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plants MDPI

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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 (CaIMP) and IMP like proteins (CaIMPL1 and CaIMPL2) from chickpea (*Cicer arietinum*). Biochemical study revealed that *CaIMP* encodes a lithium-sensitive phosphatase enzyme with broad substrate specificity. 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 3-P and D Gal 1-P. Given the relation between IMP, IMPL1 and IMPL2, the difference in substrate specificity among these highly homologous enzymes suggests that the genes encoding these enzymes have diverged evolutionarily.

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

BACKGROUND

Journal of Experimental Botany, Vol. 64, No. 18, pp. 5623–5639, 2013
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This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
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RESEARCH PAPER

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

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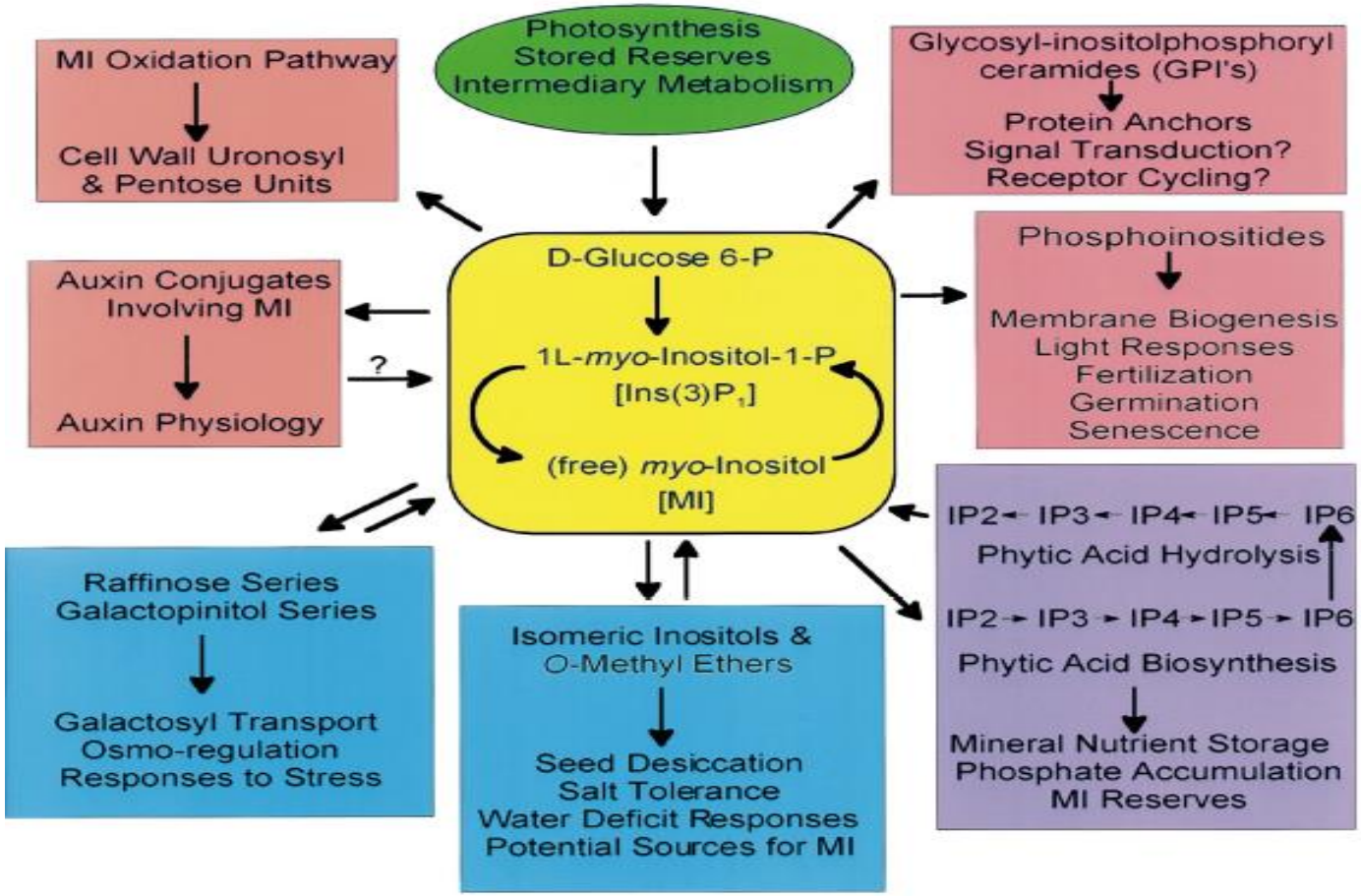
Inositol

