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Elucidating the Role of Inositol Monophosphatases Gene Family in Abiotic Stress Management †

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Abstract: *Myo*-inositol is considered as an important osmoprotectant, which is directly involved in abiotic stress management in plants. We have biochemically and functionally characterized the Inositol Monophosphatase (CaIMP1) and IMP like proteins (CaIMPL1 and CaIMPL2) from chickpea (*Cicer arietinum*). We had already reported the broad substrate specificity of CaIMP1 as determined through biochemical characterization. Our work also signifies the role of CaIMPL2 in Histidine pathway as it was able to catalyze the dephosphorylation of Histidinol 1-P, however IMPL1 was mostly involved in the hydrolysis of D-Ins 1-P and D Gal 1-P. As decoded by sequence similarity and phylogenetic study, CaIMP, CaIMPL1 and CaIMPL2 were found to be homologous enzymes but we observed very contrasting difference in their substrate specificity which may be the result of divergent evolution of these enzymes.

Keywords: *Cicer arietinum*; Inositol Monophosphatase; IMP like proteins; Broad substrate specificity; Abiotic stress

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1. Introduction

Inositol metabolism has been shown to play crucial role in chickpea under environmental stress. Increased *Myo*-inositol content as well as higher relative expression of *Myo*-inositol 1-phosphate synthase (MIPS) has also been reported in chickpea [1]. In *Myo*-inositol biosynthesis pathway, the second step of the pathway which is conversion of inositol 1-phosphate to *myo*-inositol is catalyzed by *Myo*-inositol Monophosphatase (IMP).

Chickpea being an important crop of Asia pacific region endures many environmental stresses during its growth period but is considered as relative tolerant towards abiotic stresses. Hence chickpea is widely reported as an important crop for genetic resources of tolerant genes against diverse abiotic stresses [2]. Much work has been done on MIPS gene, while little is known about detailed function and metabolic regulation of IMP in chickpea. We have isolated IMP (*CaIMP*) and two IMP like protein genes (*CaIMPL1* and *CaIMPL2*) from drought tolerant legume Chickpea (*Cicer arietinum*). We have already characterized the chickpea Inositol Monophosphatase (CaIMP1) both biochemically and functionally and also reported its peculiar broad substrate specificity [3]. Further, we have overexpressed it in *Arabidopsis thaliana* and these overexpression lines of *CaIMP1* were found to be tolerant to variety of abiotic stresses and also shown improved seed germination under these abiotic stresses [3]. In this article, we have explored the expression profiling and biochemical parameters of CaIMPL1 and CaIMPL2 recombinant proteins to get some information regarding role of these IMP family enzymes in plant metabolism.

2. Materials and Methods

2.1. Isolation of Chickpea IMPL Genes along with Its cDNA and Sequence Study

Cicer arietinum cv. BGD72 was used and was procured from the Indian Agricultural Research Institute (IARI), New Delhi, India. Leaves from chickpea plant were used to extract total RNA using Tri reagent (Sigma, St Louis, MO, USA) and cDNA was prepared using DNaseI treated RNA with random primers following the as per protocol of the cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Amplification of full length cDNA of CaIMPL1 and CaIMPL2 was done using gene specific primers. Sequence information was obtained from NCBI with Accession No. XM_004489614 (IMPL1) and XM_004494897 (IMPL2), followed by cloning into pJET 1.2 blunt vector (Thermo Scientific, USA). To get the information of genomic structure of genes (CaIMPL1 and CaIMPL2), PCR was done using genomic DNA isolated from chickpea plant. Further, PCR product was eluted and cloned in pJET cloning vector (Thermo Scientific, USA) and it was followed by subsequent sequencing for sequence confirmation. All primer sequences are provided in Table S1 (Supplementary).

