**Background**

Polygalacturonase inhibiting proteins (PGIPs) are part of the innate immune system of plants. PGIPs may specifically inhibit fungal polygalacturonases (PGs) [1-3]. The action of PGIPs on PGs during fungal attack slows down the infection rate and facilitates the prolonged existence of mid-sized oligogalacturonides (damage-associated molecular pattern molecules, DAMPs), which in turn can elicit a general defence response from the plant [4-6]. PGIPs are not specialized inhibitors of a single PG, but rather versatile proteins that capable of recognising different surface motifs of structurally variable PGs [6]. Knowledge about the structural properties of PGIPs can provide valuable insight into the nature and dynamics of these interactions with fungal PGs, and also helps to identify promising candidate PGIPs for biotechnological approaches to improve plant disease resistance.

Yao et al. [7] isolated mRNA containing the complete coding sequence region of the *MdPGIP* gene from cDNA of *M. domestica* cv Golden Delicious [GenBank: MDU77041]. Similarly, Arendse et al. [8] isolated the complete *MdPGIP1* gene from gDNA of *M. domestica* cv Granny Smith [GenBank:DQ185063]. The sequence of the *MdPGIP1* accession DQ185063 was compared to the *MdPGIP* accession MDU77041 and the results showed that the two gene sequences share a 100% identity. The *MdPGIP1* gene sequence elicited interest in its potential use as an anti-fungal agent and was subsequently used to transfer into potato [9] and tobacco [10]. *MdPGIP1* inhibits PGs from *Botryosphaeria obtusa* and *Diaporthe ambigua*, which are both apple pathogens. In
addition, further studies performed to date (Matsaunyane and Oelofse, unpublished) indicate that the protein also inhibits PGs from *Verticillium dahlia*, *Botrytis cinerea*, *Colletotrichum acutatum* and *Colletotrichum coccodes*, but not that of *Fusarium verticillioides*. To further explore the biochemical characteristics of *MdPGIP1* as a potential tool in improving disease resistance of food crops, *in silico* analyses were performed to compare the properties of *MdPGIP1* to other characterised PGIPs. This further characterization forms part of new information on the *MdPGIP1* encoded protein.

**Methods**

The *MdPGIP1* gene sequence with the GenBank accession [DQ185063] was used during *in silico* analysis in this study. The nucleotide sequence was translated into the encoding polypeptide using the http://web.expasy.org/translate/ database. The amino acid composition of the *MdPGIP1* protein was calculated using the http://wwwbiology.arizona.edu/biochemistry/biochemistry.html database. In addition, the polarity, functional group side chains and their respective charge, and the amino acids' water propensity, were also calculated on this database. The hydrophobicity plot of the *MdPGIP1* contiguous amino acid residues was determined by constructing the Kyte-Doolittle hydrophathy graph (http://gcat.davidson.edu/DGPB/kyt-doolittle.htm) [11]. The molecular weight of *MdPGIP1* was determined by compiling a ProtScale of the polypeptide using its respective constituting residues (http://web.expasy.org/cgi-bin/protscale/protscale.pl). Software from the NetNGlyc 1.0 Server (http://genome.cbs.dtu.dk/services/) was used to analyse the possible N-linked glycosylation sites of *MdPGIP1*. The putative crystal structure of *MdPGIP1* was modelled using SWISS-MODEL [12], a protein structure homology-modeling server, accessible via the ExPASy web server (www. Swissmodel.expasy.org).

**Findings**

**Amino acid composition and primary structure of *MdPGIP1* supports its folded structure and function**

Although subject to modification, the linear sequence of amino acids, as represented by the primary structure of a protein, holds the required information for protein folding, the biological and cellular processes and activities of proteins. The amino acid residues that comprise the *MdPGIP1* polypeptide were therefore analysed, based on the translated nucleotide sequence [13]. The primary structure of *MdPGIP1* with its identified domains is shown in Figure 1. Features include a putative 24 amino acid signal peptide (Figure 1A), a potential cleavage site [Alanine-Leucine-Serine (ALS)] for the signal peptide (Figure 1, ALS in pink), a 46 amino acid N-terminal domain (Figure 1B), a 238 amino acid leucine-rich repeat (LRR) domain (Figure 1C) and a 22 amino acid C-terminal domain (Figure 1D).