- *Myo*-Inositol plays a central role in growth and development.
- This is especially true in plant biology where molecular entities containing or utilizing MI are involved in structure and function.



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Inositol monophosphatase like Proteins (IMPL)

- **To date, three putative IMP encoding sequences have been identified in the Arabidopsis genome; VTC4, *Myo*-inositol monophosphatase-like1 and 2 (IMPL1& IMPL2)**
- ***vtc4* null mutants showed only a 30% reduction in *myo*-inositol content, suggesting genetic redundancy in the capacity to generate *myo*-inositol (Torabinejad et al., 2009)**
- **IMPL1 and IMPL2 were shown to have *in-vitro* IMP activity (Torabinejad et al., 2009)**
- **It has been shown that IMPL2 protein (At4g39120) was also able to catalyze the dephosphorylation of histidinol-P to histidinol (Petersen et al., 2010)**
- **we need to explore the functions of IMPL genes in plant metabolism and its regulation under various abiotic stresses and growth & development**

Objectives

1. **Isolation and cloning of IMPL genes and their corresponding cDNA from chickpea**

2. Expression pattern of *CaIMPL* genes under different environmental stresses, hormones and in different tissue /organ

3. Bacterial over expression, purification and biochemical characterization of *CaIMPL1* and *CaIMPL2* gene(s) product and their comparison

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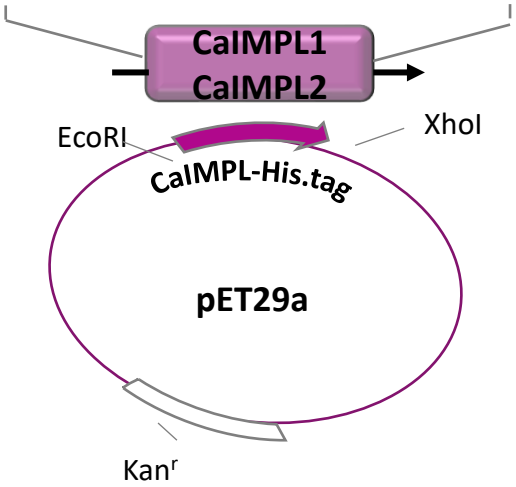
Methodology

Isolation and molecular cloning of *CaIMPL1* and *CaIMPL2* cDNA

- Isolation of Total RNA
- cDNA synthesis
- Amplification using gene specific primers
- Cloning into pJET blunt vector
- Insert confirmation using restriction digestion
- Sequencing

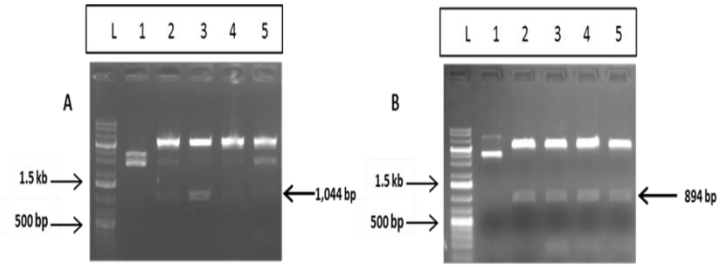
Methodology

Bacterial overexpression of *CaIMPL1* and *CaIMPL2*



CaIMPL1 **and** ***CaIMPL2*** **was sub-cloned into the** ***EcoRI/XhoI*** **sites of bacterial expression vector pET 29a (Novagen)**

Resulting plasmid was transformed into bacterial host strain ***E. coli*** **(BL21-DE3)**



Restriction digestion of pET29a:*CaIMPL1* [A] and pET29a:*CaIMPL2* [B]

Expression culture, Cells harvesting And Cell lysis

SDS-PAGE Gel Run
For checking the protein expression in the Soluble and pellet fractions

Protein Purification
using nickel charged affinity columns

Biochemical characterizations
pH optimun, temp. optimum, substrate Optimum, co-factor optimum etc.

