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Mutational analysis of the pumpkin (*Cucurbita maxima*) phloem exudate lectin, PP2 reveals Ser-104 is crucial for carbohydrate binding

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ABSTRACT

The pumpkin phloem lectin (PP2) is an RNA-binding, defense-related, chitooligosaccharide-specific, homodimeric lectin of M_r 48 kDa expressed at high concentrations in the sieve elements and companion cells of pumpkin (*Cucurbita maxima*). In the present study, PP2 was expressed in the methylotrophic yeast *Pichia pastoris* with the *Saccharomyces* α-factor sequence to direct the recombinant protein into the secretory pathway as a prerequisite for unimpaired folding and posttranslational glycosylation of recombinant PP2. Previous computational modeling and ligand docking studies predicted a putative chitooligosaccharide-binding site on the PP2 surface, which was divided into three subsites, with two amino acid residues in each subsite identified as possible candidates for interaction with chitooligosaccharides (CHOs). In this work, mutational analysis and hemagglutination assays were employed to verify the role of the predicted residues in the carbohydrate binding activity of the protein. The results obtained revealed that mutation of Ser-104 to Ala (S104A) at subsite-2 resulted in about 90% loss of agglutination activity of the protein, indicating that Ser-104 is crucial for the binding of CHOs to PP2. Also, L100A (at subsite-1) and K200A (at subsite-3) independently decreased the lectin activity by about 40%, indicating that these two residues also contribute significantly to sugar binding by PP2. Together, these findings confirm that all the three subsites contribute to varying degrees toward PP2-carbohydrate interaction, and confirm the validity of the computational model, as proposed earlier.

1. Introduction

In many plants, including those of the Cucurbitaceae family, phloem fluid is responsible for the transport of a variety of substances including proteins, phytohormones, mRNA, small RNAs and viral nucleic acids in the sieve tube [1]. Previous studies have shown that phloem proteins identified in the angiosperm sieve tube system also play an important role in long distance signaling in plants that is associated with the development and function of the vascular system [2,3]. The recent release of the complete cucumber genome sequence [4] has greatly facilitated our ability to investigate the unusual properties of the Cucurbitaceae phloem. A close observation of the transcriptome, proteome, and metabolome data on cucurbit exudate by fluorescence microscopy suggested the existence of two functionally independent phloem transport systems, namely fascicular phloem (FP) and extrafascicular phloem (EFP). Among these two, the EFP system is of greater interest, as most of the above-mentioned functions are associated with proteins present in it [5,6].

Chitin binding lectins have been identified in the vascular exudates of plants including those from many genera of the Cucurbitaceae. One of the most extensively studied structural proteins from the EFP of vascular exudates is PP2 (phloem protein 2), which can be isolated from pumpkin phloem along with the structural filamentous protein PP1 (phloem protein 1) [7]. Although it is known that phloem filament formation is due to PP1 and PP2, a molecular level understanding of their interaction is still elusive. Multigene PP2 proteins are widely distributed in the plant kingdom, indicating that they are ancient in angiosperms. Moreover, nearly 30 predicted proteins of *Arabidopsis* share up to 41% homology with PP2 indicating that PP2-like proteins fulfill important related functions [8].

Phloem specific expression was demonstrated for seven PP2 genes; these are CbmPP2, CmsLec17, CmmLec17, CmmLec26, AgPP2-1, AgPP2-A1 and AtPP2-A2. Chitin binding lectin activity has been demonstrated for all these proteins [8,9]. Chitin binding plant lectins strongly recognize carbohydrate structures present

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on fungal cell walls and the exoskeleton of invertebrates. Similarly, lectins from elderberry (*Sambucus* sp.) [10] and *Mackia amurensis* [11] bind to sialic acid, which is the major constituent of animal glycoproteins, although plants do not contain this sugar.

Cucurbitaceae phloem lectins are a very small family of proteins that bind to chitooligosaccharides (CHOs) and also recognize viral RNA [12]. The pumpkin phloem lectin, abbreviated as PP2, is a RNA binding, defense related, chitooligosaccharide-specific lectin expressed both in sieve elements and companion cells of pumpkin (*Cucurbita maxima*) at high concentration. PP2 is a 48 kDa homodimeric cysteine-rich lectin which does not contain a hevein domain and has one possible *N*-glycosylation site [7,13]. While its primary structure determined from the cDNA sequence has been known for a long time [13,14], due to the lack of three dimensional crystal structures of any of the PP2 family proteins, the carbohydrate-binding site of this lectin is not well characterized. In view of this lacuna, in a previous study we developed a 3-dimensional structural model of PP2 (also known as PPL) by computational methods and predicted the residues that are likely to be involved in the binding of chitooligosaccharides by this protein using molecular docking and molecular dynamics approaches [15].

