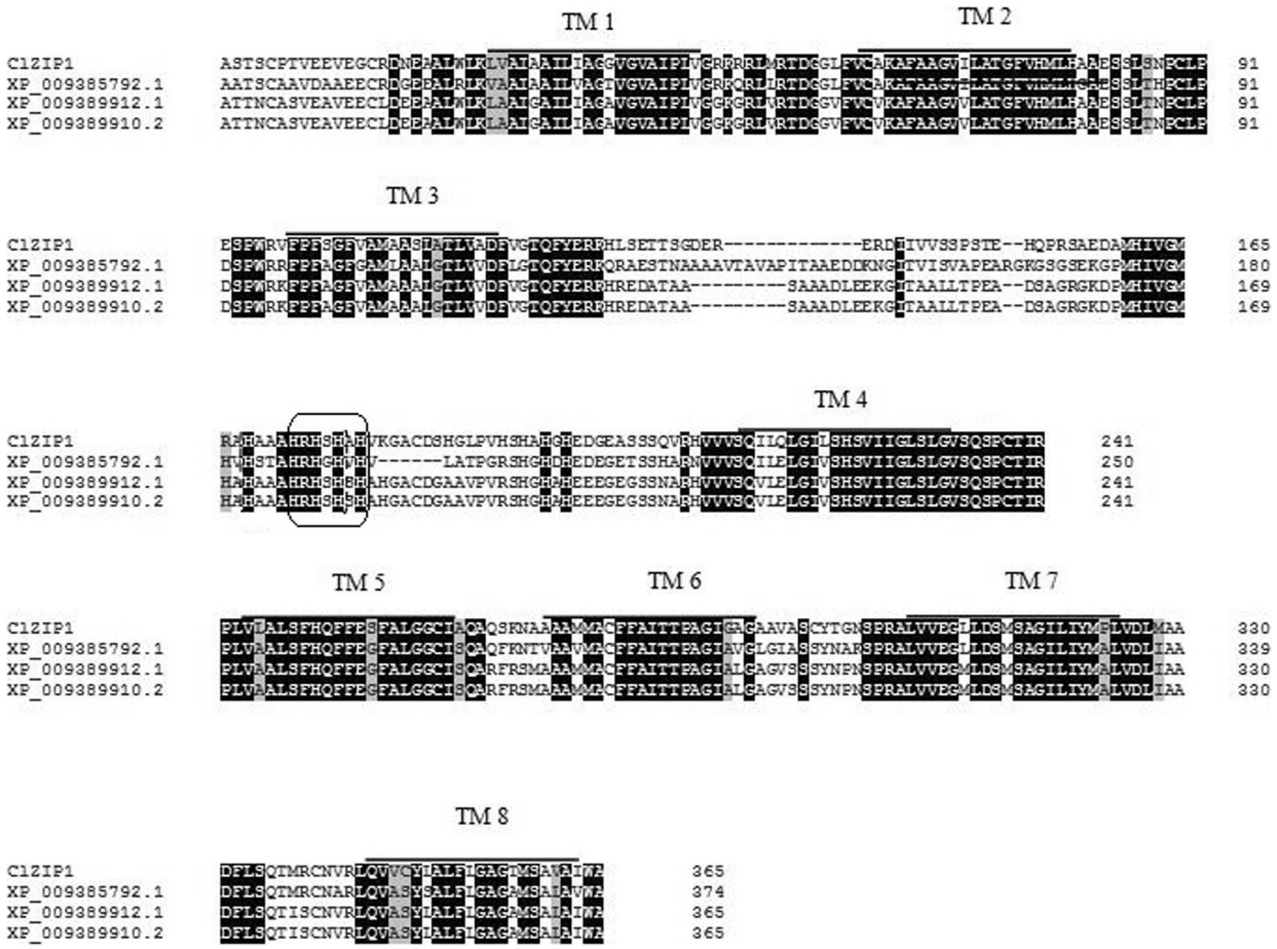

Fig. 2 Multiple sequence alignment showing 8 domains characteristic of ZIP transporters and the conserved regions among the different families. The square indicates conserved histidine motif