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Methodology

Recombinant CaIMPL1 & CaIMPL2 Protein Purification

Solubilization

Pellet is solubilized in 8M Urea Buffer

⇩

Dialysis

(Protein dialyzed to remove Urea)

6M Urea Buffer

4M Urea Buffer

3M Urea Buffer

2M Urea Buffer

1M Urea Buffer

0M Urea Buffer

⇩

Column Purification

Dialyzed Sample loaded on to the Column Matrix (nickel charged affinity columns) and purified fractions collected

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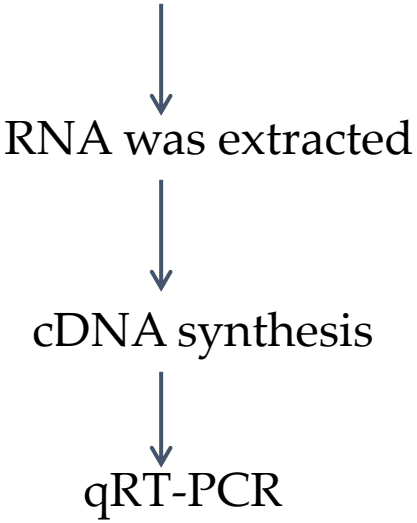
Methodology

Expression pattern of *CaIMPL* genes

Chickpea seedlings were subjected to various abiotic stress and hormone treatments



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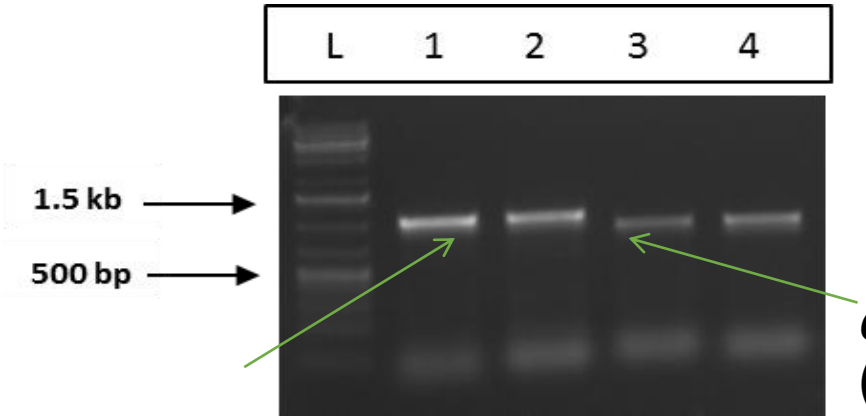
Results and Discussion

Amplification and Cloning of *CaIMPL1 and CaIMPL2*



RNA Isolation

↓

cDNA Preparation

↓

Amplification

Fig.1: PCR amplification of *CaIMPL1* & *CaIMPL2*

CaIMPL1 and *CaIMPL2* cDNAs were initially cloned in PCR cloning vector (pJET 1.2 blunt; Fermentas)

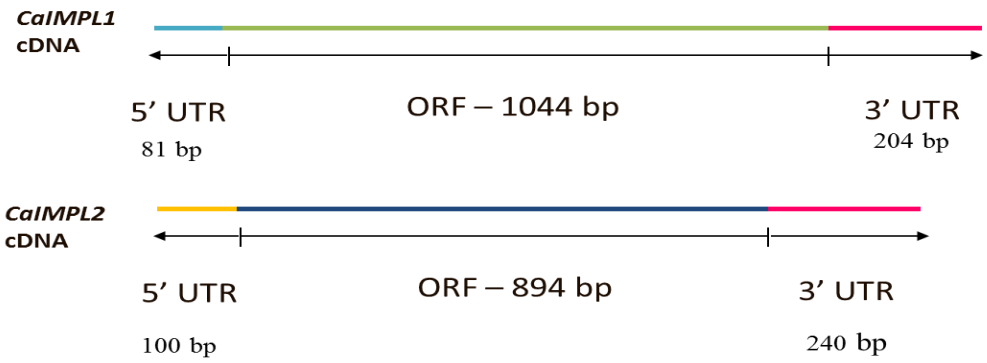


Fig. 2: Diagrammatic representation of *CaIMPL1* and *CaIMPL2* cDNA with UTR

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Results and Discussion

Sequence Analysis of CaIMPL1 and CaIMPL2

>CaIMPL1 (348 AA; 38.2 kD)