2.2. Expression Pattern of IMP Gene Family in Different Environmental Stresses and Different Tissue/Organ

Chickpea seeds were germinated and 6 days old seedlings were subjected to different abiotic stress treatment for 6 h [Salt (150 mM), dehydration, cold (4°C), heat (42°C)], Hormone treatment, 100μM each (Abscisic acid, Salisylic acid, Auxin, Gibberellic acid) for 6 h and total chickpea RNA was isolated from whole seedling using TRI reagent (Sigma, St Louis, MO, USA) from these seedlings. Method reported by Boominathan et al. (2004) was used for stress and hormone treatment [1]. 2μg total RNA was used for cDNA preparation using ABI cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). With the help of Real Time PCR (qRT-PCR), expression analysis was performed for *CaIMPL1* and *CaIMPL2* along with CaIMP. Apart from this, to get an idea for spatial expression of *CaIMPL1* and *CaIMPL2* genes in different organs of chickpea plant (root, stem, flower, seed) were used for total RNA isolation which was followed by cDNA preparation and then expression analysis was performed. Real-time PCR reactions were set up on ABI Step one real-time PCR using real time primers for CaIMPL1, CaIMPL2 and CaIMP1. Primer sequences are provided in table S1 (supplementary). The expression of *CaIMPL1*, *CaIMPL2* and *CaIMP1* mRNA was normalized to the expression of endogenous control 18S rRNA. For each real-time PCR reaction, 20 μL of a reaction mixture containing 2 μL of diluted RT product, 10 μL of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and 20 pico-molar of each forward and reverse primer was added. A negative control lacking RT enzyme was also included in each assay. The relative expression value of each transcript in all experiments was calculated using the $\Delta\Delta$CT method.

2.3. Bacterial Overexpression, Purification and Biochemical Characterization of CaIMPL1 and CaIMPL2 Gene(S) Product

For generating recombinant protein, the cDNA of *CaIMPL1* and *CaIMPL2* was sub cloned into the expression vector pET-28a (Novagen, Madison,WI, USA) with His-tag. Then, the resulting plasmid was transferred to the *E. coli* BL-21 (DE3) cells [4]. Sequence was confirmed through sequencing and restriction digestion. Transformed *E. coli* cells was induced and culture was sonicated, the extracts was analyzed for expression on SDS-PAGE [5]. Solubilization and purification of the recombinant which expressed as inclusion bodies were then solubilized by procedure reported by Majee et al. (2004) [6] and the purification of protein of interest

was done using affinity chromatography (GE Healthcare, Sweden). The purified fractions of CaIMPL1 and CaIMPL2 proteins were used for biochemical characterization using Malachite green dye based inorganic phosphate estimation assay [3].

3. Results

3.1. Molecular Cloning, Sequencing and Characterization of CaIMPL1 and CaIMPL2 Genes and cDNA(s)

Amplification of full-length cDNA of CaIMPL1 and CaIMPL2 was done using gene specific primers followed by cloning into pJET 1.2 blunt vector (Thermo scientific, USA) (Figure 1). Full length sequence (CDS) of CaIMPL1 and CaIMPL2 was obtained from NCBI with Accession No. XM_004489614 (IMPL1) and XM_004494897 (IMPL2). The insert was confirmed through colony PCR and restriction digestion. The full length sequence of CaIMPL1 including the 5′ and 3′ UTR was found to be 1329 bp, containing an coding region of 1044 bp which encodes a polypeptide of 347 aa. The full length sequence of CaIMPL2 is 1234 bp in length (including the 5′ and 3′ UTR), containing coding region of 894 bp which encodes polypeptide of 297 aa (Figure 2).

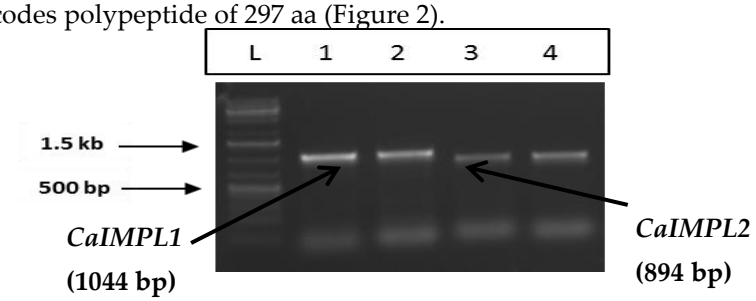

Figure 1. PCR amplification of CaIMPL1, CaIMPL2 for bacterial expression and plant expression.

