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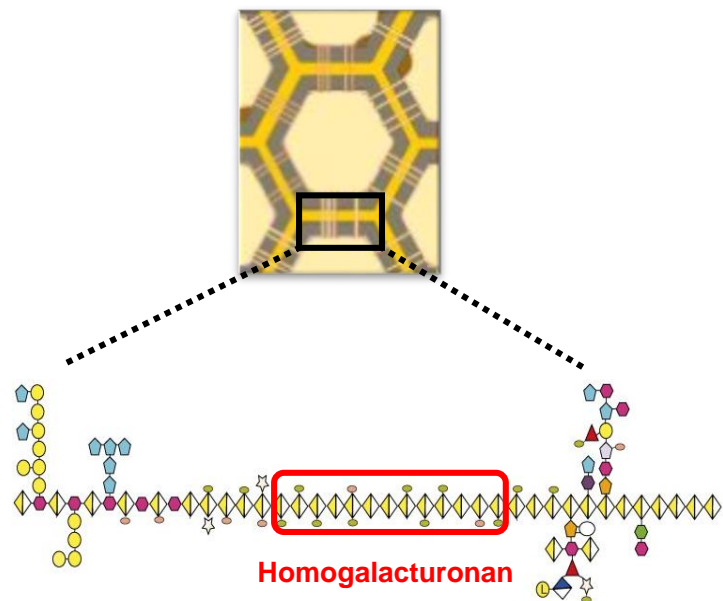
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Abstract:	<p>Pectins, complex polysaccharides and major components of the plant primary cell wall, can be degraded by pectate lyases (PLs). PLs cleave glycosidic bonds of homogalacturonans (HG), the main pectic domain, by β-elimination, releasing unsaturated oligogalacturonides (OGs). To understand the catalytic mechanism and structure/function of these enzymes, we characterized VdPelB from <i>Verticillium dahliae</i>. We first solved the crystal structure of VdPelB at 1.2Å resolution showing that it is a right-handed parallel β-helix structure. Molecular dynamics (MD) simulations further highlighted the dynamics of the enzyme in complex with substrates that vary in their degree of methylesterification, identifying amino acids involved in substrate binding and cleavage of non-methylesterified pectins. We then biochemically characterized wild type and mutated forms of VdPelB. Pectate lyase VdPelB was most active on non-methylesterified pectins, at pH8.0 in presence of Ca²⁺ ions. The VdPelB-G125R mutant was most active at pH9.0 and showed higher relative activity compared to native enzyme. The OGs released by VdPelB differed to that of previously characterized PLs, showing its peculiar specificity in relation to its structure. OGs released from <i>Verticillium</i>-partially tolerant and sensitive flax cultivars differed which could facilitate the identification VdPelB-mediated elicitors of defence responses.</p>
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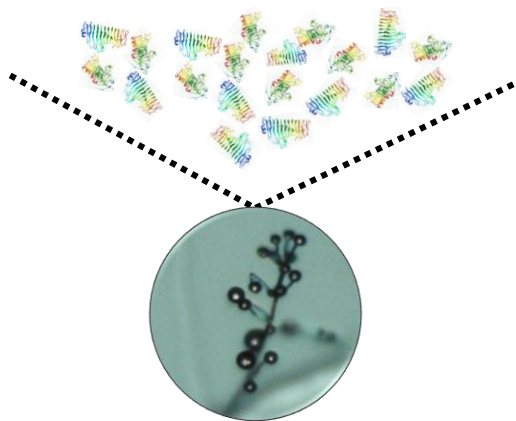
Highlights

- First crystallographic structure of *Verticillium* pectate lyase.
- Identification of key amino-acids for the activity
- Biochemical characterization of the enzyme and LC-MS/MS analysis of the oligogalacturonides (OG) produced
- Susceptible or partially resistant flax cultivars differ in OGs produced