The Zn concentration in the basal portion of turmeric and medium was analyzed both in the presence and absence of ZSB (Fig. 6). In the absence of ZSB, Zn concentration in basal portion (Fig. 6a) was lowest in control and increased inconsistently with increasing Zn levels, registering the maximum concentration at 100 ppm applied Zn (6.15 µg g^{-1}). In contrast, in the presence of ZSB, Zn concentration in the basal portion showed a significant increase with maximum concentration at 50 and 100 ppm applied Zn (39.67 µg g^{-1} and 76.67 µg g^{-1} respectively). However, the Zn concentration in the medium increased with increasing levels of applied Zn both in the presence and absence of ZSB (Fig. 6b). In the presence of ZSB, Zn concentration in the medium was higher than that without ZSB up to 25 ppm (7.1 µg mL^{-1}). At further higher levels (50 and 100 ppm), the Zn concentration in the medium with ZSB was markedly lower (17.23 and 21.77 µg mL^{-1}, respectively) than that in absence of ZSB (21.57 and 38.47 µg mL^{-1}, respectively).

Discussion

Zn uptake by plants is based on the membrane potential and Zn transporters that ferry the Zn to the cytoplasm through the plasma membrane (Olsen and Palmgren 2014; Tiong et al. 2015). The adaptive responses to Zn deficiency manifested by plants are through expression of Zn transporters (Lira-Morales et al. 2019) and these may be effectively deployed for enhancing the uptake of Zn by plants (Sasaki et al. 2016). However, information on genes associated with Zn uptake and translocation in turmeric or any such rhizomatous species is, heretofore, not available. A transcriptome-based approach was opted for identification of the Zn transporter gene from turmeric and 11 unigenes were identified from the RNA seq data. Among these, the longest unigene, showing high abundance, as determined by FPKM values was used for further studies. On BLASTn analysis, the Zn transporter sequence mined from the transcriptome showed 77.2% similarity to *Musa acuminata*,