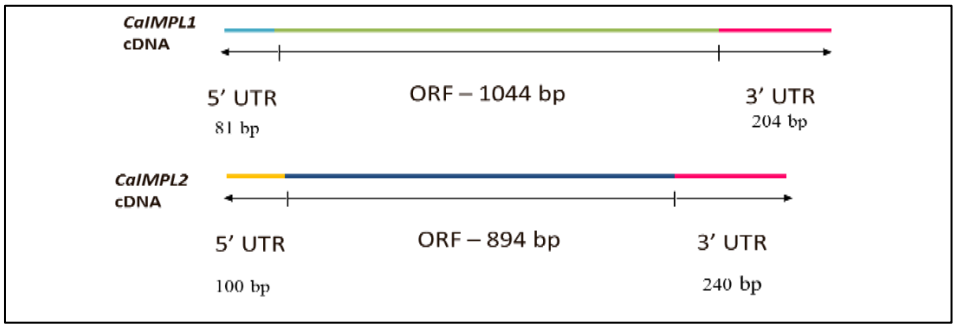

Figure 2. Diagrammatic representation of CaIMPL1 and CaIMPL2 cDNA with UTR.

3.2. IMP Gene Family Transcript Differentially Expressed in Different Environmental Stresses, in Presence of Different Hormones and in Different Tissue/Organ

The expression of Both *CaIMPL1* and *CaIMPL2* along with *CaIMP1* was found to be increased under all the abiotic stresses, while maximum expression of all the three transcripts was reported in case of dehydration stress. In comparison to *CaIMPL1*, *CaIMPL2* showed lower expression. Among *CaIMPL1*, *CaIMPL2* and *CaIMP1*, highest transcript accumulation was observed in case of *CaIMPL1*, under all the stresses (Figure 3A). Further, the expression pattern of all three genes was investigated in presence of exogenous hormones (Abscisic acid, Salicylic acid, Auxin, Gibberellic acid etc.), highest transcription induction of all these genes was observed in case of ABA treatment. Although, GA treatment could induce a slightly increased transcript accumulation in case of all three genes (Figure 3B).

Both *CaIMPL1* and *CaIMPL2* along with *CaIMP1* were found to be differentially expressed in different organs of chickpea plant (Figure 3C).