The amino acid composition of the polypeptide, the polarity of the amino acids, type of side chain found in their respective functional (R) group, charge of the amino acids, as well as their water propensity was also calculated (http://wwwbiology.arizona.edu/biochemistry/biochemistry.html). The water propensity of amino acids can be used to indicate, *in silico*, the location of the respective amino acid in the final structure of a protein during folding [11]. The interior of a globular protein normally houses hydrophobic residues, whereas the outer side is a location for hydrophilic residues. The Kyte-Doolittle hydrophathy graph was subsequently constructed (Figure 2) to obtain further insights into the effect of the different water propensities of the *MdPGIP1* residues on...
the structure of the protein (http://gcat.davidson.edu/DGBP/kd/kyte-doolittle.htm, [11]). The total number of amino acids, of the total 330, that were effective in the construction of the hydropathy plot was 322. The window size of the \textit{MdPGIP1} hydropathy plot was 9 and strong negative peaks (indicative of hydrophilic areas) were observed on the plot. This is an indication of a possible surface area of a globular protein. The hydropathy plot also indicated the existence of a repetitive region between residues 71 and 300 on the window number (x-axis), and by the strong positive (hydrophobic) peaks on the hydrophobicity score (y-axis) (Figure 2).

PGIPs are known to be peripheral membrane-associated proteins secreted to the apoplast [14]. The hydropathy plot was used to identify portions of \textit{MdPGIP1} that could be associated with the membrane. The most hydrophobic residues served as a basis for this identification and these residues were found from residue 9 to 21 on the protein [11]. The residues found at this location on \textit{MdPGIP1} are Met, Glu, Leu, Lys, Phe, Ser, Ile, Phe, Leu, Ser, Leu, Thr, Leu, Leu, Phe, Ser, Ser, Val, Leu, Lys, Pro, Ala, Leu and Ser (Figure 1). The relative hydrophobicity of this portion is confirmed in Figure 2, where the hydropathy score is the highest throughout the span of the \textit{MdPGIP1} polypeptide length at a value between one and two. This hydropathy score is thought to be a deciding factor for this portion to be membrane associated, thus confirming this aspect for \textit{MdPGIP1}, similar to other PGIPs [15].

\textbf{\textit{MdPGIP1} belongs to the Leucine Rich Repeat superfamily}

The structural features of \textit{MdPGIP1} were found to be consistent with typical PGIP features described by other authors [16-19]. Structural studies of PGIP proteins are important for it is known that the change of one or a few residues may confer new PGs recognition specificities to a PGIP and may improve its inhibitory strength [6,20]. The recognition specificity is determined by variation in the amino acids comprising the LRR domain [6,20]. This data may support the planning of mutational strategies towards improving the properties of natural PGIPs and the versatility of their recognition capabilities against the many diverse microbial PGs [21].

As mentioned, evaluation of the primary structure of \textit{MdPGIP1} indicates a LRR region of 238 amino acids containing 123 hydrophobic amino acids (Figure 1). The LRR domain spans from residues 71 to 308. The \textit{MdPGIP1} polypeptide contains 10 LRRs as indicated in Figure 1, hence the protein belongs to the LRR family [16,18,22]. All the PGIPs isolated to date comprise 10 LRRs matching the extracytoplasmic LRR consensus LxxLxxLxxLxxNxLxGxIPx, features that also apply to \textit{MdPGIP1}.

LRR motifs play an important role in the cellular functions of several proteins [23]. A typical motif contains 20 to 29 residues and these motifs have been identified in plants, animals, as well as in microorganisms [20,23]. In the case of PGIP, these leucine residues are important in the binding of PGIP to the cell wall through the interaction of the residues with the acidic pectin within the cell wall matrix [14,15].

\textbf{Physicochemical properties and glycosylation of the \textit{MdPGIP1} pre-protein}

Following the analysis of how hydrophobicity and hydrophilicity affect the \textit{MdPGIP1} structure, further analyses were performed to determine the molecular weight of \textit{MdPGIP1}. ProtScale was used to compute the profile of \textit{MdPGIP1} and produced its constituent amino acids (http://web.expasy.org/cgi-bin/protscale/protscale.pl). The molecular weight of all the residues that make up \textit{MdPGIP1} was determined per residue and mapped on the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{A hydropathy plot constructed for the \textit{Malus domestica} polygalacturonase inhibiting protein 1 (\textit{MdPGIP1}) polypeptide.}
\end{figure}
polypeptide to assist with determining the molecular weight of the protein (graph not shown).

Purified MdPGIP1 was found to have a molecular mass of between 44 to 54 kDa (cv Golden Delicious) [24] and 46 kDa (cv Granny Smith) [10]. Using the residues’ molecular mass and their mapping throughout the MdPGIP1 polypeptides, the molecular mass of the non-glycosylated apo-protein was calculated and determined to be 36.615 kDa. The bulk of the MdPGIP1 polypeptide consists of Leu and Ser at 16.1% and 10.6%, respectively (Figure 1), a combined contribution of 29% to the molecular weight of MdPGIP1.