In the present study we have expressed PP2 protein from pumpkin and its mutants in *Pichia pastoris* with the *saccharomyces* α-factor sequence to direct the recombinant protein into the secretory pathway. Subsequently, the recombinant proteins were characterized and their lectin activity analyzed by a hemagglutination assay. The results obtained revealed that S-104, L-100 and K-200 are important for carbohydrate binding to PP2. To the best of our knowledge, this is the first report identifying the amino acid residues crucial for the chitooligosaccharide binding activity of Cucurbitaceae phloem exudate lectins.

2. Materials and methods

2.1. Expression of PP2 sequences in P. pastoris

Cloning and expression of native and mutant forms of PP2 were performed using the EasySelect *Pichia* Expression Kit from Invitrogen (Carlsbad, CA, USA). The coding sequence for PP2 was amplified by PCR from the Bluescript vector containing cDNA encoding PP2 (GenBank accession number Q42383) by using primers PP2 Forward (5′-CTCGAGATGGACAACAA AGAGAAGGAAG-3′) and PP2 Reverse (5′-TCTAGATGCGCAACCACATCCCTTTG-3′). The PCR conditions were as follow: 5 min denaturation at 94 °C, 25 cycles of 20 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C. The amplified PP2 sequence was cloned as an EcoRI/XbaI fragment in the *Pichia* vector pPICZα A and transformed into *Escherichia coli* Top10F cells using heat shock transformation. Afterwards *E. coli* transformants were selected on LB agar plates containing Zeocin (25 μg mL^{-1}). The plasmids were purified using the Zyppy mini prep kit. Finally, the sequence of the fusion construct was verified by sequencing using 5′ and 3′AOX1 specific *Pichia* primers (AOX1 Forward (5′-GACT GGTTCCAATTGGACAAGC-3′), and AOX1 Reverse (5′-GCAAATGGC ATTCTGACATCC-3′). The plasmid DNA was linearized with the restriction enzyme PmeI (Fermentas, St. Leon-Rot, Germany), 10 μg of the expression vector was directly introduced into the *Pichia* strain X-33 by using EasyComp™ chemical transformation as described by the supplier. The transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% Agar) containing 100 μg mL^{-1} Zeocin. The integration of the PP2 gene sequence in the chromosomal AOX1 locus of *P. pastoris* was confirmed by PCR using the AOX1 primers, and the following parameters: 2 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and ending with an elongation step of 1 min at 72 °C. For expression analysis several colonies were inoculated in 5 ml BMGY medium, i.e. 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate and without amino acids, 4×10^{-5}% biotin, 100 mM potassium phosphate buffer (pH 6) and 1% glycerol, and grown at 30 °C in a shaker incubator at 200 rpm for 24 h.

Afterwards, *Pichia* cells were washed with sterilized water and transferred to the BMMY medium (BMGY medium supplemented with 1% of methanol instead of 1% of glycerol). Induction of the culture was achieved by adding 100% methanol (2% final concentration) for three successive days, once in the morning and once in the evening. Protein profiles in the medium and cell pellet were analyzed after trichloroacetic acid precipitation (10% final concentration) by SDS–PAGE and Western blot analysis.

2.2. Large-scale culture and purification of PP2

Transformed *P. pastoris* X-33 colonies were inoculated into 5 mL BMGY medium and grown for 24 h at 30 °C in a rotary shaker at 200 rpm. Subsequently, cultures were transferred to 50 mL BMGY in 250 mL Erlenmeyer flasks and allowed to grow until the culture reached an optical density between 2 and 6 at 595 nm. *Pichia* cells were washed with sterilized water and resuspended in 200 mL of BMMY medium. The culture was allowed to grow for 72 h in a 1.0 L Erlenmeyer flask under the same conditions as described above. During this incubation, 100% methanol was added to the culture twice a day as indicated above (2% final concentration). After 3 days of methanol induction, the culture was centrifuged for 10 min at 3000×*g* and the supernatant was brought to 80% ammonium sulfate for protein precipitation and stored at 4 °C. One liter of *Pichia*-culture were precipitated for one purification of His tagged recombinant PP2 using a GE Äkta protein purifier. Precipitated proteins were resuspended in a small volume of Ni^{2+}-NTA binding buffer (300 mM NaCl, 20 mM imidazole and 20 mM sodium dihydrogen phosphate, pH 8) with several buffer changes. The sample was then loaded on a Ni^{2+}-NTA agarose column equilibrated with the same binding buffer. After extensive washing with the binding buffer, bound proteins were eluted with the Ni^{2+}-NTA gradient elution buffer (20–500 mM imidazole in 20 mM sodium dihydrogen phosphate, pH 8, 500 mM NaCl). Fractions from the column were collected and examined by SDS–PAGE and Western blot analysis.

2.3. Site directed mutagenesis

Site-directed mutagenesis of the PP2 coding sequence was done using QuikChange lightning site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). Residues selected for mutations are based on ligand docking and simulation studies of PP2 from our previous report [15].