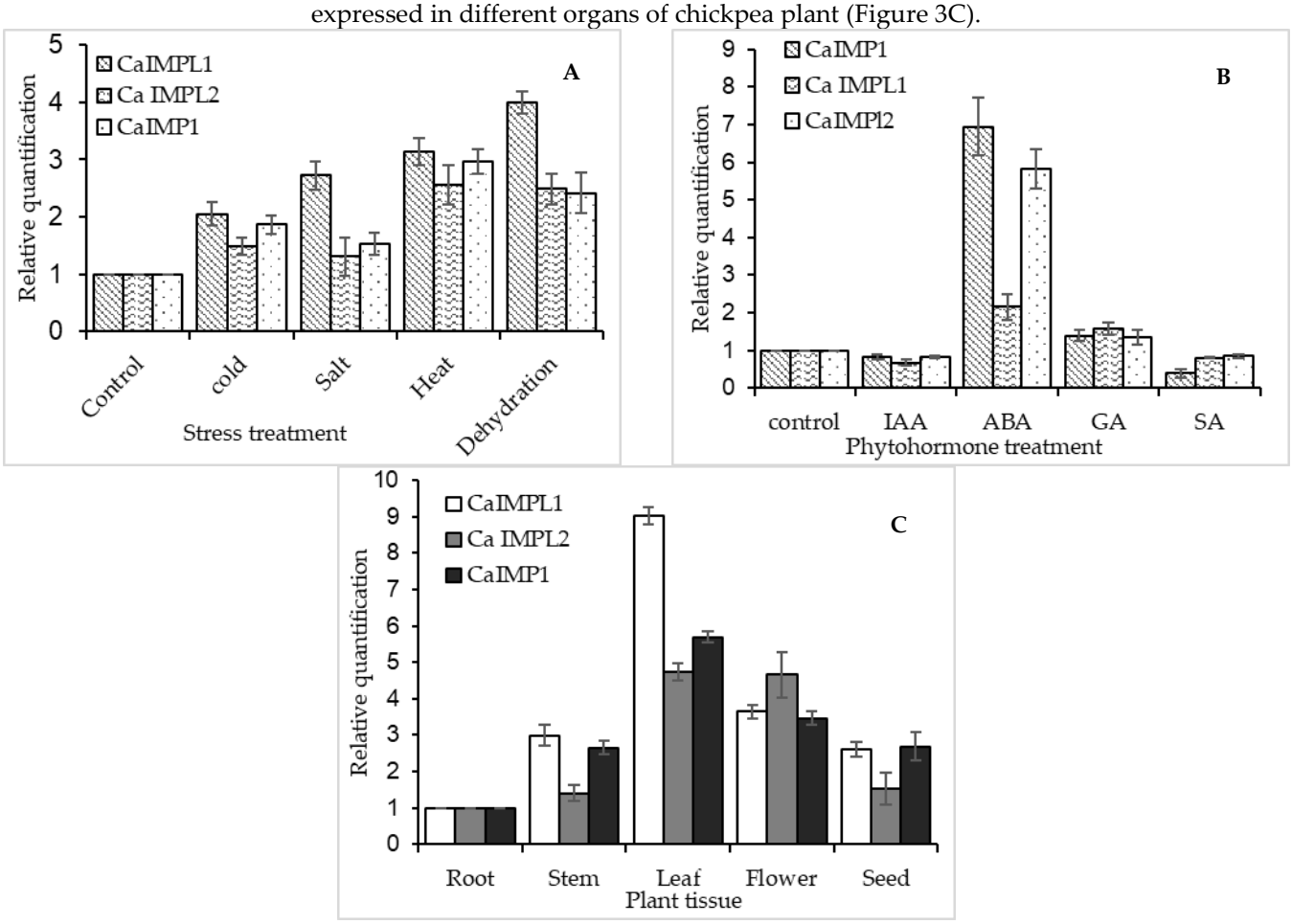

Figure 3. Relative expression analysis of *CaIMP1*, *CaIMPL1*, and *CaIMPL2* transcripts using Real-time PCR (A) seedlings with different abiotic stress treatment, (B) under treatment of seedlings with exogenous hormones, and (C) in different organs of chickpea plant. 18S rRNA was used as an endogenous control for normalizing the relative expression value of each transcript in all experiments and calculated using the $\Delta\Delta$CT method (Applied Biosystems, Foster City, CA, USA). Data depicted in the charts are obtained from triplicate analysis of three replicates. Error bars designate ± SD standard deviation.

3.3. CaIMPL1 and CaIMPL2 Proteins Exhibit Distinct Biochemical Properties

Culture of transformed cells (pET28a:CaIMPL1:BL21-DE3 and pET28a:CaIMPL2:BL21-DE3) was given and culture was then induced by 0.5 mM IPTG as soon as the absorbance reached 0.6–0.8 at 600 nm. Cells were harvested using centrifugation, sonicated and finally analyzed on 12% SDS-PAGE (Figure 4). CaIMPL1 and CaIMPL2 were expressed as a 32.7 and 38.1 kDa protein respectively mainly in the inclusion bodies as evident by SDS-PAGE analysis. Solubilization of the recombinant protein was performed [6] and then expressed protein was serially dialyzed which was followed by purification using affinity chromatography (GE Healthcare, Sweden) and used for biochemical characterization study (Figure 5)

The purified fractions of CaIMPL1 and CaIMPL2 proteins were used for biochemical characterization using Malachite green dye based inorganic phosphate estimation assay [3] (Table 1).

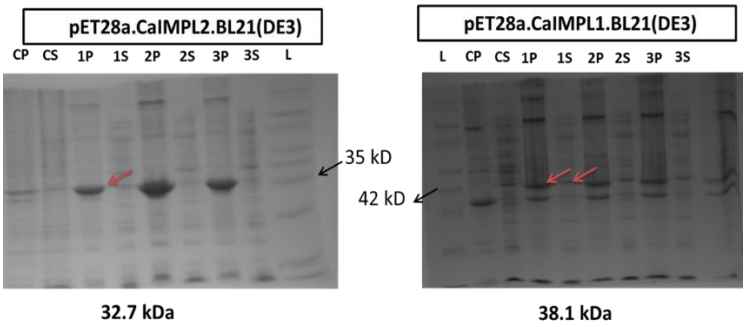

Figure 4. SDS PAGE analysis of CaIMPL1 and CaIMPL2 overexpressed recombinant protein in *E. coli* BL21 (DE3). 12% SDS-PAGE gel was used. [CP, CS- Control having pET28a empty vector transformed induced cells; 1P-3S- CaIMPL1 and CaIMPL2 transformed cells; L, molecular weight ladder; P-pellet fractions; S-soluble fractions/supernatant fraction.].