Lastly, the theoretical isoelectric point value (pl) of MdPGIP1 was calculated to be 6.98. The relatively high pl of the mature MdPGIP1 polypeptide is attributed to the presence of the positively charged Lys and Arg residues. These residues are believed to interact with the acidic pectin in the cell wall matrix, supporting the cell wall association of PGIP [16]. Addition of amino sugars during glycosylation of the pre-protein can further increase the pl to 8.0 [10], generating a basic functional protein.

PGIPs have been reported to be glycoproteins [4,22,25], undergoing post-translational glycosylation [26,27]. Glycosylation enables proteins to participate in biological processes, such as attaching to the extracellular matrix, as well as protein-ligand interactions, and has been shown to contribute to protein stability and increase resistance to protease digestion [27]. These are important features related to the function of PGIP in an extracellular environment.

N-glycosylation initially occurs in the endoplasmic reticulum and the target residue is Asp that is found in the sequence Asn-X-Ser/Thr, where X can be any residue except Pro [28,29]. The N-linked glycosylation sites of MdPGIP1 were analysed using the NetNGlyc 1.0 Server (http://genome.cbs.dtu.dk/services/) and the analysis is shown in Table 1. The typical consensus glycosylation sequence required for this modification (Asn-X-Ser/Thr) was identified in MdPGIP1, supporting its characterization as a glycoprotein. Aspects of the glycosylation process that can be modified and thus affect biological function, include glycan composition, glycan structure and glycan length [30].

Sequence comparison of the MdPGIP1 encoded protein

PGIPs have been identified in many dicot and monocot plants. PGIP genes are often found as small gene families that encode PGIP isoforms with different specificities and affinities towards secreted fungal PGs [6]. The phylogenetic relationship between MdPGIP1 was compared with PGIPs from other plants as shown in Figure 3. Amino acid comparisons between the different PGIPs showed that PGIPs from fruit bearing trees share a high percentage of similarity with each other when compared to other plants. MdPGIP1 shares a 99.4%, 98%, and 98% amino acid identity with the PGIPs from Malus pumila, Malus hupehensis and Pyrus communis (members of the Rosaceae family), respectively.

The MdPGIP1 amino acid sequence was also compared with PGIPs from non-fruit bearing Eucalyptus tree species (members of the Myrtaceae family), namely, E. grandis, E. saligna, E. nitens, E. urophylla and E. camaldulensis. Interestingly, MdPGIP1 was found to share a 97% amino acid identity with E. grandis, and a 96% amino acid identity with E. saligna, and E. nitens. E. urophylla and E. camaldulensis share a 95% amino acid identity with MdPGIP1. In contrast, MdPGIP1 shares only a 55%, 48% and 53% identity with PvPGIP1, PtPGIP2 and GmPGIP (members of the Fabaceae family) respectively.

An analysis of PGIP sequences from different eudicotyledonous species (Fabaceae, Brassicaceae, Rosaceae and Rutaceae) indicated that diversification of PGIP genes during evolution has been driven by positive selection [31], limited to a small number of PGIP residues that are mostly solvent exposed and located in the β-sheet B1 corresponding to the concave surface of the protein (below).

Structural modeling: MdPGIP1 shares a similar structure with PtPGIP2

Two types of repeats, types A and B, are found in plant LRR domains [23]. Eight repeats of 28 amino acids were found in type A and 29 amino acids in type B. Short β-strand, βa loop, a helix and βa loop regions are formed by the type A repeats. This leads into repeats that form a parallel β-strand. The type A formation is repeated until the C-terminal, ending with a β-strand. β-Sheets are formed parallel to the α-helix on the face of the protein. These sheets are formed by stabilising residues found on adjacent repeats. The formation of β-sheets creates curvature to the protein and gives it a horseshoe shape.

| Position | Possibility | N-Glycosylation |
|----------|-------------|-----------------|
| 106 NLTG | 9/9         | ++              |
| 130 NLSG | 8/9         | +               |
| 144 NLTF | 5/9         | -               |
| 154 NLTG | 8/9         | +               |
| 238 NKT | 8/9         | +               |
| 254 NLSK | 9/9         | ++              |
| 251 NVSY | 9/9         | ++              |

The position of the receptor sites for N-glycosylation is included. The possibility of glycosylation occurring at that position is indicated with a + if it is positive, and - for highly unlikely.
The molecular structure of PGIP2 from *Phaseolus vulgaris* (PvPGIP2) was elucidated using X-ray crystallography (Figure 4A) [20,32]. Although the presence of a single β-sheet in PvPGIP2 was predicted, it was shown that two β-sheets (sheet B1 and B2) were present in PvPGIP2. In addition to the two β-sheets, helices were also found on the LRR domain of the PvPGIP2 molecule. The protein structure was found to be curved and elongated which is typical of other PGIPs [23]. The residues found in the β-strand/β-turn motif of PGIP were reported to be critical in the protein’s affinity and specificity towards PG ligands [20,32].