MSIVFSAASNLSWHKDCRQSSPPIGSWRLKSRIQSCKNSLQSDIYTQHRVGARSTGPIQPTHLIQVATTAAQTGAQV
VMDAVNKPRNITYKGLTDLVTETDKMSEAAILEVVKKNFDDHLILGEEGGIIGEAASDYLWCIDPLDGTTNFA
HGYPSFAVSVGVLYRGNPAAATVVEFVGGPMCWNTRIFTATAGGGAFCNGQRIHVSATNQVEQSLLVTGFGYEHDEA
WATNIELFKEFTDVSRGVRRLGAAAVDMCHVALGIVEAYWEYRLKPWDMAAGVLMVEEAGGTVSRMDGGKFCV
FDRSVLVSNGVLHTELLERIGPATEELKSKGIDFSLWYKPEDYRADV*

>CaIMPL2 (298 AA; 32.7 kD)

MLSQCHLHCYSNNLSIRSPKLRLRAMSSSSSPHQFNHFADVANKAADAAGDVIRKYFRKNFDIIHKHDLSPVTIADQ
TAEEAMVSIILDNFPSHAVYGEEKGWRCRQDSADYVWVLDPIDGTKSFITGKPLFGTLIALLQNGTPILGIIDQPVL
RERWIGMTGKRTTLNGQEVSTRTCADLSQAYLYTTSPHLFSGDAEEAFIRVRDKVKIPLYGCDCYAYALLSSGFVDL
VVESGLKPYDFLALVPVIEGSGGVITDWEGHQLRWEASPLSIAISFNVVAAGDKQIHQQALDSLQR*

Fig. 3: Polypeptide sequence of CaIMPL1 and CaIMPL2 showing characteristic motifs.

The polypeptide sequence of CaIMPL1 possesses three characteristic signature motifs (Motif 'A' – PIDGT, Motif 'B'- WDXAAG, and Motif 'C'-GEES) of the lithium-sensitive phosphatase super family enzymes including IMP, inositol polyphosphate 1-phosphatase, and fructose 1,6-bisphosphatase, though CaIMPL2 was lacking the 'Motif B', but possesses other two motifs viz. 'Motif A' and 'Motif C'.

Results and Discussion

Parameters	CaIMPL1	CaIMPL2
Genome sequence length	2791bp	2781bp
CDS length	1044bp	894
No. of exons	10	9
No. of introns	9	8
Chromosome location	2	3

Results and Discussion

Phylogenetic Tree

Fig. 4: Radial format phylogenetic tree describing the evolutionary relationship between prokaryotic IMPL, yeast IMPL, Human IMPL proteins and Fabaceae family IMPL1 and IMPL2 proteins.

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Results and Discussion

Cell lysis (DE3 cells overexpressing pET28a:CaIMPL1,L2) by sonication and analysis on SDS PAGE