PRIMARY CELL WALL

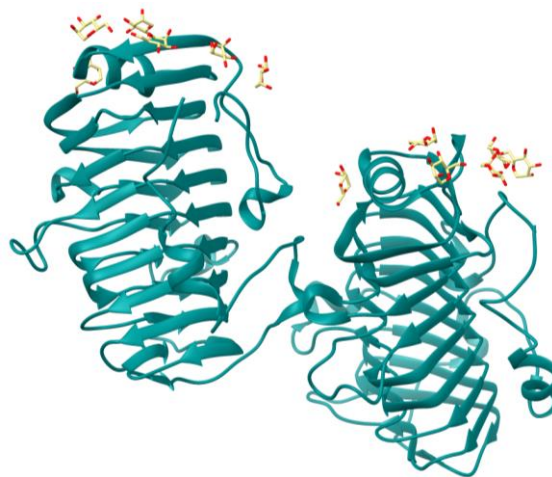


Pectate lyase like (PLLs) degrading enzymes

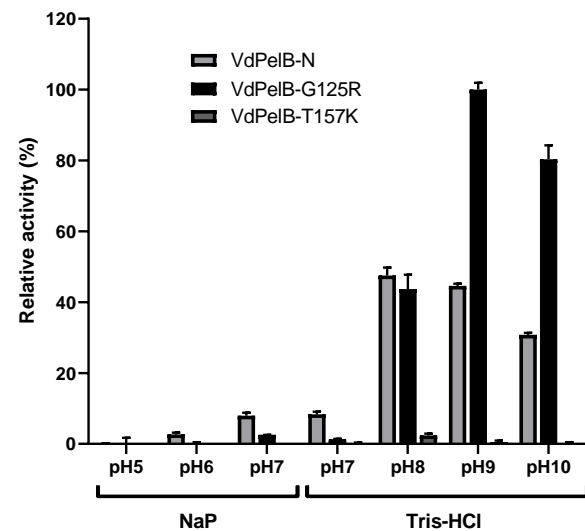


VERTICILLIUM DAHLIAE

VdPeIB CRYSTAL STRUCTURE

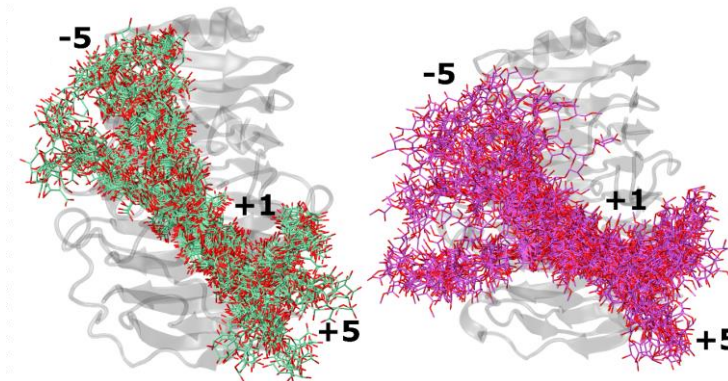


BIOCHEMICAL CHARACTERIZATION

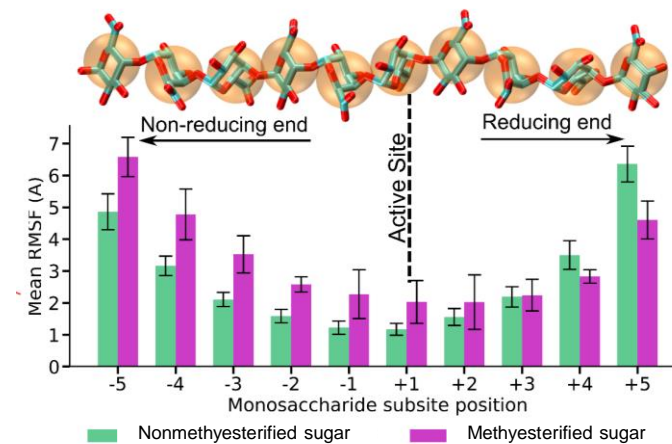


MD SIMULATIONS

VdPeIB substrate dynamics



RMSF analysis



Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Abstract

Pectins, complex polysaccharides and major components of the plant primary cell wall, can be degraded by pectate lyases (PLs). PLs cleave glycosidic bonds of homogalacturonans (HG), the main pectic domain, by β -elimination, releasing unsaturated oligogalacturonides (OGs). To understand the catalytic mechanism and structure/function of these enzymes, we characterized VdPelB from *Verticillium dahliae*. We first solved the crystal structure of VdPelB at 1.2Å resolution showing that it is a right-handed parallel β -helix structure. Molecular dynamics (MD) simulations further highlighted the dynamics of the enzyme in complex with substrates that vary in their degree of methylesterification, identifying amino acids involved in substrate binding and cleavage of non-methylesterified pectins. We then biochemically characterized wild type and mutated forms of VdPelB. Pectate lyase VdPelB was most active on non-methylesterified pectins, at pH8.0 in presence of Ca^{2+} ions. The VdPelB-G125R mutant was most active at pH9.0 and showed higher relative activity compared to native enzyme. The OGs released by VdPelB differed to that of previously characterized PLs, showing its peculiar specificity in relation to its structure. OGs released from *Verticillium*-partially tolerant and sensitive flax cultivars differed which could facilitate the identification VdPelB-mediated elicitors of defence responses.

