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In silico analysis of the germinlike protein multigene family members of tomato with predicted oxalate oxidase activity

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Abstract

Germins and germin like proteins (GLPs) have been reported as plant glycoproteins belonging to the 'cupin' superfamily. They have been documented to possess enzymatic activities leading to the generation of H_2O_2, a reactive oxygen species (ROS). Hence, members of the GLP family have been proposed to play major role in plant disease resistance through ROS-mediated signaling. Interestingly, the functional characterization of GLP(s) in terms of their suitability as a key player in plant disease resistance has remained under-explored in case of tomato (Solanumlycopersicum L.). In the present study, 15 tomato GLPs, predicted to have oxalate oxidase activity, have been investigated in silico. Deduced multiple amino acid sequence alignment-based clustering of these proteins was carried out to classify them into 3 sub-families. All the proteins were found to contain the conserved amino acid stretches, representing the BoxA, B and C, and an inter-motif region of variable length. It was observed through homology modeling and structural alignment that the active sites of all except 3 of these proteins have Mn^{2+} bound at the active site involving the three conserved histidine and one conserved glutamate residue(s). The active site architecture was analyzed with a comparative view in order to examine the metal binding capacity of tomato GLPs. Thus the present work makes a platform for further genetic, molecular biological and functional genomics studies in the field of tomato GLPs, the possible key players for conferring biotic and/or abiotic stress tolerance, in future.

Highlights

- In tomato, 15 genes encoding germin like proteins (GLPs) with predicted oxalate oxidase activity were found to be distributed in 5 different chromosomes.
- The 15 tomato GLPs were found to have structural distinctness with a basic conserved 'jelly roll' structure.
- Three out of the 15 proteins were predicted not to bind Mn^{2+}.
- The distinct active site architecture of the tomato GLPs might explain the differential Mn^{2+} binding.

Keywords: Active site architecture, biotic stress, homology modeling, Mn^{2+} binding, point mutation.

Germin and germin like proteins (GLPs) constitute a large family of proteins, reported to be expressed at a higher rate during germination and pathogen attack (Zhang *et al.*, 1995; Berna and Bernier, 1999).The GLPs have been documented to be structurally related but functionally diverse members of the 'cupin' superfamily, capable of playing a wide variety of roles as enzymes, structural proteins, or receptors (Bernier and Berna, 2001). These glycoproteinshave been documented to have significant tolerance to heat, detergent treatment and degradation by proteases. Though first reported in germinating wheat seedlings (Thompson and Lane, 1980; Grzelczak and Lane, 1984), due to their inherent enzymatic

properties, this class of proteins has been considered quite crucial for plants' resistance to several biotic stresses. Several GLPs have been demonstrated to have oxalate oxidase (OXO) (Dumas *et al.,* 1993; Lane *et al.,* 1993) or superoxide dismutase (SOD) (Yamahara *et al.,* 1999; Carter and Thornburg 2000; Tabuchi *et al.,* 2003; Christensen *et al.,* 2004) activities. The OXO and/or SOD activity-generated H_2O_2 has been reported to be involved in disease resistance of plants (Lamb and Dixon, 1997) through reaching a toxic level that can hinder the growth of the microbes (Peng and Ku´c, 1992). At the same time, the elevated level of H_2O_2, a reactive oxygen species (ROS), has been documented to cause structural modification of the plant cell wall (Bolwell *et al.,* 1995), induce lipid peroxide, salicylic acid, ethylene synthesis and finally to activate the programmed cell death (PCD) in plant cells (van Breusegem *et al.,* 2001). Furthermore, $H_2O_2$is supposed to play important role in signal transduction leading to induction of pathogenesis-related proteins, polyphenol oxidase and hypersensitive response.