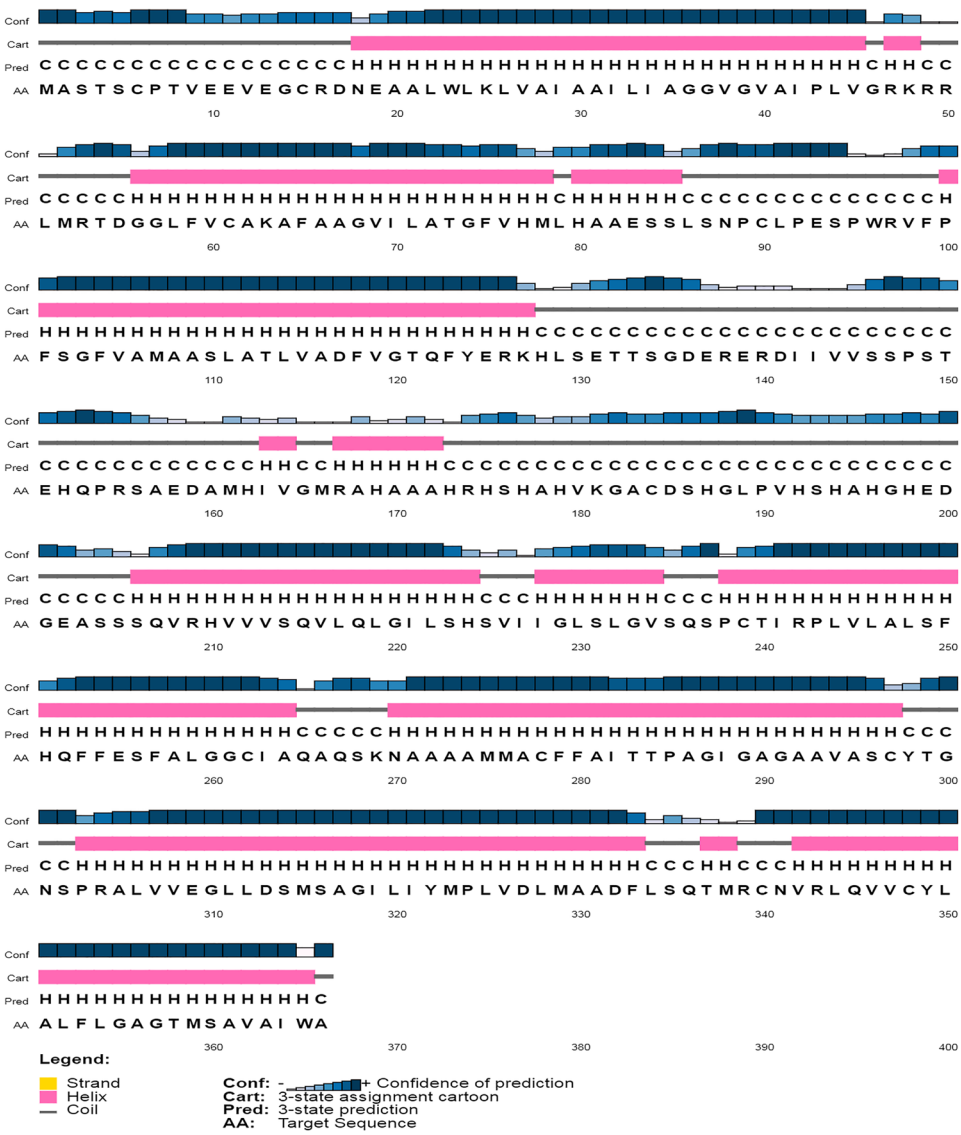

Fig. 3 Prediction of secondary structure of *C. longa* zinc transporter (ClZIP1*)* by PSIPRED

while the cloned sequences showed 76.74% identity. This is the first comprehensive study on Zn transporters from *C. longa* L. based on RNA seq and the putative Zn transporter gene (*ClZIP1*) is reported for the first time, albeit reports on Zn transporter genes in other crops (Williams et al. 2000; Ishimaru et al. 2011; Bashir et al. 2016).

While it is important to precisely classify the genes into gene families in order to assign gene function and to gain information on gene evolution, it is common knowledge that genes belonging to the same family share among them major features like functional domains and sequences and also molecules and elements that interact with them (Singh et al. 2014). The sequence similarities with a close or distantly related species or kingdom are indicators of evolutionary characteristics of the gene. On phylogenetic analysis, *ClZIP1* was found to cluster with Zn transporters from *Musa acuminata* and form a clade with members belonging to the order Zingiberales that included family Zingiberaceae and family Musaceae (Fig. 1). Though multiple sequence alignment of the transporters from the cluster revealed highly conserved regions between the *ClZIP1* and members of the Musaceae family, a few regions were specifically conserved among members of the family Musaceae.

Identification of genes and gene families is cumbersome especially when it is de novo. Plant ZIP family transporters are predicted to have 6–9 transmembrane (TM) domains (alpha helices) with 8 being the most characteristic feature of ZIP family transporters (Guerinot 2000). The molecular weight has been reported to range from 33.1 to 51.4 kDa and protein sequence ranged from 322 to 478 amino acids (Vatansever et al. 2016). We found that ClZIP1 was 366 amino acids in length and had a weight of 38.47 kDa. NCBI conserved domain search also suggested that it was a member of the ZIP family with accession numbers