2.4. Agglutination activity of native and mutant forms of PP2

Hemagglutination assays were performed in 96 well ELISA micro titer plates using rabbit erythrocytes. To each well containing 100 μL of serially diluted wild type PP2 or a mutant protein, 100 μL of 4% rabbit erythrocytes were added and mixed. The plate was incubated at 4 °C for one hour and then the agglutination titer was visually scored. Hemagglutination-inhibition assays were performed in a similar way by using 1 mM stock solutions of CHOs. The solutions were mixed in the following manner: 50 μL of saccharide solution was placed in the first well and serially 2-fold diluted. Then 50 μL of purified PP2 (30 μg/mL) was added to each well. After keeping the microtitre plate at 4 °C for 15 min, 100 μL of 4% rabbit erythrocytes were added, the plate was again incubated for one hour at 4 °C and the titer was scored visually. The percent loss of activity of wild type and mutant forms was detected

by estimating the resultant concentration at IC_{50} of each recombinant protein.

3. Results

3.1. Identification of residues involved in carbohydrate binding and construction of mutants

In an earlier study, we reported 3-dimensional models of PPL and its complexes with various chitooligosaccharides, derived from homology modeling, molecular docking and molecular dynamics simulations [15]. These studies revealed an extended binding cleft on the protein surface which can accommodate various CHOs. Within the binding cleft, three subsites were identified, and at each subsite, at least two amino acids were predicted to contribute to ligand binding by either hydrophobic interactions or hydrogen bond formation with the CHOs (Fig. 1). To explore the role of the amino acids involved in CHOs binding, double and single mutants were generated at each subsite. Hemagglutination assays were used to compare the activity of the wild type and mutant forms of PP2 in order to identify the amino acid residues that make crucial contributions to ligand binding.

The primary sequence of PP2 is shown in Fig. 2A, and the alignment of several proteins from Cucurbitaceae which show significant homology and high conservation of the predicted binding site for CHOs is shown in Fig. 2B. The amino acid residues predicted by molecular docking and molecular dynamics studies [15] to be involved in binding for PP2 were selected for the mutations. The binding pocket extends from 100-Leu-Ile-Glu-Val-Ser-Trp-105 and 199-Trp-Lys-200 (Fig. 2A). Based on our docking studies, this extended binding region was divided into three subsites, consisting of major interacting residues Leu-100 and Glu-102 (subsite-1), Ile-101, Ser-104 and Trp-199 (subsite-2), and Trp-199 and Lys-200 (subsite-3). In the present study, mutations were carried out at these three subsites. The residues which were selected for double mutations are: Leu-100 and Ile-101 (subsite-1), Ser-104 and Trp-105 (subsite-2) and Trp-199 and Lys-200 (subsite-3). In addition to the above double mutations, single mutants were also generated at each subsite; Leu-100 at subsite-1, Ser-104 at subsite-2 and Lys-200 at subsite-3. The Trp residues were mutated to Leu and all other residues were mutated to Ala.

3.2. Purification of recombinant proteins and their characterization

Nine different mutants (M1–M9) were generated by modifying specific amino acid residues in the primary structure of PP2, employing the Quick change Site-directed mutagenesis kit (Table 1). The resulting constructs were transformed into *P. pastoris* strain X-33. Transformed colonies were grown and the recombinant proteins were purified from the culture medium.

The recombinant PP2 (rPP2) and its mutants were characterized by SDS–PAGE. Coomassie-staining of the recombinant proteins showed two closely spaced bands with a molecular mass of ~26 kDa and ~28 kDa (Fig. 3A). The molecular weight of the polypeptides corresponding to these bands is in agreement with the calculated mass corresponding to the primary sequence of PP2 considering that rPP2 contains an additional *c-myc* epitope and a hexaHis tag as compared to the native PP2 from pumpkin. Further characterization of the purified proteins was done by Western blot analysis, using a monoclonal antibody directed against the C-terminal hexaHis tag (Fig. 3B) and C-terminal *myc* tag (Fig. 3C). The upper band at ~28 kDa probably corresponds to a glycosylated form of the protein, resulting from *N*-glycosylation at Asn-113, the only potential *N*-glycosylation site in the primary structure of PP2.