The GLPs have been well studied and characterized in different plant species. Thirty two *GLP* genes have been identified from *Arabidopsis* (Carter *et al.,* 1998; Carter and Thornburg 1999), 14 have been documented from *Hordeumvulgare* (Wu *et al.,* 2000; Druka *et al.,* 2002; Zimmermann *et al.,* 2006), and around 8 different *GLP* genes have been reported to be expressed in rice (Membré and Bernier, 1998). The GLPs have also been studied in grape vine (Godfrey *et al.,* 2007), pea (Gucciardo *et al.,* 2007), wheat (Segarra *et al.,* 2003) and many other plant systems. Several germins and GLPs have been described at functional level through transgenic research. Transgenic soybean (Donaldson *et al.,* 2001) poplar (Liang *et al.,* 2001) and sunflower (Hu *et al.,* 2003) plants expressing the wheat germin have been reported to have enhanced tolerance to pathogen attack. Similarly, transgenic maize plants over-expressing wheat germinhave been documented to exhibit enhanced resistance to corn borer (Ramputh *et al.,* 2002). Recently, it has been reported that silencing the germin like protein in *Nicotianaattenuata* results in improvement of the performance of herbivores (Lou and Baldwin, 2006). In case of rice, the GLP multigene family has been characterized to act as a complex quantitative trait loci (QTL) for disease resistance (Manosalva *et al.,* 2009). Among the rice GLPs, *OsGLP1* has been documented to play a role in determining plant architecture (plant height) as well as disease resistance (Banerjee and Maiti, 2010). The *OsGLP1* has been found to possess superoxide dismutase (SOD) activity and to be involved in cell wall reinforcement in transgenic tobacco plants expressing the same (Banerjee *et al.,* 2010). In case of *Brassica*, the GLP family members has been reported to be involved in initiating oxidative burst to impede pathogenesis of the pathogen *Sclerotiniasclerotiorum* (Rietz *et al.,* 2012). All these observations make GLPs an interesting class of protein to work on.

Among the major vegetable crops, tomato (*Solanumlycopersicum* L.) is one with a global importance. Recent decoding of the genome sequence in tomato has broadened the scope of plant molecular biological research in this important crop plant. Taking the help of the decoded genome, the present study documents the in silico characterization of a multigene family of tomato encoding GLPs with predicted oxalate oxidase activity. Through homology-based structural analysis, the metal binding active sites of these proteins are analyzed to explain the predicted differential metal binding capacity. Along with that, point mutations leading to the same are identified. As a detailed bioinformatics analysis is rendered as a pre-requisite for any molecular biological research, this attempt might pave the way for further research on tomato GLPs in near future.

Materials and Methods

Retrieving the sequence information of the 15 tomato GLPs with predicted oxalate oxidase activity was doneusing the SolGenomics database (http://solgenomics.net/). Annotation of them wasdone as SlGLPA…SlGLPO in this study. The accession numbers of them are SlGLPA (Solyc01g088280.1), SlGLPB (Solyc01g088290.1),

SlGLPC (Solyc01g088300.2), SlGLPD (Solyc 01g102890.2), SlGLPE (Solyc01g102900.2), SlGLPF (Solyc01g102910.2), SlGLPG (Solyc03g123410.1), SlGLPH (Solyc07g041720.1), SlGLPI (Solyc07g 065330.2), SlGLPJ (Solyc09g089990.2), SlGLPK (Solyc09g090000.1), SlGLPL (Solyc09g090010.2), SlGLPM (Solyc09g090020.2), SlGLPN (Solyc09g090040.2) and SlGLPO (Solyc11g068600.1). The GenBank accession numbers of the barley (*Hordeumvulgare*) GLPs, used during cluster analysis are HvGER1a (ABG46232), HvGER2a (ABG46233), HvGER3a (ABG46234), HvGER4c (ABG46235), HvGER5a (ABG46237) and HvGER6a (ABG46238). Multiple amino acid sequence alignment and analysis were done through the Clustal Omega web server (https://www.ebi.ac.uk/Tools/msa/clustalo/) and pictorial representation was done using the EsPript web-server (http://espript.ibcp.fr).Analysis of different parameters associated with the protein sequences were carried out using the ExPasy web-resources (www.expasy.org). Performance of homology-based structural modeling was done in the SWISS MODEL web-server (http://swissmodel.expasy.org/) and pictorial representation was done through PyMol (www.pymol.org).