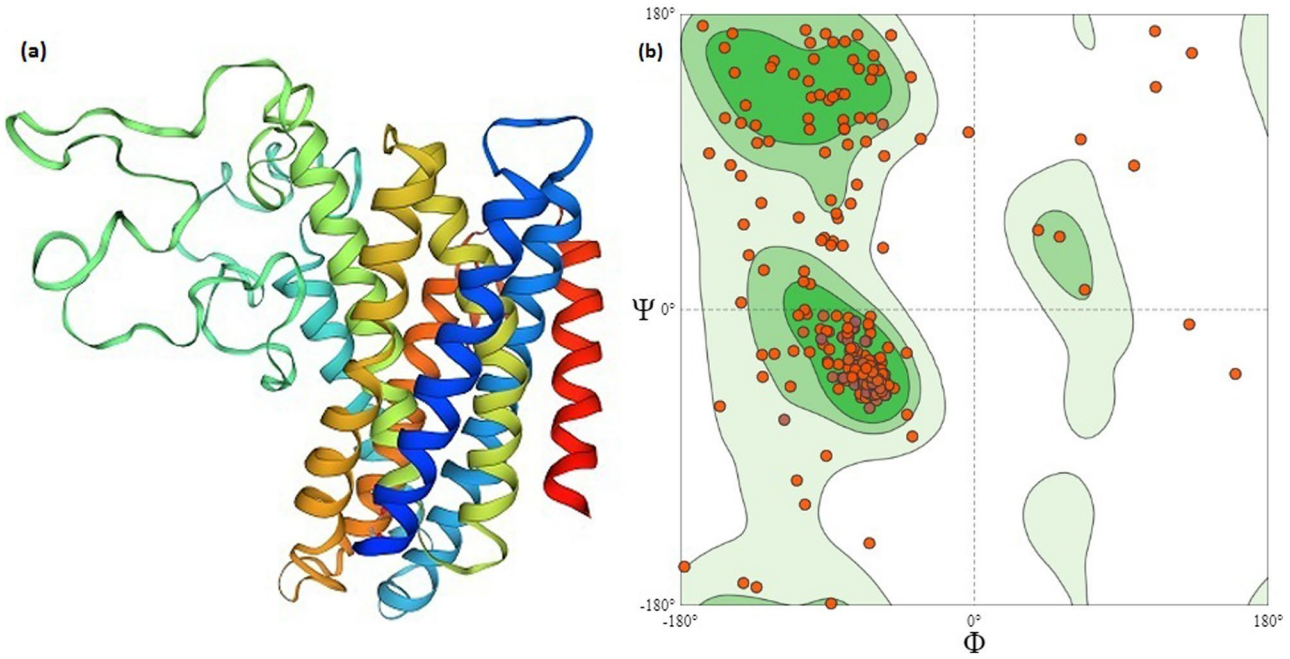

Fig. 4 (**a**) 3D structure of *C. longa* zinc transporter (ClZIP1) modeled using SWISS-MODEL server. (**b**) Ramachandran plot

cl00437 and pfam02535. The 3D homology model of ClZIP1 revealed 8 TM domains and a long hydrophobic loop as seen in most of the ZIP proteins. A variable region was also identified between TM domains 3 and 4. A highly conserved histidine-rich ZIP signature motif with amino acid sequence HRHSHAHV was also identified within this variable region. Further, a 10-bp ZDRE *cis*-element was predicted in the promoter of *ClZIP1* gene with two mismatches from the consensus described by Assunção et al. (2010a). ZDRE is noteworthy among the promoters of the genes upregulated by low Zn, but not in genes that are not Zn deficiency inducible. Collectively, these findings confirmed that *ClZIP1* from *C. longa* L. belonged to the ZIP family. ZIP protein of model species *Arabidopsis* had comparable properties (Alagarasan 2017; Fu et al. 2017), as that of ClZIP1. Multiple sequence alignment of ClZIP1 with closely related ZIP from *Arabidopsis thaliana* showed high similarity (Fig. S3).

Further, the physicochemical analysis of the novel ClZIP1 protein showed that it is acidic in nature as indicated by pI < 7 and presence of more negatively charged amino acids. According to Grotz et al. (1998), the conserved acidic amino acids in the transmembrane domains of ZIP proteins are crucial for substrate transport. The instability index which is the measure of in vivo half-life indicated that the Zn transporter is unstable. A protein with in vivo half-life of < 5.0 h has instability index of > 40 and the one with half-life of > 16 h has