The crystal structure of PvPGIP2 served as a template to which the MdPGIP1 was modelled. The putative structure of MdPGIP1 was modelled using SWISS-MODEL [12] and the modeling results are shown in Figure 4B. The structure of MdPGIP1 was also found to be curved and elongated. In addition, sheet B1, sheet B2 and 3_10-helices were also identified on the LRR domain of the MdPGIP1 molecule. Sheet B1 of the MdPGIP1 LRR domain is located near the N-terminal in the concave inner side of the LRR region (Figure 4). The MdPGIP1 sheet B1 comprises 38 residues, of which 19 are hydrophobic, and these are located at residues 75, 77–78, 99, 101, 123–124, 126, 147–148, 171–172, 197, 220, 243–244, 267, 289 and 290 on the MdPGIP1 polypeptide (Figure 5). The hydropathy plot confirmed the observation where the hydrophobicity scores at these residue positions are relatively high. Sheet B2 is located on the convex outer side of the LRR region and comprises 29 residues, with 16 of those being hydrophilic (Figure 5). These hydrophilic residues are found at position 85, 108, 132, 134, 154, 156, 180, 182, 203, 205, 226, 228, 275, 296, 298 and 300 on the MdPGIP1 polypeptide. This water propensity of the sheet B2 residues is confirmed on the hydropathy plot. Sheet B2 determines the folding of PGIPs by connecting β-sheet B1 and 3_10-helices [32]. In addition, it is thought to form an additional surface on the PGIP for interaction with PGs [33].
Glycine residues found in sheet B2 of PGIPs are thought to be responsible for the bending of the sheet [32,33] and these residues were identified in the analysis of _MdPGIP1_ (Figure 5, green highlighted residues). Further analysis of the glycine residues revealed that they are located, in sheet B2 of _MdPGIP1_ (Figure 5, green highlighted residues), at positions similar to those identified on _PvPGIP2_. The speculation is that the _MdPGIP1_ sheet B2 glycine residues are also responsible for the bending of the sheet, as was described for _PvPGIP2_ [20,32]. However, the _MdPGIP1_ molecule does have a curve that is more relaxed compared to _PvPGIP2_.

The amino acids within sheet B2 of _PvPGIP2_ also comprise several aromatic and hydrophobic residues that contribute to the bending of the _PvPGIP2_ molecule [32,33]. These residues were identified as Phe-133, Phe-156, Phe-172, Phe-176, Phe-194, and Tyr-169. During analysis of the _MdPGIP1_ polypeptide, amino acids identified at the same positions were Leu-133, Leu-156, Phe-172, Val-176, Phe-194, and Leu-169. Leucine and valine are hydrophobic aliphatic residues with smaller side chains and these residues may be the cause of the more relaxed curve observed on the _MdPGIP1_ molecule.

While sheet B2 is thought to form an additional surface on the PGIP protein for interaction with PGs [32,33], sheet B1 residues determine the affinity and specificity of _PvPGIP2_. Asparagine residues have been found to form an Asn-ladder on _PvPGIP2_ and these were found to form
hydrogen bonds with amide groups and the main-chain carbonyl. This quality also influences the bending of the protein [32,33]. Twelve of the 20 Asn residues observed on the PvPGIP2 molecule were also observed on MdPGIP1 (Figure 5, blue highlighted residues).

**Conclusion**

Although phylogenetically distant from the archetypical PvPGIP2, the overall properties of the MdPGIP1 protein are broadly similar to that of the PGIPs thus far characterised. However, even slight structural differences may confer new or broader recognition specificities to a PGIP or may improve its inhibitory strength. Based on the foundation laid in the present study, future studies of the detected differences will add support to the biotechnological use of MdPGIP1 in recombinant transgenic applications as a targeted inhibitor of fungal PGs. Moreover, it may assist in the identification of promising candidate PGIPs for crop protection, and in improving the properties of natural PGIPs and thus the versatility of their recognition capabilities against the many diverse microbial PGs.

**Abbreviations**

A-L-S: Signal peptide cleavage site; DAMP: Damage-associated molecular pattern; LRR: Leucine-rich repeat; N-X-S/T: N glycosylation site; PG: Polygalacturonase; PGIP: Polygalacturonase inhibiting protein; UPGMA: Unweighted pair group method with arithmetic mean.

**Competing interests**

LBTM and DO are staff members of the South African ARC. The ARC partially financed the publication of this manuscript. The authors declare that they have no competing interest regarding the publication of this article.

**Authors’ contributions**

All authors conceptualised the research. LBTM performed the analyses. DO and IAD supervised the research and contributed to writing of the manuscript. All authors read and approved the final manuscript.

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