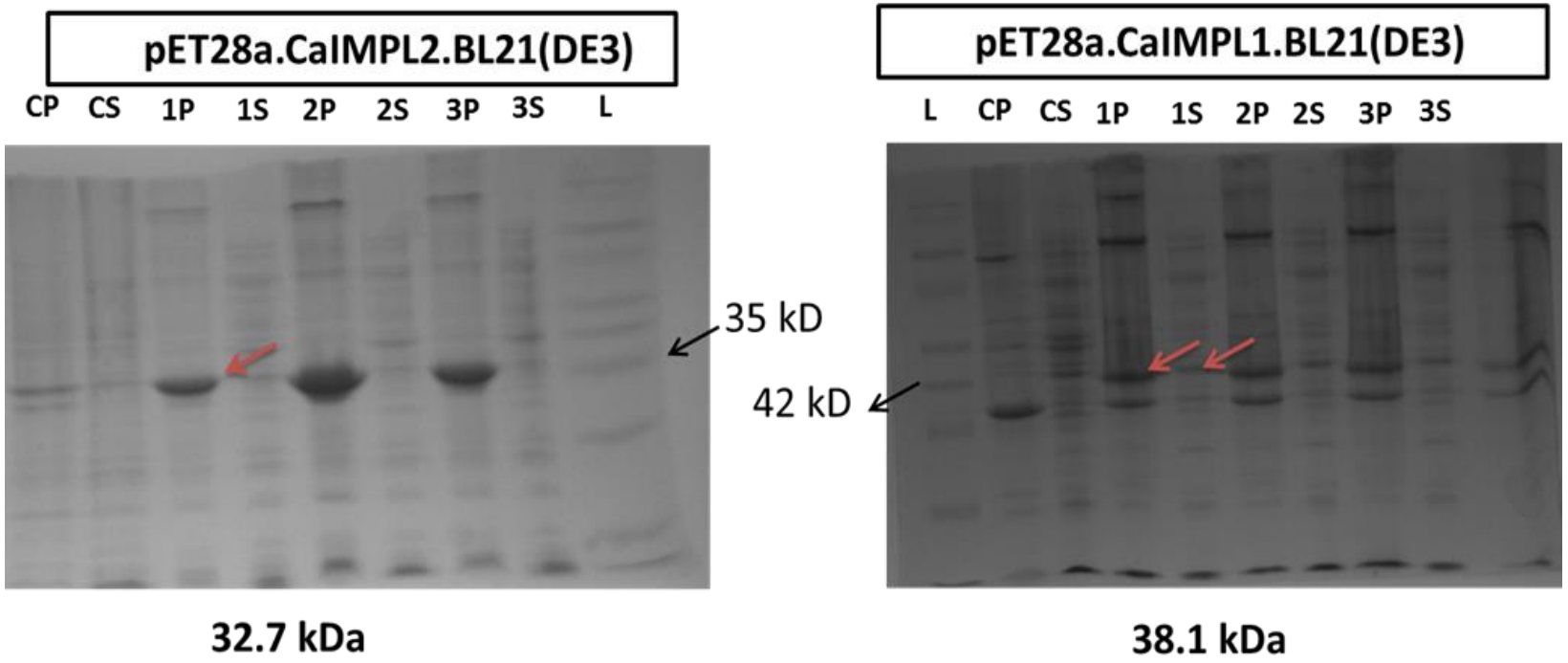

Fig.5: 12% SDS PAGE analysis of CaIMPL1 and CaIMPL2 over-expressed protein in *E. coli* BL21 (DE3). [Control, pET23d empty vector transformed induced cells; CaIMP, *CaIMP* transformed induced cells; M, molecular weight marker; P, pellet fractions; S, soluble fractions.]