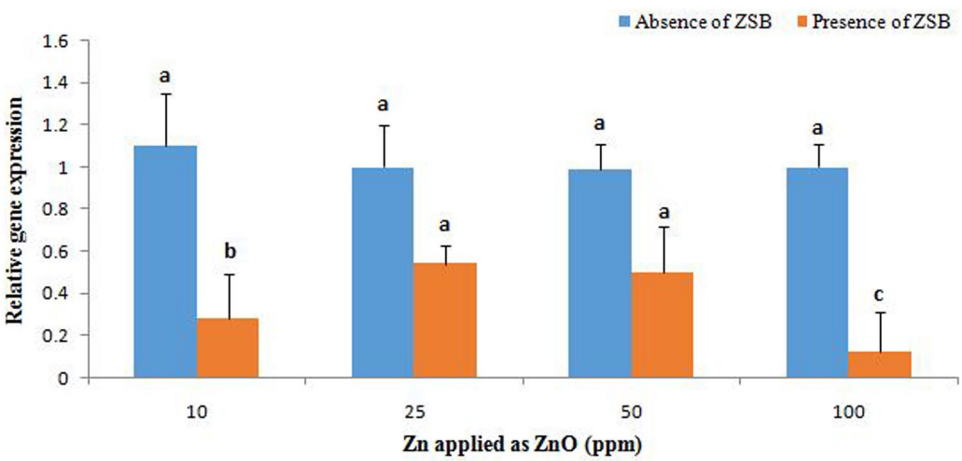

Fig. 5 Relative gene expression of *ClZIP1* in basal portion of in vitro plantlets at different concentrations of applied Zn in the presence and absence of ZSB (*B. safensis*) (vertical bars represent standard errors; different letters indicate significant difference at $P < 0.05$ (LSD))

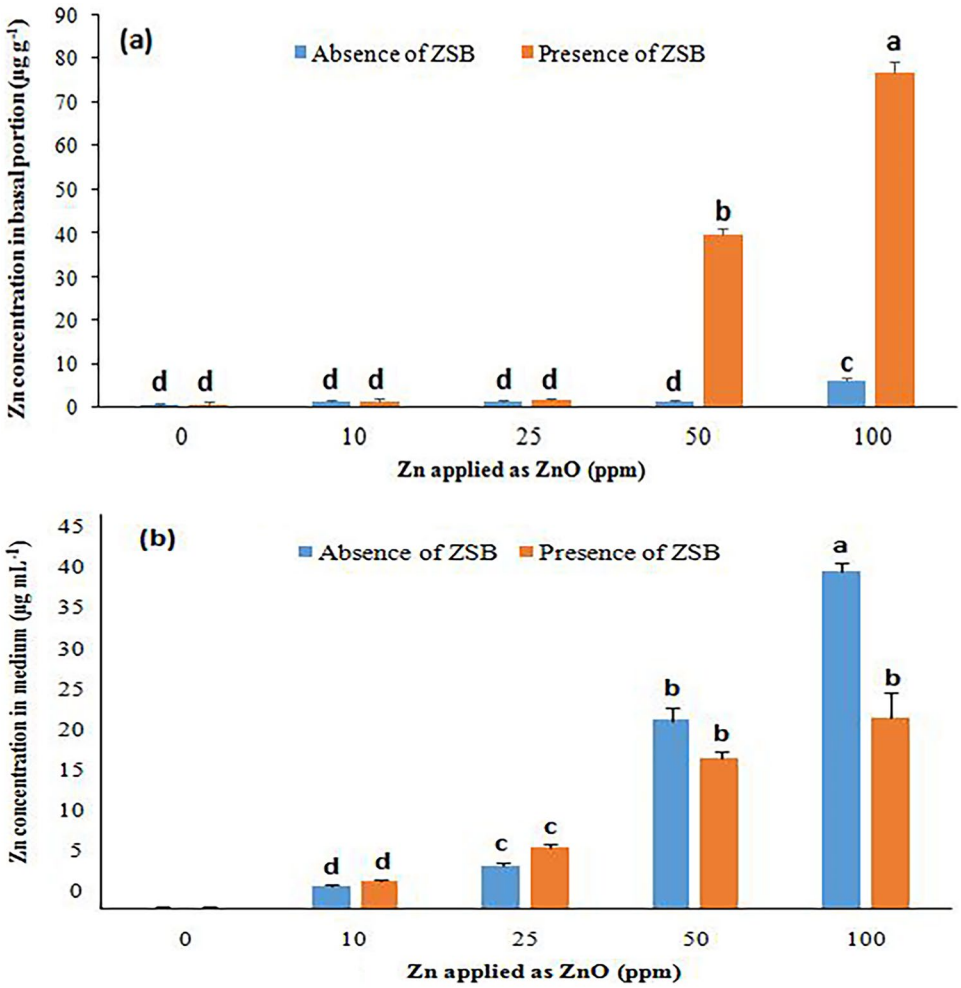

Fig. 6 Zn concentration in (**a**) basal portion and (**b**) medium at different concentrations of applied Zn in the presence and absence of ZSB (*B. safensis*) (vertical bars represent standard errors; different letters indicate significant difference at $P < 0.05$ (LSD))