Table 1. Properties of the 15 tomato *GLP* genesand their deduced amino acid sequences

Name	Located on (chromosome number)	Gene length (bp)	No. of introns	CDS* length (bp)	Predicted molecular weight of polypeptide (kDa)	Predicted theoretical pI of polypeptide	Predicted signal peptide cleavage site (between amino acid residue numbers)
SlGLPA	1	688	1	639	23.95	9.21	-
SlGLPB	1	546	1	405	14.86	8.49	-
SlGLPC	1	789	1	678	24.55	7.79	23-24
SlGLPD	1	774	1	687	24.52	5.61	22-23
SlGLPE	1	770	1	687	24.39	7.80	22-23
SlGLPF	1	793	1	687	24.63	6.16	22-23
SlGLPG	3	627	0	627	21.57	5.83	**17**-18
SlGLPH	7	636	0	636	22.03	6.25	19-20
SlGLPI	7	919	1	660	24.09	8.64	20-21
SlGLPJ	9	1,323	1	675	24.06	5.21	22-23
SlGLPK	9	2,174	2	654	24.30	7.76	21-22
SlGLPL	9	1,445	1	675	24.83	7.75	21-22
SlGLPM	9	1,189	2	846	30.85	8.62	21-22
SlGLPN	9	1,341	1	687	24.93	6.12	21-22
SlGLPO	11	621	0	621	21.91	5.51	**26**-27

* Coding DNA Sequence
Smallest/lowest and largest/highest values are indicated in bold

Table 2. Analysis of the homology models of 15 GLPs from tomato

Name	Stretch of amino acid residues modeled	Template PDB ID	Sequence identity with template (%)	R.M.S.D C^{α} with template*	Ligand present in the model
SlGLPA	39 to 212	1fi2A	31	0.502	-
SlGLPB	2 to 132	1fi2A	33.59	0.098	-
SlGLPC	24 to 225	1fi2A	40.89	0.095	Mn^{2+}
SlGLPD	23 to 227	1fi2A	48.30	0.077	Mn^{2+}
SlGLPE	23 to 227	1fi2A	48.30	0.072	Mn^{2+}
SlGLPF	23 to 227	1fi2A	48.06	0.082	Mn^{2+}
SlGLPG	14 to 208	1fi2A	34.33	0.080	Mn^{2+}
SlGLPH	16 to 211	1fi2A	34.33	0.097	Mn^{2+}
SlGLPI	21 to 214	1fi2A	30.96	1.017	Mn^{2+}
SlGLPJ	23 to 224	1fi2A	43.07	0.085	Mn^{2+}
SlGLPK	22 to 206	1fi2A	38.31	0.356	Mn^{2+}
SlGLPL	75 to 222	3qacA	21.43	4.594	-
SlGLPM	22 to 215	1fi2A	44.62	0.092	Mn^{2+}
SlGLPN	22 to 228	1fi2A	42.03	0.090	Mn^{2+}
SlGLPO	27 to 206	1fi2A	28.14	0.204	Mn^{2+}

* Root mean square deviation of the α carbon backbone
Smallest and highest values are indicated in bold

Results and Discussion

Genomic organization and sequence analysis of the GLP gene family members in tomato

In the present study, 15 tomato GLPs with predicted oxalate oxidase activity were identified using the tomato genome sequence available in the SolGenomics database. Analysis of the corresponding genes was carried out to find out the genomic organization of these 15 genes in tomato genome. It was found that these15 genes are located on 5 different chromosomes in tomato. Among the 5 chromosomes, chromosome 1 contains 6 GLP genes (annotated in this study as SlGLPA, SlGLPB, SlGLPC, SlGLPD, SlGLPE and SlGLPF) in a~8.5 Mb spanning region. All the genes, except SlGLPF were found to be located in reverse orientation. Chromosome 3 was found to contain 1 GLP gene (SlGLPG) in reverse orientation and chromosome 7 was found to contain 2 GLP genes (SlGLPH and SlGLPI) in forward and reverse orientation, respectively. On the other hand, 5 GLP genes (SlGLPJ, SlGLPK, SlGLPL, SlGLPM and SlGLPN) were observed to be very closely located on chromosome 9, all in reverse orientation, in a ~17.2 kb spanning region. The SlGLPO gene was found to be present in forward orientation on chromosome 11. The schematic image of the genomic organization of all these 15 genes is presented in Figure 1A.