Results and Discussion

CaIMPL1 and CaIMPL2 Protein Purification

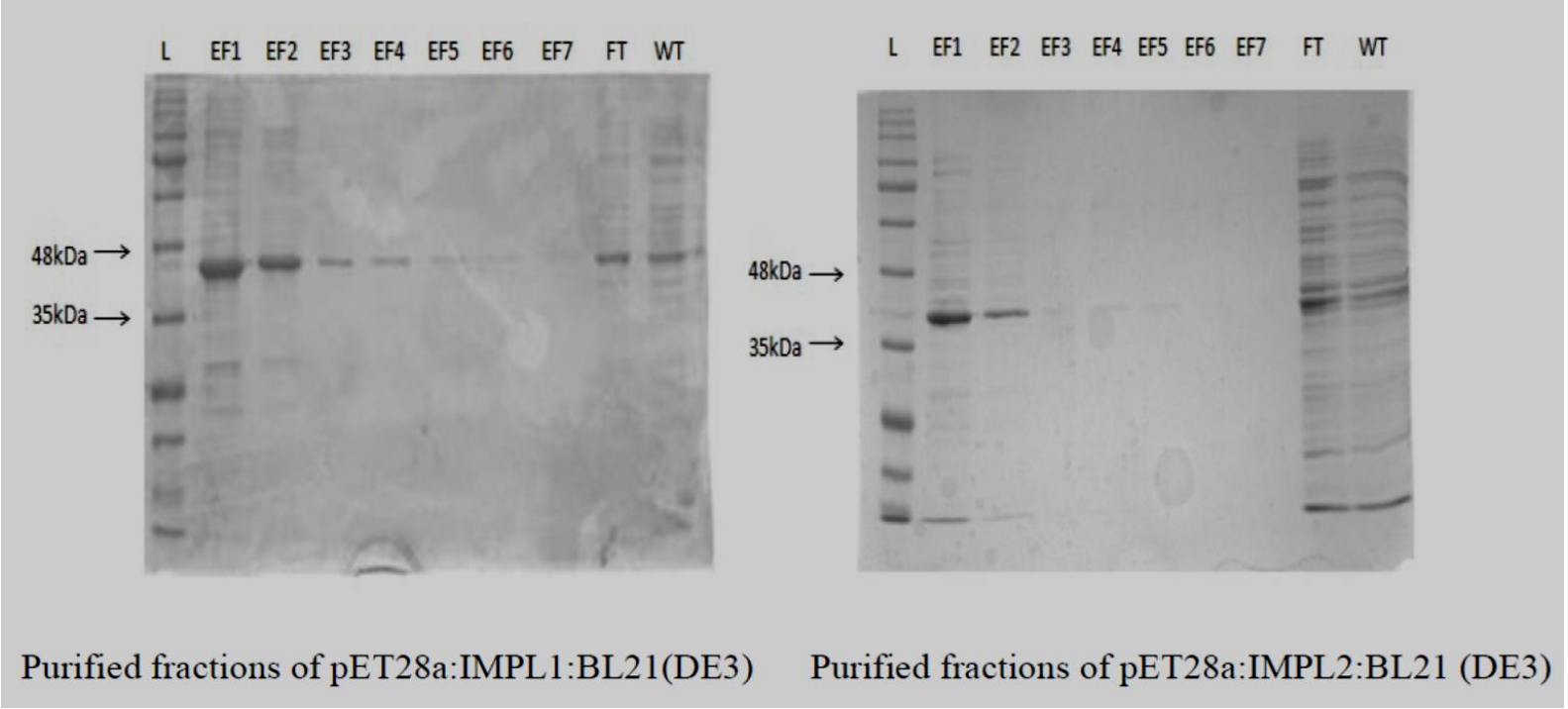

Fig. 6: Purified fractions of CaIMPL1 and CaIMPL2 proteins on 12% SDS PAGE

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Results and Discussion

Phosphatase Activity of purified CaIMPL1 & CaIMPL2 protein with different substrates

IMP activity was assayed by colorimetric estimation of released inorganic phosphate after enzymatic hydrolysis of L-myo inositol 1 phosphate with Malachite Green