index of < 40.0. A high aliphatic index is indicative of greater thermostability (Gasteiger et al. 2005). The ClZIP1 protein is highly thermostable as evident from the high score. Also, it had a positive GRAVY value indicating its hydrophobic nature, which is a common feature of membrane spanning proteins (Grotz et al. 1998). N-Myristoylation is an important post translational change known to affect the conformational stability as well as the propensity of the protein to interact with membranes or the hydrophobic domains of other proteins. *N*-Myristoylated proteins are considered key players of cellular pathways like signal transduction, membrane transport, and apoptosis. The presence of 12 myristoylation sites in ClZIP1 protein suggested that they might play a vital role in the transport of Zn in plants (Mondal et al. 2014; Zaun et al. 2012). Secondary structure analysis indicated that the protein contained high percentage of helix which is a characteristic of alpha-helical TM spanning proteins. The low MolProbity score and high overall quality factor confirmed that it was a good model. Basically, lower MolProbity score and a quality factor > 50 are accepted for good-quality models (Messaoudi et al. 2013). The Ramachandran plot of the predicted model further reaffirmed that the model is of good quality.

Functional analysis of *ClZIP1* was done in the presence and absence of ZSB at all levels of applied Zn (Fig. S4). In the absence of ZSB, the gene expression remained upregulated and on par at all levels of applied Zn indicating that enough labile Zn was not available to the plant due to the sparingly soluble nature of ZnO. This is consistent with findings of Ramesh et al. (2003) and Ishimaru et al. (2005) who reported that deficiency of Zn enhanced the root expression of ZIP gene. Likewise, in rice, *OsZIP8* was reported to be stimulated in Zn-deficient shoots and roots (Yang et al. 2009). In contrast, *MtZIP1* was found to be over expressed in Zn-deficient roots and leaves in *Medicago truncatula* (Lopez-Millan et al. 2004). The enhanced expression of transporters under low Zn has been implicated to play an important role in accumulation of Zn in plant tissues (Nishiyama et al. 2012), suggesting their role in Zn uptake under Zn-deficient conditions (Li et al. 2013). It has also been envisaged that transcription factors regulate the adaptation to Zn deficiency by increasing the transcription of ZIPs and related genes (Assunção et al. 2010a; Pedas et al. 2009). The transporter gene (*ClZIP1*) identified in this study was induced by low Zn conditions similar to those observed in rice (Ramesh et al. 2003; Ishimaru et al. 2005; Lee et al. 2010).

The gene expression in control (no applied Zn) remained unaltered, irrespective of the presence of ZSB. However, in all the other treatments, the gene expression was downregulated with a maximum of 88.0% at 100 ppm Zn. This could be attributed to enrichment of available Zn pool following solubilization of ZnO by *B. safensis*. This agreed with the report that the expression levels of ZIP genes in rice (*OsZIP1*, *OsZIP4*, and *OsZIP5*) were altered due to Zn sufficiency and ZSB inoculation (Krithika and Balachandar 2016). In this study, different degrees of Zn complementary capability were displayed by *ClZIP1* similar to earlier reports in *Arabidopsis*, rice, and maize ZIPs (Li et al. 2013).

The data on Zn concentration in the basal portion revealed that the intrinsic Zn levels were very low in the absence of ZSB, even at high levels of applied Zn in the medium. It is apparent that Zn was not available in the medium for uptake by the plant due to the sparingly soluble nature of ZnO. However, in the presence of ZSB, greater intrinsic Zn concentrations were registered in the tissue at all levels of applied Zn with maximum at 50 and 100 ppm (39.67 and 76.67 μg g^{-1}, respectively), suggesting 82.0% and 74.5% increase, respectively, in comparison to the corresponding treatments without ZSB. A concomitant reduction in Zn in the medium was observed at these levels, indicating 24.0% and 50.0% reduction in the presence of ZSB. This clearly indicated that in treatments with ZSB, the Zn concentration in the plant tissue was enhanced. The overall increase in intrinsic Zn levels in the tissue could be due to improved solubilization of ZnO by ZSB, resulting in increased availability of labile Zn, which eventually led to improved transportation of Zn.