Keywords: Pectate lyase, pectins, homogalacturonan, oligogalacturonides, *Verticillium dahliae*.

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2 **structural, dynamical and biochemical characterizations**

3

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21

22 **Abstract**

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43 *dahliae*.

44

45 1. Introduction

46 Primary cell wall, a complex structure of proteins and polysaccharides, cellulose and
47 hemicelluloses, is embedded in a pectin matrix. Pectins, are complex polysaccharides
48 composing up to 30% of cell wall dry mass in dicotyledonous species [1]. Pectin is mainly
49 constituted of homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan
50 II (RG-II) domains, but its composition can differ between plant organs and among species.
51 The most abundant pectic domain is HG, a linear homopolymer of α -1,4-linked galacturonic
52 acids (GalA), which represents up to 65% of pectins [2]. During synthesis, HG can be O-
53 acetylated at O-2 or O-3 and/or methylesterified at C-6 carboxyl, before being exported at the
54 cell wall with a degree of methylesterification (DM) of ~80% and a degree of acetylation (DA)
55 of ~5-10%, depending on species [3]. At the wall, HG chains can be modified by different
56 enzyme families, including pectin acetyltransferase (PAEs; EC 3.1.1.6), pectin methylesterases
57 (PMEs; CE8, EC 3.1.1.11), polygalacturonases (PGs; GH28, EC 3.2.1.15, EC 3.2.1.67, EC
58 3.2.1.82), and pectin lyases-like (PLLs), which comprise pectate lyases (PLs; EC 4.2.2.2) and
59 pectin lyase (PNLs, EC 4.2.2.10). All these enzymes are produced by plants to fine-tune pectin
60 during development [4–8], but they are also secreted by most phytopathogenic bacteria and
61 fungi during plant infection [9–13]. PMEs and PAE hydrolyse the O6-ester and O2-acetylated
62 linkages, respectively, leading to a higher susceptibility of HG to PG- and PLL-mediated
63 degradation [14]. PLL are pectolytic enzymes that cleave HG via a β -elimination mechanism
64 leading to the formation of an unsaturated C4-C5 bond [15], and can be divided into two
65 subfamilies depending on their biochemical specificities: i) PLs have a high affinity for non- or
66 low-methylesterified pectins and an optimal pH near 8.5. Their activity requires Ca^{2+} ions. ii)
67 PNLs are most active on high-DM pectins at acidic pH values [16]. Both type of enzymes can
68 degrade HG chains, and release oligogalacturonides (OGs), but their mode of action can differ.
69 For PLs both endo and exo modes of action have been described, while only endo-PNL have
70 been characterised so far [17]. For the latter, it was notably shown that endo-PNLs from *B.*
71 *fuckeliana*, *A. parasiticus* and *Aspergillus* sp., can first release OGs with degrees of
72 polymerisation (DP) 5–7, that are subsequently used as substrates, generating OGs of DP3 and
73 DP4 as end-products [9,18,19]. Despite having the same DP, the final products can differ in
74 their degrees and patterns of methylesterification and acetylation (DM/DA) depending on the
75 enzymes' specificities; implying potential differences in substrate binding, and therefore in
76 PLLs fine structures. Several crystallographic structures of bacterial and fungal PLL have been
77 reported [15,20–24]. Overall, the PLL fold resemble that of published PME, PGs and

78 rhamnogalacturonan lyases [25–27], and is composed of three parallel β -sheets forming a right-
79 handed parallel β -helix. The three β -sheets are called PB1, PB2 and PB3 and the turns
80 connecting them T1, T2 and T3 [28]. The active site features three Asp, localized on the PB1
81 β -sheet and, in the case of PLs, accommodates Ca^{2+} [29]. In PNLs, Ca^{2+} is, on the other hand,
82 replaced by Asp [15]. Additionally, the PL binding site is dominated by charged acidic and
83 basic residues (Gln, Lys, Arg) which can accommodate negatively charged pectate substrates.
84 In contrast, the PNL binding site is dominated by aromatic residues [15,29], which have less
85 affinity for lowly methylesterified pectins. These differences in structure could translate into
86 distinct enzyme dynamics when in complex with substrates of varying degrees of
87 methylesterification.