Among the 15 genes, SlGLPK was found to be the longest (2,174 bp), whereas SlGLPB was found to be smallest (546 bp) (Table 1). Except SlGLPG, SlGLPH and SlGLPO, all the genes were found to contain introns. TheSlGLPK and SlGLPM genes contained 2 introns each, whereas the rest 10 genes were observed to possess single intron (Figure 1A). Among the introns, the 2nd intron present in the SlGLPK gene was found to be the largest (1,269 nt, data not shown). The Coding DNA Sequence (CDS) was longest in case of the SlGLPM gene (846 bp)and smallest (405 bp) in case of the SlGLPB gene (Table

1).

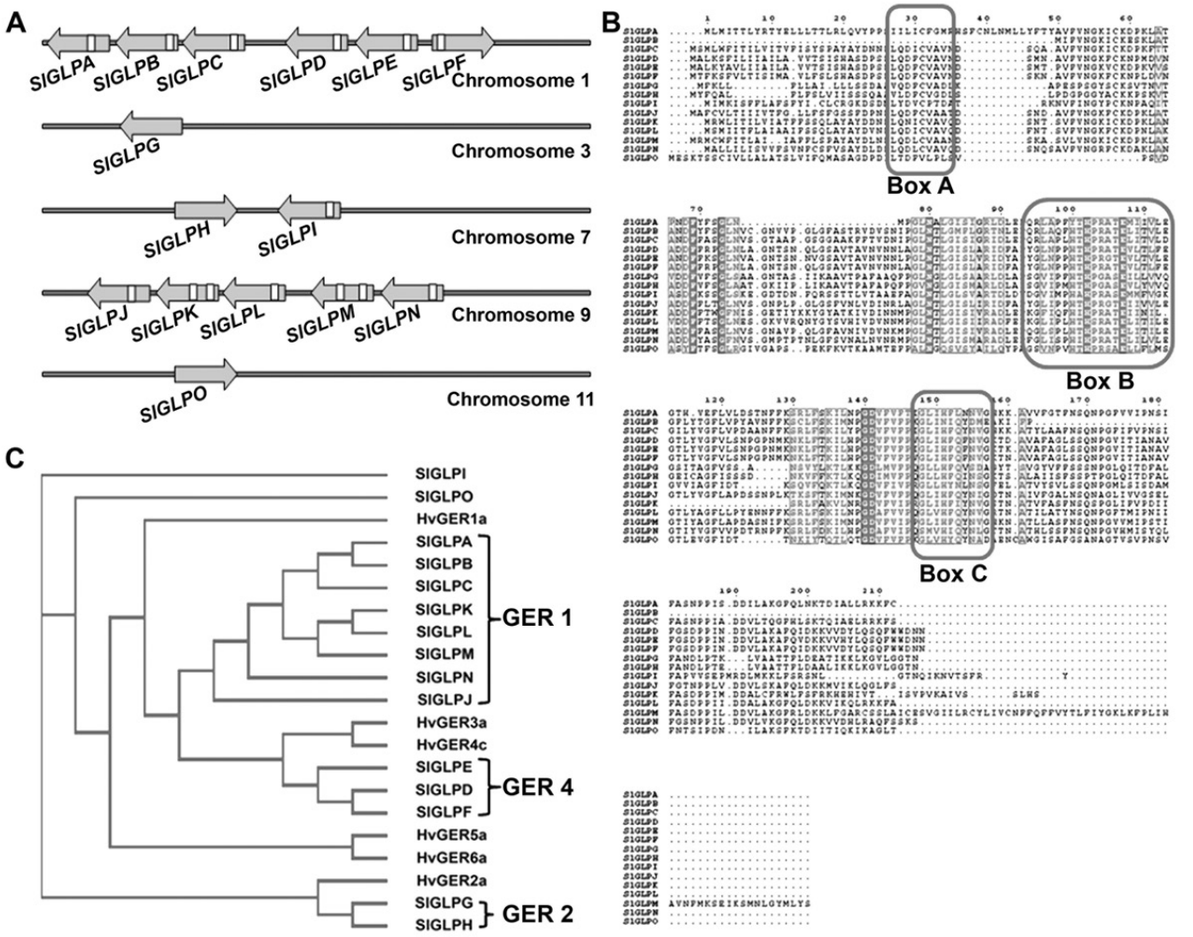

Figure 1:A.Genomic organization of the 15 tomato genes encoding GLPs with predicted oxalate oxidase activity. Presence of introns are indicated by small white boxes. B. Multiple sequence alignment of the 15 tomato GLPs. Amino acid residues in the 3 conserved regions (Box A, Box B and Box C) are shown inside boxes. C. Clustering of tomato GLPs along with GLPs from barley in order to identify the members belonging to the GER 1, GER 2 and GER 4 sub-family.