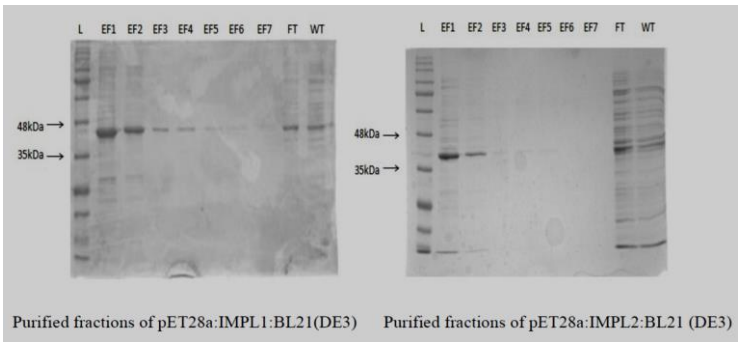

Figure 5. Purified fractions of CaIMPL1 and CaIMPL2 proteins on 12% SDS PAGE.

Table 1. Biochemical characterization of recombinant CaIMP1, CaIMPL1 and CaIMPL2.

	Enzyme (Substrate)	*Km* (μM)	Vmax (μmol min^{-1} mg^{-1})	MgCl2 (mM)	Optimum Temperature	Optimum pH
1	CaIMPL2 (Histidinol 1-P)	29	3.8	3	37°C	8
2	CaIMPL1 (D-Inositol 1-Phosphate)	24	4.1	3	37°C	8
3	CaIMPL1 (D-Galactose 1-Phosphate)	18	4.9	3	37°C	8
4	CaIMP (D-Inositol 1-Phosphate)	25	4.4	3	37°C	8
5	CaIMP (D-Galactose 1-Phosphate)	16	5.3	3	37°C	8

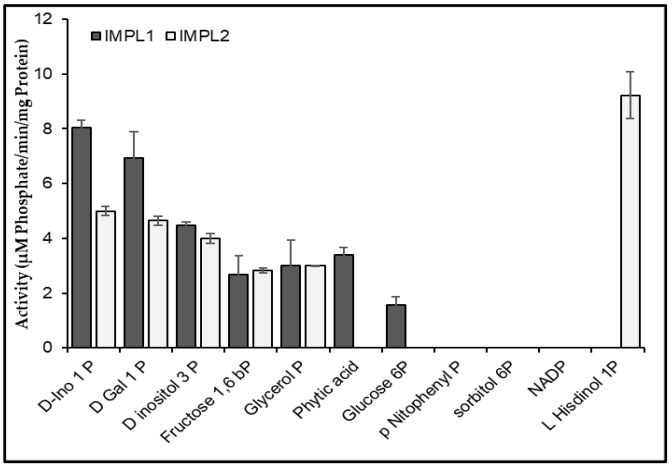

Figure 6. Activity of recombinant CaIMPL1 and CaIMPL2 with different substrates. Data depicted in the charts are obtained from triplicate analysis of three replicates. Error bars designate ± SD standard deviation.

Purified protein samples were initially used to test the substrate specificity using different sugar phosphates including the general substrate *myo*-inositol 1-phosphate and D-Galactose 1-phosphate. Then, temperature optima, pH optima and optimum co factor concentration were estimated. Our data supports that CaIMPL2 protein chiefly catalysed the hydrolysis of histidinol 1-phosphate (which is one of step of Histidine biosynthesis pathway) in contrast to the CaIMPL1 protein which mainly showed the phosphatase activity with D-Ins 1-P in addition to other substrates like reported CaIMP1. This diverse substrate specificity needs to be further explored to get clarity about role and regulation of these enzymes in plant metabolism. Various kinetic parameters have been summarized in Table 1.

4. Conclusion

Biochemical characterization of IMPL and activity comparisons of the CaIMPL1 and CaIMPL2 enzymes from *Cicer arietinum* were done with various inositol and other phosphate substrates.