88 In fungi, PLLs are encoded by large multigenic families which are expressed during
89 infection. *Verticillium dahliae* Kleb., a soil-borne vascular fungus, targets a large number of
90 plant species, causing Verticillium wilt disease to become widespread among fiber flax, with
91 detrimental effects to fiber quality [30–32]. *V. dahliae* infects plants by piercing the root surface
92 using hyphae. When Verticillium reaches the vascular tissue, hyphae start to bud and form
93 conidia which progress in xylem vessels before germinating and penetrating adjacent vessel
94 elements to start another infection cycle. [33,34]. During its colonization and proliferation
95 Verticillium produces and secretes a number of pectinolytic enzymes, including thirteen PLLs.
96 Considering the role of PLLs in determining pathogenicity, it is of paramount importance to
97 determine their biochemical and structural properties [30,35,36]. This could allow engineering
98 novel strategies to control or inhibit, the pathogen's pectinolytic arsenal. For this purpose, we
99 characterized, via combined experimental and computational approaches, one *V. dahliae* PLL
100 (VdPelB, VDAG_04718) after its heterologous expression in *P. pastoris*. The obtention of the
101 3D structure of VdPelB after X-ray diffraction and the analysis of enzyme dynamics when in
102 complex with substrates of distinct DM, allowed the identification of the residues favouring
103 pectate lyase (PL) activity. Experiments confirmed the importance of these residues in
104 mediating PL activity showing that VdPelB is a *bona fide* PL, that releases peculiar OG as
105 compared to previously characterized PLs. More importantly, the OGs released from roots of
106 *Verticillium*-partially tolerant and sensitive flax cultivars differed, paving the way for the
107 identification of VdPelB-mediated OGs that can trigger plant defence mechanisms.

108

109 **2. Material and methods**

110 **2.1. Bioinformatical analysis**

111 *Verticillium dahliae* PLL sequences were retrieved using available genome database
112 (ftp.broadinstitute.org). SignalP-5.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) was
113 used for identifying putative signal peptide and putative glycosylation sites were predicted
114 using NetOGlyc 4.0 Server (<http://www.cbs.dtu.dk/services/NetOGlyc/>). Sequences were
115 aligned and phylogenetic analysis was carried out using MEGA multiple sequence alignment
116 program (<https://www.megasoftware.net/>). Homology models used for molecular replacement
117 were created using I-TASSER structure prediction software
118 (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) and UCSF Chimera
119 (<http://www.cgl.ucsf.edu/chimera/>) was used for creation of graphics.

120 **2.2. Fungal strain and growth**

121 *V.dahliae* was isolated from CALIRA company flax test fields (Martainneville, France) and
122 was kindly provided by Linéa-Semences company (Grandvilliers, France). Fungus was grown
123 as described in Safran et al. [37]. Briefly, fungus was grown in polygalacturonic acid sodium
124 salt (PGA, P3850, Sigma) at 10 g.L⁻¹ and in pectin methylesterified potassium salt from citrus
125 fruit (55–70% DM, P9436, Sigma) solutions to induce expression of *PLLs*. After 15 days of
126 growth in dark conditions at 25°C and 80 rpm shaking, mycelium was collected and filtered
127 under vacuum using Buchner flask. Collected mycelium was frozen in liquid nitrogen,
128 lyophilized and ground. Isolation of RNA and cDNA synthesis was realized as previously
129 described in Lemaire et al.[38].

130 **2.3. Cloning, heterologous expression and purification of VdPelB**

131 *V. dahliae* PelB gene (VdPelB, UNIPROT: G2X3Y1, GenBank: EGY23280.1,
132 *VDAG_04718*) consists of 1 single exon of 1002 bp length. The coding sequence, minus the
133 nucleotides encoding signal peptide, was PCR-amplified using cDNA and gene-specific
134 primers while VdPelB mutants were generated by PCR mutagenesis, a method for generating
135 site-directed mutagenesis, using specific primers carrying mutations (Table S1). Cloning of the
136 PCR-amplified sequences in pPICZαB, sequencing and expression in *P. pastoris* heterologous
137 expression system as well as purification steps were done as previously described in Safran et
138 al. [37].