Amino acid sequence-based analysis of tomato GLPs

The predicted molecular weight of the 15 GLPs of tomato ranged from 14.86 kDa (SlGLPB) to 30.85 kDa (SlGLPM) (Table 1). The predicted theoretical pI of the proteins ranged from 5.21 (SlGLPJ) to 9.21 (SlGLPA). Except SlGLPA and SlGLPB, signal peptides were predicted for all the proteins, where longest (26 amino acids) signal peptide was predicted in case of SlGLPO and smallest (17 amino acids) signal peptide was predicted for SlGLPG.

Amino acid sequences of these 15 GLPs were subjected to multiple sequence alignment. It was found that, in spite of having N-terminal and C-terminal unique sequences, all these proteins contained the conserved amino acid residues designated as the Box B and Box C, characteristic to plant GLPs (Figure 1B). However, the Box A was found to have a less extent of sequence

conservation in all the tomato GLPs. Apart from SlGLPK, variation in the length of the inter-motif hinge region between the two boxes (Box B and Box C)in all the tomato GLPs were found to be not more than 6 amino acids. However, the hinge region of SlGLPK was found to be significantly smaller and almost absent (Figure 1B).

In order to classify the tomato GLPs, sequence comparison was done with GLPs from barley. Six GLPs from HordeumvulgareL. (HvGER1a, HvGER2a, HvGER3a, HvGER4c, HvGER5a and HvGER6a), belonging to separate classes (GER1, GER2, GER3, GER4, GER5 and GER6) were incorporated during cluster analysis.Among the 15 tomato GLPs, SlGLPG and SlGLPH were found to share maximum amount of sequence similarity with HvGER2a and hence they were classified as GER2 sub-family members (Figure 1C). On the other hand, the SlGLPD, SlGLPE and SlGLPF proteins shared significant sequence similarity with HvGER4c and were classified in the GER4 sub-family.Among rest of the tomato GLPs, SlGLPA, SlGLPB, SlGLPC, SlGLPJ, SlGLPK, SlGLPL, SlGLPM and SlGLPN belonged to a cluster that was found to have sequence similarity with HvGER1a. Hence, they were classified in the GER1 sub-family. The SlGLPI and SlGLPO proteins failed to show significant sequence similarity with the reported Hordeum GLPs and hence remained unclassified in this study.