Our findings suggest that IMPL2 enzyme from chickpea could hydrolyse histidinol 1-phosphate, which indicates its role in Histidine biosynthesis pathway. While IMPL1 enzyme from chickpea could mainly hydrolyze D-Ins 1-P substrate

Both CaIMPL1 & CaIMPL2 like CaIMP1 showed broad substate specificity.

IMPL1 and IMPL2 genes whose function is not yet completely known were observed to be induced under various environmental stress conditions.

Appendix A

(Elucidating the role of Inositol monophosphatases gene family in abiotic stress management)

Table S1. Primer details.

Primer No.	Primer sequence (5′-3′)	Purpose
CaSS1F	ATGTTGTCACAGTGCCATCT	For cloning full length CDS of *CaIMPL2* sequence
CaSS2R	GCTCTAGATTCATTACAACGGTAA	
CaSS3F	ATGTCAATTGTATTCTCCGCAGC	For cloning full length CDS of *CaIMPL2* sequence
CaSS4R	GAGGACTACAGGGCAGACGTTTAA	
CaSS5F	GAATTCATGTTGTCACAGTGCCATCT	To clone full length *CaIMPL*2 in bacterial expression vector
CaSS6R	CTCGAGCCGTTGTAATGAATCTAGAGC	
CaSS7F	GAATTCATGTCAATTGTATTCTCCGCAGC	To clone full length CDS of *CaIMPL*1 in bacterial expression vector
CaSS8R	CTCGAGAACGTCTGCCCTGTAGTCCTC	
CaSS9F	GTGTACGCCAATCCTTGTGAAC	For Real time PCR in chickpea for *CaIMPL2*
CaSS10R	CGACTACTCACGATTCCGCATA	
CaSS11F	CTCGGATGGATGGAGGAAAA	For Real time PCR in chickpea for *CaIMPL1*
CaSS12R	GGAGGACGCCGTTTGAAA	
CaSS13F	AGCGTGTAGCTGCTTCAAACC	For Real time PCR in chickpea for *CaIMP*
CaSS14R	GTTTGGCGCAGAGCATCA	
CaSS15F	GCCCGCGACGTTGTGA	For Real time PCR for chickpea 18S (Endogenous control)
CaSS16R	CCTTGTTACGACTTCTCCTTCCTCTA	

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Primer details.

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Conflicts of Interest: "The author declares no conflict of interest." "The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

References

1. Boominathan, P.; Shukla, R.; Kumar, A.; Manna, D.; Negi, D.; Verma, P.K.; Chattopadhyay, D. Long Term Transcript Accumulation during the Development of Dehydration Adaptation in Cicer arietinum. *Plant Physiol.* **2004**, *135*, 1608–1620, doi:10.1104/pp.104.043141.
2. Genetic Resources, Chromosome Engineering, and Crop Improvement. *Genetic Resources, Chromosome Engineering, and Crop Improvement* 2006. https://doi.org/10.1201/9780203489260
3. 1. Saxena, S.C.; Salvi, P.; Kaur, H.; Verma, P.; Petla, B.P.; Rao, V.; Kamble, N.U.; Majee, M. Differentially expressed myo-inositol monophosphatase gene (CaIMP) in chickpea (Cicer arietinum L.) encodes a lithium-sensitive phosphatase enzyme with broad substrate specificity and improves seed germination and seedling growth under abiotic stresses. *J. Exp. Bot.* **2013**, *64*, 5623–5639, doi:10.1093/jxb/ert336.
4. Sambrook, J. and Russell, D.W. Expression of cloned genes in *Escherichia coli*. in *Molecular cloning: a laboratory manual*, 3rd ed.; Cold Spring Harbor Laboratory press: Cold Spring Harbor, NY, USA, 2001, vol 3, pp 15.23–15.24.
5. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685, doi:10.1038/227680a0
6. Majee, M.; Maitra, S.; Dastidar, K.G.; Pattnaik, S.; Chatterjee, A.; Hait, N.C.; Das, K.P.; Majumder, A.L. A Novel Salt-tolerant l-myo-Inositol-1-phosphate Synthase from Porteresia coarctata (Roxb.) Tateoka, a Halophytic Wild Rice. *J. Biol. Chem.* **2004**, *279*, 28539–28552, doi:10.1074/jbc.m310138200.