139 **2.4. Crystallization of VdPelB**

140 Pectate lyase VdPelB was concentrated at 10 mg.mL⁻¹ in Tris-HCl pH-7.5 buffer.
141 Crystallization conditions were screened using the sitting-drop vapor-diffusion method. Pectate

142 lyase VdPelB (100 nL) was mixed with an equal volume of precipitant (1:1) using Mosquito
143 robot (STP Labtech). The crystals that resulted in best diffraction data were obtained with 0.1
144 M MIB (Malonic acid, Imidazole, Boric acid system) at pH8.0, with 25 % PEG 1500 as the
145 precipitant (condition B5 from the PACT premier kit, Molecular Dimensions, Sheffield, UK)
146 after 1 month. Optimization was realized using the hanging drop vapor-diffusion method
147 forming the drop by mixing 1 μ L of precipitant solution with 1 μ L of the enzyme. The crystals
148 (120 μ m x 30 μ m) were cryo-protected by increasing PEG 1500 concentration to 35%, before
149 mounting them in a loop and flash-cooling them in liquid nitrogen.

150 **2.5. VdPelB X-ray data collection and processing**

151 X-ray diffraction data were collected at the PROXIMA-2a beamline of the Soleil
152 synchrotron (Saint Aubin, France), at a temperature of -173°C using an EIGER 9M detector
153 (Dectris). Upon a first data collection to 1.3 Å resolution, three more data sets were collected
154 from the same crystal in order to obtain a complete data set. Thereby the kappa angle was tilted
155 once to 30°, once to 60° and finally a helical data set was collected at 1.2 Å resolution. The
156 reflections of each data set were indexed and integrated using XDS [39], scaled and merged
157 using XSCALE [40]. The VdPelB crystal has a primitive monoclinic lattice in the P 1 2₁ 1 space
158 group, with two molecules contained in asymmetric unit [41].

159 **2.6. Structure solution and refinement**

160 The structure of VdPelB was solved by molecular replacement using *Phaser* [42]. The data
161 were phased using pectate lyase BsPelA (PDB: 3VMV, Uniprot D0VP31), as a search model
162 [43]. Model was build using *Autobuild* and refined using *Refine* from PHENIX suite [44]. The
163 model was iteratively improved with *Coot* [45] and *Refine*. The final structure for VdPelB has
164 been deposited in the Protein Data Bank (PDB) as entry 7BBV.

165 **2.7. VdPelB biochemical characterization**

166 Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United
167 States) was used to determine the protein concentration, with Bovine Serum Albumin (A7906,
168 Sigma) as a standard. Deglycosylation was performed using Peptide-N-Glycosidase F (PNGase
169 F) at 37 °C for one hour according to the supplier's protocol (New England Biolabs, Hitchin,
170 UK). Enzyme purity and molecular weight were estimated using a 12% SDS-PAGE and mini-
171 PROTEAN 3 system (BioRad, Hercules, California, United States). Gels were stained using

172 PageBlue Protein Staining Solution (Thermo Fisher Scientific) according to the manufacturer's
173 protocol.

174 The substrate specificity of VdPelB was determined using PGA (81325, Sigma) and citrus
175 pectin of various DM: 20–34% (P9311, Sigma), 55–70% (P9436, Sigma) and >85% (P9561,
176 Sigma), with 0.5 μM CaCl_2 or 5 μM EDTA (Sigma) final concentrations. Enzyme activity was
177 measured by monitoring the increase in optical density at 235 nm due to formation of
178 unsaturated uronide product using UV/VIS Spectrophotometer (PowerWave Xs2, BioTek,
179 France) during 60 min. The optimum temperature was determined by incubating the enzymatic
180 reaction between 20 and 70°C for 12 min using PGA as a substrate (0.4%, w/v). The optimum
181 pH was determined between pH5.0 and 10.0 using sodium phosphate (NaP, pH5.0 to 7.0) and
182 Tris-HCl buffer (pH7.0 to 10.0) and 0.4% (w/v) PGA as a substrate. VdPelB and VdPelB-
183 G125R kinetic parameters, K_m , V_{\max} and k_{cat} , were calculated using GraphPad Prism (8.4.2).
184 The reactions were performed using 0.75 to 15 $\text{mg}\cdot\text{mL}^{-1}$ PGA concentrations at 50 mM Tris-
185 HCl pH8.0 (native) and 9.0 (G125R) during 12 min at 35°C. All experiments were realized in
186 triplicate. The statistical analysis was done using the Welch's t-test.