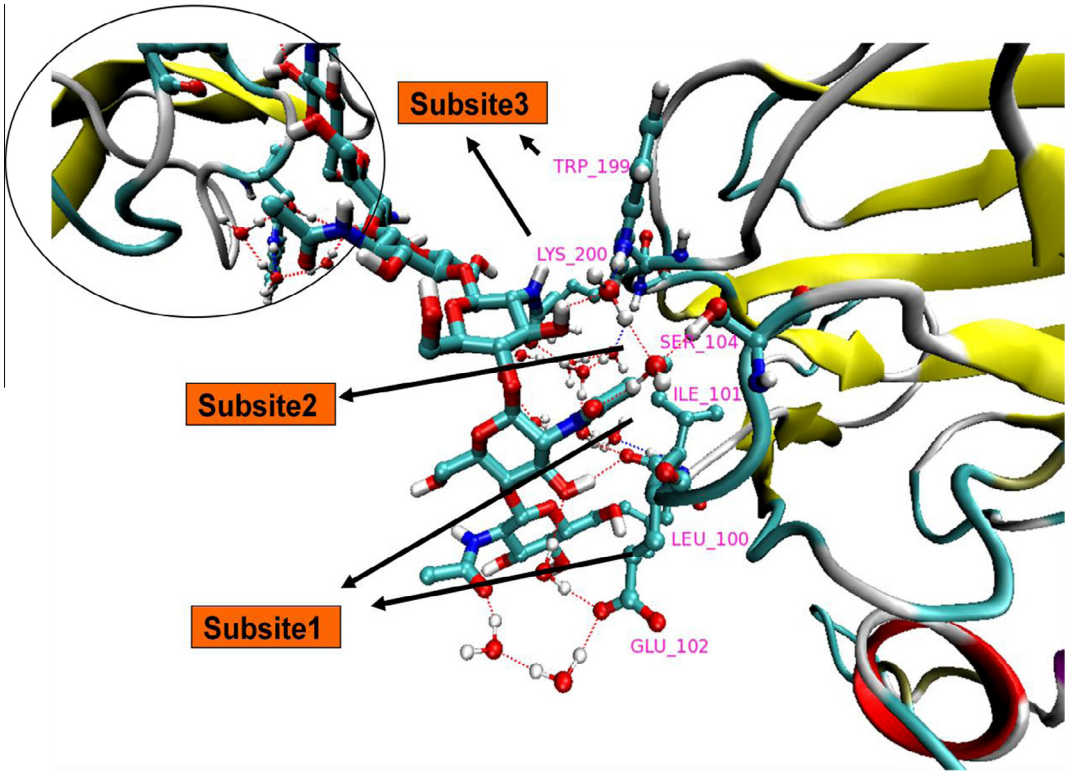

Fig. 1. Interaction of chitohexaose with the carbohydrate binding cleft of PP2, showing the amino acid residues involved in carbohydrate binding (adapted from Narahari et al. [15] and modified). The binding site was divided into three subsites as indicated by arrows: subsite-1 (L100, I101), subsite-2 (S104, W105) and subsite-3 (W199, K200). Only three GlcNAc residues from the non-reducing end of chitohexaose are seen to interact with the binding cleft. GlcNAc residues at the reducing end interact with another PP2 monomer (shown in the circle at the top left corner).