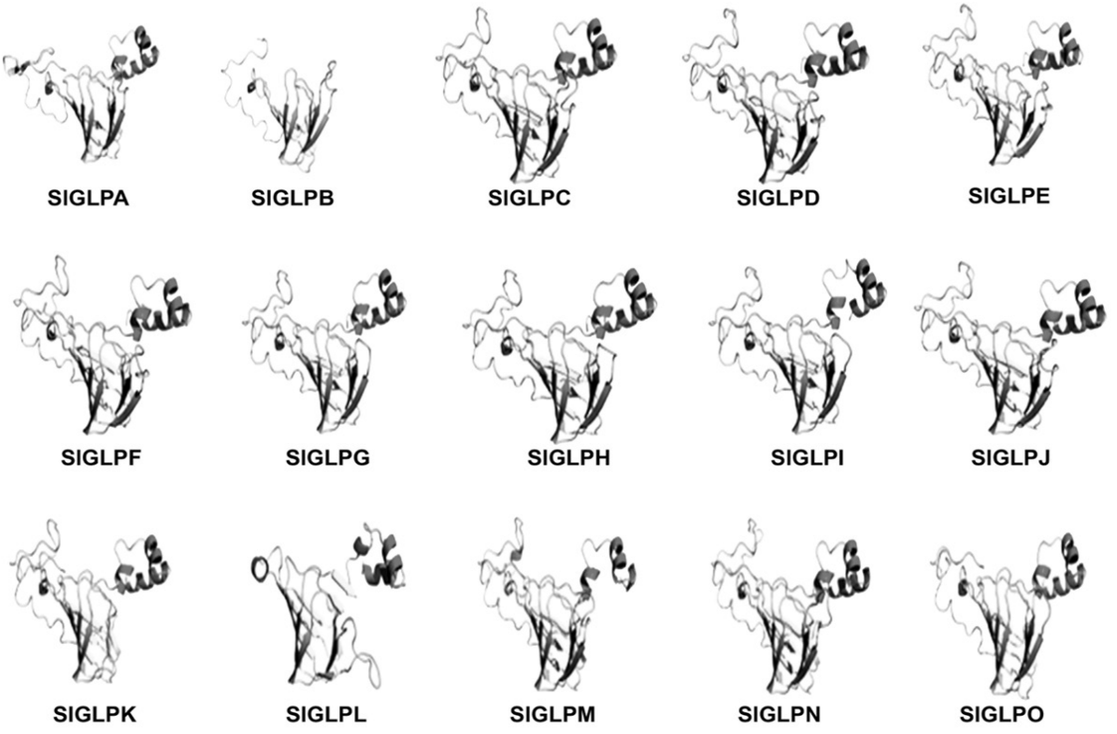

Figure 2:Comparative view of the structures of the 15 tomato GLPs with predicted oxalate oxidase activity. Structural distinctness along with the basic conserved 'jelly roll' structure, characteristic feature of plant GLPs, should be noted.

Structural modeling and analysis of tomato GLPs

Homology-based structural models of all these 15 tomato GLPs were prepared and analyzed in this study. Except SlGLPL, all the other GLP models were prepared using the solved crystal structure ofHordeumvulgare oxalate oxidase protein (PDB ID: 1fi2A). For the SlGLPL model, solved crystal structure of Amaranthus 11s proglobulin seed storage protein (PDB ID: 3qacA), which shared 21.43% sequence identity with SlGLPL (Table 2) was used as template.All the other GLPs shared 21.48% (SlGLPO) to 48.30% (SlGLPD and SlGLPE) sequence identity with the Hordeumvulgareoxalate oxidase protein.Length of the stretch of amino acid residues modeled was found to be highest in case of SlGLPN (207 amino acids) and lowest in case of SlGLPB (131 amino acids) (Table 2). All the tomato GLPs were found to have distinguishable structural models, having similarity with the characteristic β 'jelly roll' structure of plant GLPs (Figure 2). The root mean square deviations of the α-carbon backbone (R.M.S.D Cα, indictor of the structural similarity between the model and its template) of the tomato GLP structural models with their corresponding template were analyzed through structural superimposition. The R.M.S.D Cα value was found to be lowest (0.072) in case of SlGLPE and highest (4.594) in case of SlGLPL (Table 2). Interestingly, all the tomato GLP homology models, except SlGLPA, SlGLPB and SlGLPL, were observed to contain Mn2+ as ligand (Table 2).