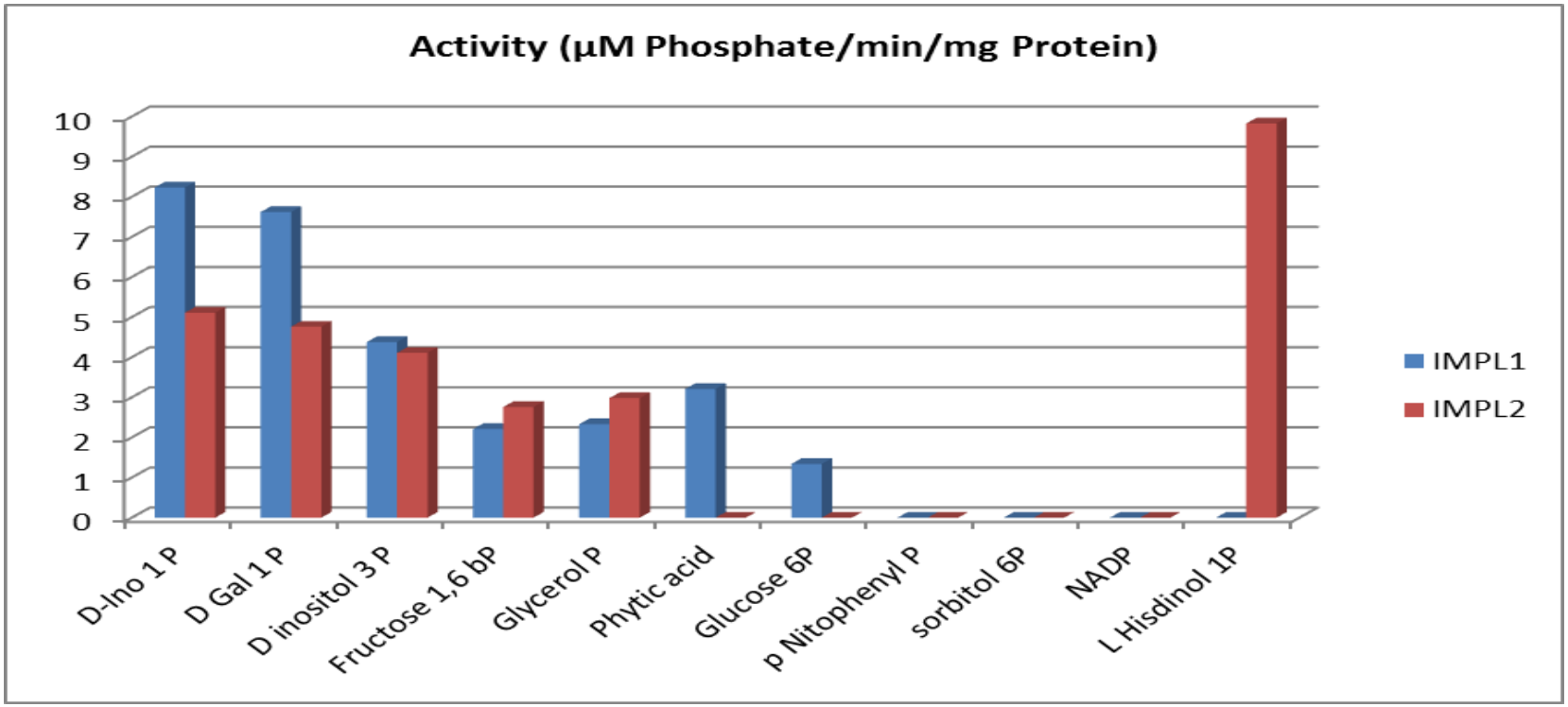

Fig. 7: Activity of CaIMPL1 and CaIMPL2 with different substrates

Results and Discussion

Biochemical characterization of recombinant CaIMP, CaIMPL1 and CaIMPL2

Table A. Kinetic Parameters of IMPL1 and IMPL2 Recombinant Proteins.

	Enzyme (Substrate)	K_m (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-Ins 3-P)	24	4.1	3	37°C	8
3	CaIMPL1 (D-Gal 1-P)	18	4.9	3	37°C	8
4	CaIMP (D-Ins 3-P)	25	4.4	3	37°C	8
5	CaIMP (D-Gal 1-P)	16	5.3	3	37°C	8