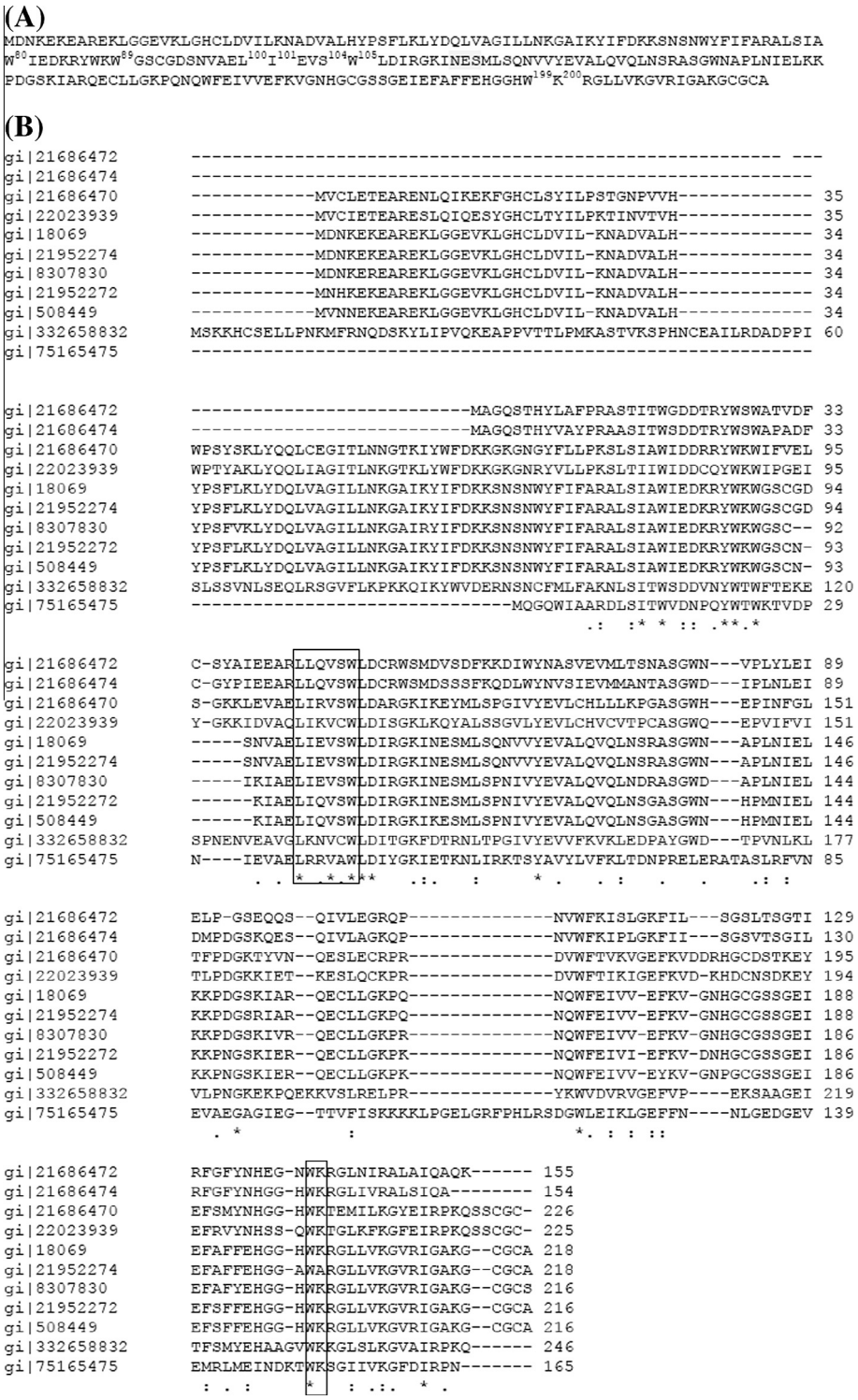

Fig. 2. (A) Primary structure of PP2 derived from the cDNA sequence (Bostwick et al. [14]). The amino acids selected for mutations are numbered and highlighted. The possible *N*-glycosylation site (NES) is highlighted. (B) Multiple sequence alignment of amino acid sequences of the PP2 domain from *Cucurbitaceae*, *Arabidopsis thaliana* and *Nictaba*. The carbohydrate binding regions indicated in the boxes were selected from computational modeling (Narahari et al. [15]). Proteins that were aligned are: from top to bottom *Cucumis sativus* 17 kDa phloem lectin, *Cucumis melo* 17 kDa phloem lectin (Lec17-1), *Cucumis melo* 24 kDa phloem lectin, *Cucumis sativus* 24 kDa phloem lectin, *Cucurbita maxima* phloem lectin, *Cucurbita digitata* phloem lectin, *Cucurbita moschata* phloem lectin, *Cucurbita argyrosperma subsp. Sororia* phloem lectin, *Cucurbita argyrosperma* phloem lectin, *Arabidopsis thaliana* phloem protein 2–A1 (AtPP2–A1) and Nictaba. The sequences were obtained from the protein database of NCBI.

3.3. Hemagglutination activity of recombinant proteins

Analysis of agglutination activity of native and recombinant forms of PP2 suggested that the lectin activity of rPP2 is similar to that of the native protein even though rPP2 is partially glycosylated. Mutation of Trp-80 and Trp-89 in the PP2 sequence independently (mutants M1 and M2) or together (M3) did not significantly change the apparent agglutination activity of the protein (Fig. 4). However, mutation of Ser-104 and Trp-105 at subsite-2 (M2) drastically reduced the agglutination activity of the protein,

Table 1
List of primer sequences used to amplify the original and mutant forms of PP2.

Mutant	Mutagenic primer sequence[a] (5′-3′)
PP2-wild	Fwd: ctcgagatggacaacaaagagaaggaag Rev: cttccttctctttgttgtccatctcgag
PP2-M1 W80L	Fwd: tgcaagagctctctcaatagcttgattgaagataagagatactggaaat Rev: atttccagtatctcttatcttcaatcaaagctattgagagagctcttgca
PP2-M2 W89L	Fwd: attgaagataagagatactggaaattgggatcctgtggcgatagcaac Rev: gttgctatcgccacaggatcccaatttccagtatctcttatcttcaat
PP2-M3* W80L and W89L	M1 + M2
PP2-M4 L100A and I101A	Fwd: ggcgatagcaacgttgcagaggctgctgaagtatcttggctggacatt Rev: aatgtccagccaagatacttcagcagcctctgcaacgttgctatcgcc
PP2-M5 S104A and W105L	Fwd: acgttgcagagcttattgaagtagctttgctggacattcgtggaaagatc Rev: gatctttccacgaatgtccagcaaagctacttcaataagctctgcaacgt
PP2-M6 W199L and K200A	Fwd: ctttttttgaacatggagggcatttggctaggggggctgctcgtgaaagg Rev: cctttcacgagcagccccctagccaaatgccctccatgttcaaaaaag
PP2-M7 L100A	Fwd: ggcgatagcaacgttgcagaggctattgaagtatcttggctggacatt Rev: aatgtccagccaagatacttcaatagcctctgcaacgttgctatcgcc
PP2-M8 S104A	Fwd: cgttgcagagcttattgaagtagcttggctggacattcgtggaa Rev: ttccacgaatgtccagccaagctacttcaataagctctgcaacg
PP2-M9 K200A	Fwd: cgttgcagagcttattgaagtagcttggctggacattcgtggaa Rev: ttccacgaatgtccagccaagctacttcaataagctctgcaacg