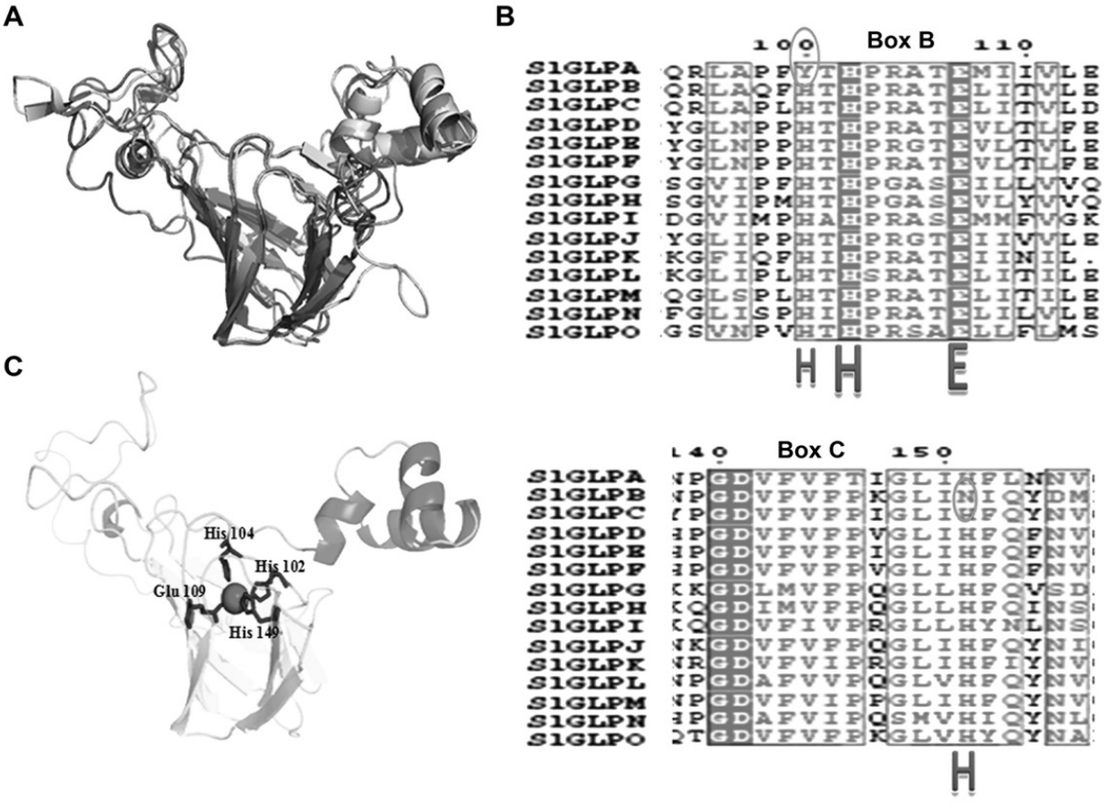

Fig.3:A.Structural super-imposition of the 15 tomato GLPs to reveal the conserved β barrel. B. Conserved His and Glu residue(s) in the Box B and Box C of tomato GLPs, documented to be involved in Mn^{2+} binding. Change in these amino acids, as observed in case of SlGLPA and SlGLPB are shown inside small circles. C. View of the active site architecture, constituted by the conserved His and Glu residues' side-chains, in Mn^{2+} binding tomato GLPs. Position of Mn^{2+} is indicated as sphere.

Analysis of the Mn^{2+} binding active sites of tomato GLPs

Structural superimposition of all the tomato GLP models revealed the presence of the conserved β barrel in these proteins (Figure 3A). From the comparison of amino acid residues present in Box B and Box C, it was found that the predicted amino acid residues (2 His and 1Glu residue in Box B; 1 His residue in Box C) involved in metal ion binding to be highly conserved among the tomato GLPs (Figure 3B).However, in case of SlGLPA, the first conserved His residue of Box B was found to be replaced by Tyr (Figure 3B, upper part) and in case of SlGLPB, the conserved His residue of Box C was observed to be replaced by Asn (Figure 3B, lower part). Orientation of the side-chains of the 3 His and 1Glu residues in the conserved β barrel portion of the Mn2+ binding SlGLPC protein was found to form the active site pocket suitable for Mn2+ binding (Figure 3C). But, the amino acid replacements in case of SlGLPA and SlGLPB were found to alter the architecture of the active site for Mn2+ binding. Comparative study of the active site architecture, in reference to the Mn2+ binding SlGLPC protein revealed that in case of SlGLPA, projection of the Tyr side-chain is supposed to reduce the space required for binding Mn2+ (Figure 4A). On the other hand, projection of the Asn side-chain in the active site of SlGLPB is supposed to reduce the required compactness of the active site for Mn2+ binding (Figure 4A).