Results and Discussion

Quantitative RT PCR analysis of CaIMP, CaIMPL1 and CaIMPL2

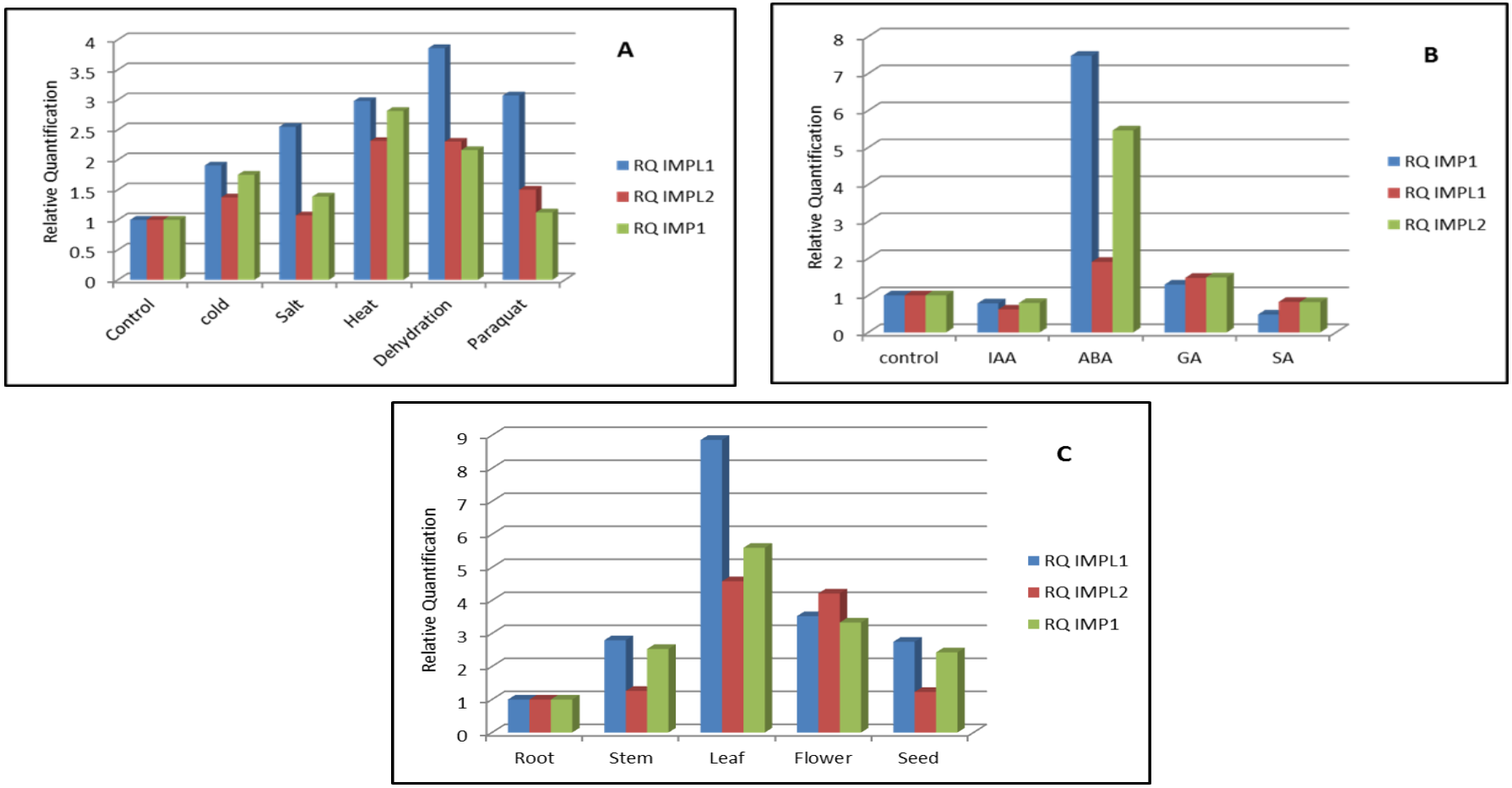

Fig. 8: Quantitative RT PCR analysis of *CaIMP*, *CaIMPL1*, and *CaIMPL2* (A) under different abiotic stresses, (B) in the presence of exogenous hormones, and (C) in different organs

Conclusions

- The research of present project emphases on the characterization of IMPL1 and IMPL2, and their functional roles in plant growth and development along with stress tolerance.
- Biochemical characterization of IMPL and kinetic comparisons of the *Cicer arietinum* recombinant IMPL1 and IMPL2 enzymes were done with various inositol phosphate substrates.
- Our data supports that IMPL2 gene encodes an active histidinol 1-phosphate phosphatase enzyme in contrast to the IMPL1 enzyme which has the ability to hydrolyze D-Ins 3-P substrate and both CaIMPL1 & CaIMPL2 like CaIMP showed broad substate specificity.
- IMPL1 and IMPL2 genes whose function is not completely yet known were found to be expressed under various abiotic stress conditions.
- Few stress responsive domains were found in amino acid sequence of CaIMPL1 and CaIMPL2

Acknowledgments

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(Grant no: YSS/2014/001012/LS).