[a] Underlined sequence encodes altered amino acid.
* Mutant M3 was obtained by a further mutation of M1 using the M2 primers.

with the mutant protein exhibiting approximately 10% activity as compared to rPP2. When only Ser-104 was changed to alanine at subsite-2 (M8), the loss of activity was comparable to that of the double mutant (M2). On the other hand, mutation of Leu-100 and Ile-101 at subsite-1 (M4) led to a moderate decrease in the agglutination activity (~40%). When only Leu-100 was changed to alanine (M7), the loss of activity was similar to that seen with M4. Furthermore, mutation of Trp-199 and Lys-200 at subsite-3 (M6) impaired its activity by 40%. Mutation of only Lys-200 to alanine (M9) also led to a 40% decrease in the lectin activity.

4. Discussion

Phloem in plants contains a variety of defense related proteins [3] and serves as a crucial barrier for invading pathogens [16]. In plants, long distance RNA transport occurs through the phloem pathway [17]. Besides its involvement in the wound sealing process, which protects loss of sieve elements and helps to maintain the turgor pressure for the mass flow [18], PP2 is involved in multiple functions, e.g., in RNA binding [12], chitin binding [8,15,19] and their translocation [20,21]. PP2 isolated from pumpkin phloem is a homodimeric lectin, which forms higher order structures in the presence of CHOs and can also bind to high mannose *N*-glycans [15,22]. Although PP2 like proteins are found in almost all kingdoms of plants and several of them are well characterized [8], no three-dimensional structure of a protein from this family is available yet.

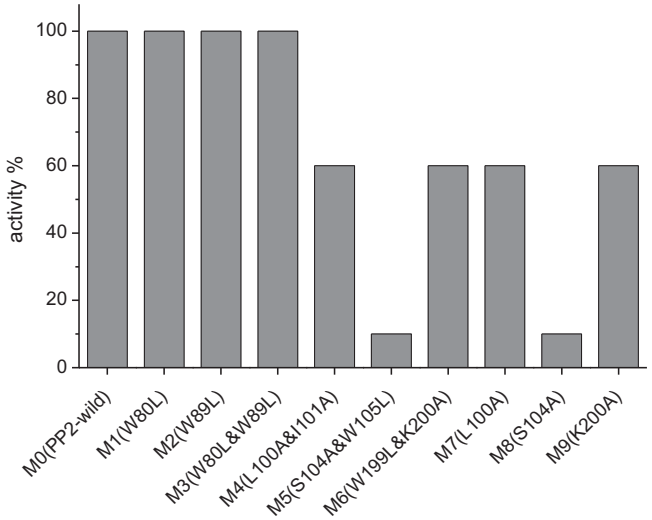

Fig. 4. Bar diagram depicting the hemagglutination activity of wild type PP2 (M0) and its mutants (M1–M9).

Analysis of the hemagglutination activity of various mutants of PP2 suggested that at subsite-1 Leu-100 makes an important contribution to carbohydrate binding by PP2 as mutation of this residue to Ala decreased the lectin activity by about 40%. Mutation of Ser-104 to Ala at subsite-2 resulted in a near complete loss (>90%) of lectin function, establishing that Ser-104 contributes the most to carbohydrate binding by PP2. Mutation of Lys-200 to Ala at subsite-3 also led to about 40% loss of lectin activity, emphasizing the significant contribution of this residue to the sugar binding activity of the lectin. These observations, taken together clearly suggest that Ser-104 is crucial for the interaction of CHOs with PP2, whereas