Nevertheless, the levels of Zn or presence of ZSB did not induce any specific changes in the expression pattern of *ClZIP1* in the leaves. Such specific spatiotemporal expression pattern in *ClZIP1* can possibly be explained by the differential Zn requirements of basal portion and leaves causing induction of expression in the basal portion while remaining subdued in the leaves. This is consistent with the reports that the ZIP family genes displayed various expression profiles with respect to tissues as well as environmental Zn concentration (Lee et al. 2010; Yang et al. 2009), and in tissues with higher requirement of Zn, the expression of the transporter gene was found to be high (Uchiyama et al. 2002). Conversely, ZSB promoted higher intrinsic Zn concentration in the basal portion of turmeric with concomitant reduction in the medium and a downregulation of the *ClZIP1* gene expression. Apparently, ZSB solubilized recalcitrant ZnO and enhanced the labile Zn pool resulting in more efficient and effective uptake and translocation of Zn from the medium, which consequently led to under expression of *ClZIP1*. Improved grain Zn concentration under Zn-deficient conditions through upregulation of $HvZIP_{13}$ was reported in barley in presence of AM fungi (Watts-Williams and Cavagnaro 2018).

It is, therefore, evident that *ClZIP1* gene could play a vital role in Zn translocation to the rhizome of turmeric and can be used as a biomarker for Zn availability in soil. We have attempted a simple, non-invasive, and economic way of validating gene expression, and function of any nutrient transporter gene can be unraveled with the help of the corresponding nutrient-solubilizing microorganism. Moreover, the novel Zn transporter identified in the current study (*ClZIP1*) can be used as a bait gene to identify other ZIP family members from turmeric and related species. Enhancing the Zn content in edible rhizomes is important for improving nutrition. However, a major tenet of such metal transporters is their rate-limiting nature. Hence, it is important to learn the mechanism of their regulation during translocation of such micronutrients to storage or sink organs in crops. There are several reports in crops like barley, wherein overexpression of ZIP transporter *AtZIP1* has resulted in increased Zn uptake as well as higher accumulation of Zn in seeds (Ramesh et al. 2004). Therefore, a tissue-specific overexpression of ZIP genes may provide an alternative strategy for bio fortification of crops with essential nutrients like Zn.

Conclusion

In the present investigation, we have identified a novel Zn transporter gene in turmeric (*ClZIP1*) by RNA seq strategy, characterized the gene, and studied its expression under varying Zn levels in the presence of a Zn-solubilizing bacteria (ZSB). The gene was found to belong to the ZIP transporter family based on the typical domains and signature sequences. The study further revealed that inoculation of ZSB enhanced the available Zn pool in the medium and Zn uptake by the plant, while simultaneously downregulating the expression of *ClZIP1*. Contrarily, in the absence of ZSB, the available Zn pool was markedly lowered creating low Zn stress and upregulation of *ClZIP1*expression.

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Author Contribution TE Sheeja: investigation, editing, resources. R. Praveena: investigation, resources. I.P. Vijesh Kumar: investigation. C. Sarathambal: visualization, validation. O. Shajina: investigation. Hridya Vijay: investigation. Nandana Rajeev: validation. P. Prashina Mol: validation. C.P Sreena: validation, editing. V. Srinivasan: software, data curation, formal analysis. R. Dinesh: visualization, supervision, writing—original draft preparation.

Availability of Data and Material The datasets presented in this study can be found in online repositories. The repositories are NCBI SRA: accession number SRR13594120 under the Bioproject ID PRJNA698442. (http://www.ncbi.nlm.nih.gov/sra), GenBank: accession number MT192800 and MZ818774 (https://www.ncbi.nlm.nih.gov).

Declarations

Conflict of Interest The authors declare no competing interests.

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