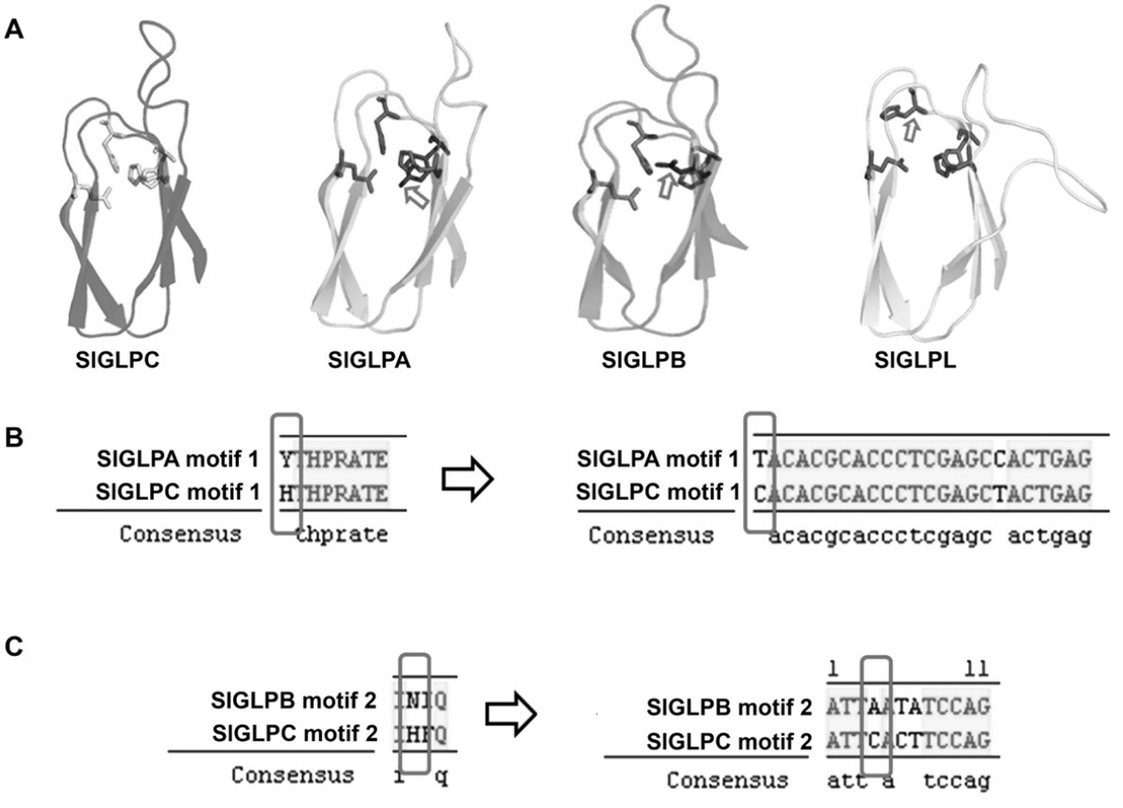

Figure 4:A.Comparative view of the active site architecture in Mn2+ binding (SlGLPC) and not binding (SlGLPA, SlGLPB and SlGLPL) tomato GLPs. Change in architecture due to the changed or differentially projected amino acid side chain is indicated by arrow marks. B. Identification of the corresponding point mutation (C to T) in SlGLPA (right), leading to the change in amino acid residue (H to Y, left) in the active site, shown inside boxes. C. Identification of the corresponding point mutation (C to A) in SlGLPB (right), leading to the change in amino acid residue (H to N, left) in the active site, shown inside boxes.

Interestingly, SlGLPL was found to contain all the conserved amino acid residues required for Mn^{2+} binding; but the side chain projection of the conserved second His residue of Box B of SlGLPLwas found to be highly altered in comparison to that of the Mn^{2+} binding SlGLPC protein (Figure 4A).However, keeping the limitations of homology-based structural modeling in mind, Mn^{2+} binding potential of SlGLPL might be examined in wet-lab condition in order to make confirm comment in this regard.

Comparison of the CDS region of SlGLPA and SLGLPB with that of SlGLPC revealed the point mutations resulting in the replacement of the aforementioned conserved amino acid residues. In case of SlGLPA, one C to T point mutation (transition) resulted in His to Tyr replacement in the Box B (Figure 4B). On the other hand, one C to A point mutation (transversion) caused the His to Asn replacement in the Box C of SlGLPB (Figure 4C). These point mutations might further be explored to design specific primers for checking the expression profile of Mn2+ binding and non-binding GLPs of tomato under different physiological conditions.

Conclusion

In the present study, the *in silico* analysis of 15 tomato GLPs with predicted oxalate oxidase activity has been documented. Through homology-based structural modeling and analyses of the tomato GLPs, their structural conservation has been depicted. Out of the 15 proteins, 3 were predicted not to bind metal ion (Mn^{2+}) and might have some other distinct physiological role. Through analysis of the active site architecture, attempt has been made to explain the differential binding of Mn^{2+} by these proteins. At the same time, the point mutations leading to the alteration in the metal binding active site of 2 (SlGLPA and SlGLPB) of the 3 proteins predicted not to bind Mn^{2+} has been identified. Hence, this work might serve as the platform for further molecular biological works targeting the functional characterization of the tomato GLPs.

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