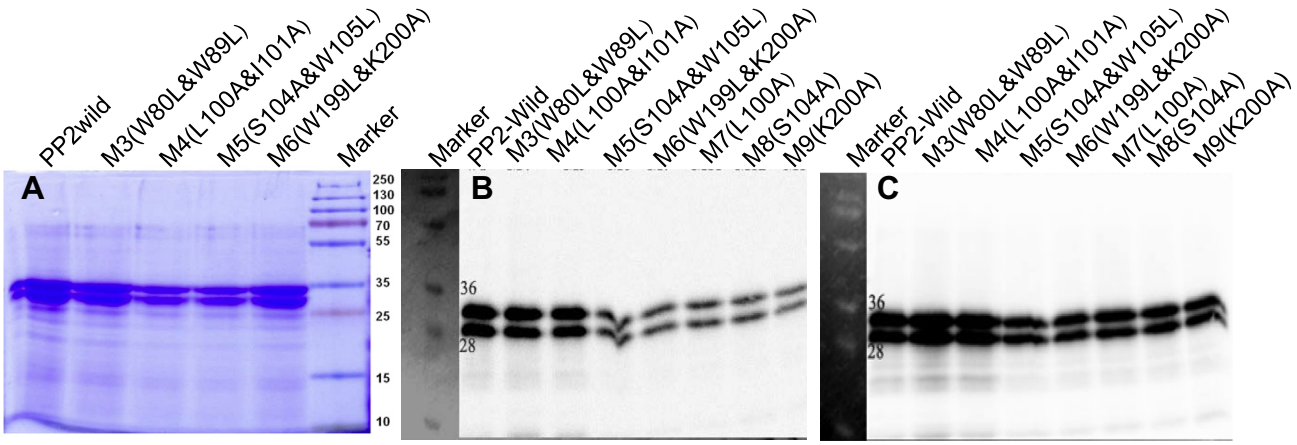

Fig. 3. (A) SDS–PAGE of recombinant PP2 and its mutants purified by Ni-NTA affinity chromatography. The gel was stained with Coomassie brilliant blue R-250. Two intense bands are seen in each lane, corresponding to the glycosylated (upper band) and non-glycosylated (lower band) forms of the protein. Lanes corresponding to different mutants are labeled. Molecular weight markers are shown in the extreme right lane. Western blot analysis of secreted PP2-wild type and its mutants with c-terminal $(His)_6$ and *myc* tags detected with fluorescent anti-His monoclonal antibody (B) and anti-*myc* antibody (C) are also shown.

Leu-100 and Lys-200 provide additional interactions that stabilize the binding of chitooligosaccharides to the lectin. Thus subsite-2 constitutes the primary binding site, whereas subsites 1 and 3 could be considered as secondary binding sites.

Interestingly hevein, another chitooligosaccharide binding lectin also has a serine at its binding pocket which forms hydrogen bonding interactions with the acetamido moiety of GlcNAc residue [23]. The well characterized chitin binding lectins UDA and WGA also contain a conserved serine at their binding pockets, which influences the carbohydrate binding activity significantly [24,25]. Thus the involvement of serine residues in the carbohydrate binding activity appears to be a general feature of chitooligosaccharide-specific lectins.

In many cases, protein-carbohydrate interactions are stabilized by aromatic residues proximal to the binding pocket. For example, studies on two chitin binding lectins, hevein and WGA, revealed that aromatic residues at positions proximal to the carbohydrate binding site stabilize the binding by C–H–π interactions and Van der Waals' contacts with CHOs [23,26]. Studies of the carbohydrate binding properties of Nictaba lectin, which exhibits high homology with Cucurbitaceae phloem lectins, also suggested the involvement of tryptophans in the carbohydrate binding pocket [27]. To verify if the tryptophan residues of PP2 play a similar role in stabilizing the interaction of the protein with CHOs we mutated Trp-80 and Trp-89 – which are highly conserved among PP2 proteins of Cucurbitaceae – to leucines. These mutations did not result in any change in the agglutination activity of the protein, suggesting that these tryptophan residues are not directly involved in the carbohydrate binding. This is consistent with the docking studies [15]. However, the double mutation of Trp-199 to leucine and Lys-200 to alanine at subsite3 has shown significant loss of binding activity (~40%). When Lys-200 alone was mutated to alanine, it resulted in similar kind of loss of activity suggesting probably the role of Trp-199 is insignificant in the binding pocket. These results are in good agreement with recent fluorescence quenching studies, which suggested that tryptophan residues are only partially exposed to the aqueous environment and are probably not involved in the ligand interaction [28].

It is known that chitin binding interactions can be further strengthened by non-covalent interactions of the ligand with residues that are proximal to the binding pocket. In this regard the amino acid sequence **LIE** (residues 100–102) found in the binding region of PP2 appears to play a significant role as modification of Leu-100 reduced the lectin activity considerably. Interestingly this sequence was also found in the well studied LysM domains and it was reported that they play a role in chitin binding by Ecp6 with picomolar affinity and its ligand-induced dimerization [29].

In summary, the present results strongly support the predicted three-dimensional structure of PP2 proposed in our previous study [15] and provide experimental validation for the extended nature of its carbohydrate binding pocket, which can accommodate up to three GlcNAc residues in the three subsites. The present results identify Ser-104 as crucial for the interaction of chitooligosaccharides with PP2 whereas Leu-100 and Lys-200 provide additional stability to the binding.

Acknowledgments

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References

[1] K.J. Oparka, S. Santacruz, The great escape: phloem transport and unloading of macromolecules, Annu. Rev. Plant Physiol. Plant Mol. Biol. 51 (2000) 323–347.
[2] C. Walz, P. Giavalisco, M. Schad, et al., Proteomics of curcurbit phloem exudate reveals a network of defence proteins, Phytochemistry 65 (2004) 1795–1804.
[3] M.-K. Lin, Y.-J. Lee, T.J. Lough, et al., Analysis of the pumpkin phloem proteome provides insights into angiosperm sieve tube function, Mol. Cell. Proteomics 8 (2) (2009) 343–356.
[4] S. Huang, R. Li, Z. Zhang, et al., The genome of the cucumber, *cucumis sativus* L, Nat. Genet. 41 (2009) 1275–1281.
[5] B. Zhang, V. Tolstikov, C. Turnbull, et al., Divergent metabolome and proteome suggest functional independence of dual phloem transport systems in cucurbits, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 13532–13537.
[6] R. Turgeon, K. Oparka, The secret phloem of pumpkins, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 13201–13202.
[7] S.M. Read, D.H. Northcote, Subunit structure and interactions of the phloem proteins of *Cucurbita maxima* (pumpkin), Eur. J. Biochem. 134 (1983) 561–569.
[8] S. Dinant, A.M. Clark, Y. Zhu, et al., Diversity of the super family of phloem lectins (phloem protein 2) in angiosperm, Plant Phys. 131 (2003) 114–128.
[9] D.D. Sabnis, J.W. Hart, The isolation and some properties of a lectin (Hemagglutinin) from *cucurbita* phloem exudate, Planta 142 (1978) 97–101.
[10] N. Shibuya, I.J. Goldstein, W.F. Broekaert, et al., The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(alpha 2–6)Gal/GalNAc sequence, J. Biol. Chem. 262 (1987) 1596–1601.
[11] R.N. Knibbs, I.J. Goldstein, R.M. Ratcliffe, et al., Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. Comparison with other sialic acid-specific lectins, J. Biol. Chem. 266 (1991) 83–88.
[12] G. Gómez, H. Torres, V. Pallás, Identification of translocatable RNA binding phloem proteins from melon, potential components of the long distance RNA transport system, Plant J. 41 (2005) 107–116.
[13] D.E. Bostwick, J.M. Dannenhoffer, M.I. Skaggs, et al., Pumpkin phloem lectin genes are specifically expressed in companion cells, Plant Cell 4 (1992) 1539–1548.
[14] D.E. Bostwick, M.I. Skaggs, G.A. Thompson, Organization and characterization of *Cucurbita phloem* lectin genes, Plant Mol. Biol. 26 (1994) 887–897.
[15] A. Narahari, H. Singla, P.K. Nareddy, et al., Isothermal titration calorimetric and computational studies on the binding of chito-oligosaccharides to pumpkin (*Cucurbita maxima*) phloem exudate lectin, J. Phys. Chem. B 115 (2011) 13710–13717.
[16] T. Will, A.C. Furch, M.R. Zimmermann, How phloem-feeding insects face the challenge of phloem-located defenses, Front. Plant Sci. 4 (2013) 336.
[17] V. Pállas, G. Gómez, Phloem RNA-binding proteins as potential components of the long-distance RNA transport system, Front. Plant Sci. 4 (2013) 130.
[18] R.F. Evert, W. Eschrich, S.E. Eichhorn, P-protein distribution in mature sieve elements of *Cucurbita maxima*, Planta 109 (1973) 193–210.
[19] A.K. Allen, A lectin from the exudate of the fruit of the vegetable marrow (*Cucurbita pepo*) that has a specificity for beta-1,4-linked *N*-acetylglucosamine oligosaccharides, Biochem. J. 183 (1979) 133–137.
[20] B. Golecki, A. Schulz, G.A. Thompson, Translocation of structural P proteins in the phloem, Plant Cell 11 (1999) 127–140.
[21] R.A. Owens, M. Blackburn, B. Ding, Possible involvement of the phloem lectin in long-distance viroid movement, Mol. Plant Microbe Interact. 14 (2001) 905–909.
[22] J. Beneteau, D. Renard, L. Marche, et al., Binding properties of the *N*-acetyl glucosamine and high-mannose *N*-glycan PP2-A1 phloem lectin in *Arabidopsis*, Plant Physiol. 153 (2010) 1345–1356.
[23] J.L. Asensio, F.J. Cañada, H.C. Siebert, et al., Structural basis for chitin recognition by defense proteins: GlcNAc residues are bound in a multivalent fashion by extended binding sites in hevein domains, Chem. Biol. 7 (2000) 529–543.
[24] F.A. Saul, P. Rovira, G. Boulot, et al., Crystal structure of *Urtica dioica* agglutinin, a super antigen presented by MHC molecules of class I and class II, Structure 8 (2000) 593–603.
[25] C.S. Wright, The crystal structure of wheat germ agglutinin at 2.2 Å resolution, J. Mol. Biol. 111 (1977) 439–457.
[26] W.I. Weis, K. Drickamer, Structural basis of lectin carbohydrate recognition, Annu. Rev. Biochem. 65 (1996) 441–473.
[27] D. Schouppe, P. Rougé, Y. Lasanajak, et al., Mutational analysis of the carbohydrate binding activity of the tobacco lectin, Glycoconj. J. 27 (2010) 613–623.
[28] A. Narahari, M.J. Swamy, Tryptophan exposure and accessibility in the chitooligosaccharide-specific phloem exudate lectin from pumpkin (*Cucurbita maxima*). A fluorescence study, J. Photochem. Photobiol., B 97 (2009) 40–47.
[29] A. Sánchez-Vallet, R. Saleem-Batcha, A. Kombrink, et al., Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerisation, eLife 2 (2013) e00790.