187 **2.8. Digestion of commercial pectins and released OGs profiling**

188 OGs released after digestions by recombinant VdPelB or commercially available
189 Aspergillus PL (named AsPel) were identified as described in Voxeur et al., 2019 [9], using a
190 novel in-house OGs library. Briefly, DM 20–34% (P9311, Sigma) and sugar beet pectin with
191 DM 42% and degree of acetylation (DA) 31% (CP Kelco, Atlanta, United States) were prepared
192 at 0.4 % (w/v) final concentration diluted in 50 mM Tris-HCl buffer (pH8.0) and incubated
193 with either VdPelB or AsPel (E-PCLYAN, Megazyme). For each substrate, enzyme
194 concentrations were adjusted to have enzymes at iso-activities (Table S2). For each substrate
195 two dilutions, were used for analysing OGs released in early, VdPelB-2 and AsPel-2, and late
196 phase, VdPelB-1 and AsPel-1, of digestions. Digestions were performed overnight. Non-
197 digested pectins were pelleted by centrifugation and the supernatant dried in a speed vacuum
198 concentrator (Concentrator plus, Eppendorf, Hamburg, Germany). Separation of OGs was done
199 as previously described using an ACQUITY UPLC Protein BEH SEC column (125Å, 1.7 μm ,
200 4.6 mm x 300 mm) at a flow rate of 0.4 $\text{ml}\cdot\text{min}^{-1}$. MS detection was performed using an
201 ACQUITY UPLC H-Class system coupled to a SYNAPT G2-Si-Q-TOF hybrid quadrupole
202 time-of-flight instrument (Waters) equipped with an electrospray ionization (ESI) source (Z-
203 spray) and an additional sprayer for the reference compound (lock spray). Samples were
204 analysed by ESI-high-resolution MS (HRMS) and MS/MS in negative ionization mode. Data

205 acquisition and processing were performed with MASSLYNX software (v.4.1; Waters).
206 [9,37,46].

207 The intensities were defined as the area under the curve, for each OG. Peak areas were clustered
208 by hierarchical clustering with complete linkage on the euclidian distance matrix and visualized
209 in the heatmap-package using R version 3.6.0. The statistical analysis was done using the
210 Welch's t-test.

211 **2.9. Molecular Dynamics simulations**

212 Two sets of molecular dynamics (MD) simulations were conducted on the VdPelB protein:
213 one in complex with a fully non-methylesterified polygalacturonate deca-saccharide, and the
214 other with a fully methylesterified polygalacturonate deca-saccharide. Parameters specified by
215 the AMBER14SB_parmbsc1 forcefield [47] were used to create the molecular topologies of
216 the complexes. Each complex was set up in a cubic box with solute-box distances of 1.0 nm
217 and solvated with water molecules specific to the TIP3P water model [48]. Na⁺ and Cl⁻ ions
218 were added to neutralise the system's net charge and reach a salt concentration of 0.165 M.
219 Using a steep-descent algorithm with a step size of 0.01, energy minimisation was performed
220 to resolve clashes between particles, with convergence being established at a particle-particle
221 force of 1000 kJ mol⁻¹ nm⁻¹. Particle-particle forces were calculated by considering van der
222 Waals and electrostatic interactions occurring up to 1.0 nm, as well as long-range electrostatics
223 treated in the Fourier space using the Particle Mesh Ewald (PME) summation method. Solvent
224 equilibration was attained post minimisation in two stages: through the NVT and NPT
225 ensembles, to reach constant temperature and pressure, respectively. Equilibration of the
226 solvent under the NVT ensemble was conducted for 1 ns, integrating the equation of motion at
227 a time step of 2 fs. The target reference temperature was 310.15 K, coupled every 0.1 ps using
228 the V-rescale thermostat³. Based on the Maxwell-Boltzmann distribution [49] at 310.15 K,
229 random velocities were then assigned to each particle in the system. Finally, solvent
230 equilibration under the NPT ensemble was conducted for 1 ns, continuing from the last step of
231 the previous equilibration, in terms of particle coordinates and velocities, at a reference
232 temperature of 310.15 K, coupled every 0.1 ps using the V-rescale thermostat [50]. Here,
233 pressure coupling was isotropically coupled every 2.0 ps, at 1 bar, using the Parrinello-Rahman
234 barostat [51]. Particle-particle interactions were computed by constructing pair lists using the
235 Verlet scheme. Short-range van der Waals and electrostatic interactions sampled through a
236 Coulomb potential, were calculated at a cutoff of 1.0 nm. The PME algorithm [52] was used to
237 compute long-range electrostatic interactions beyond this cut-off in the Fourier space, utilising

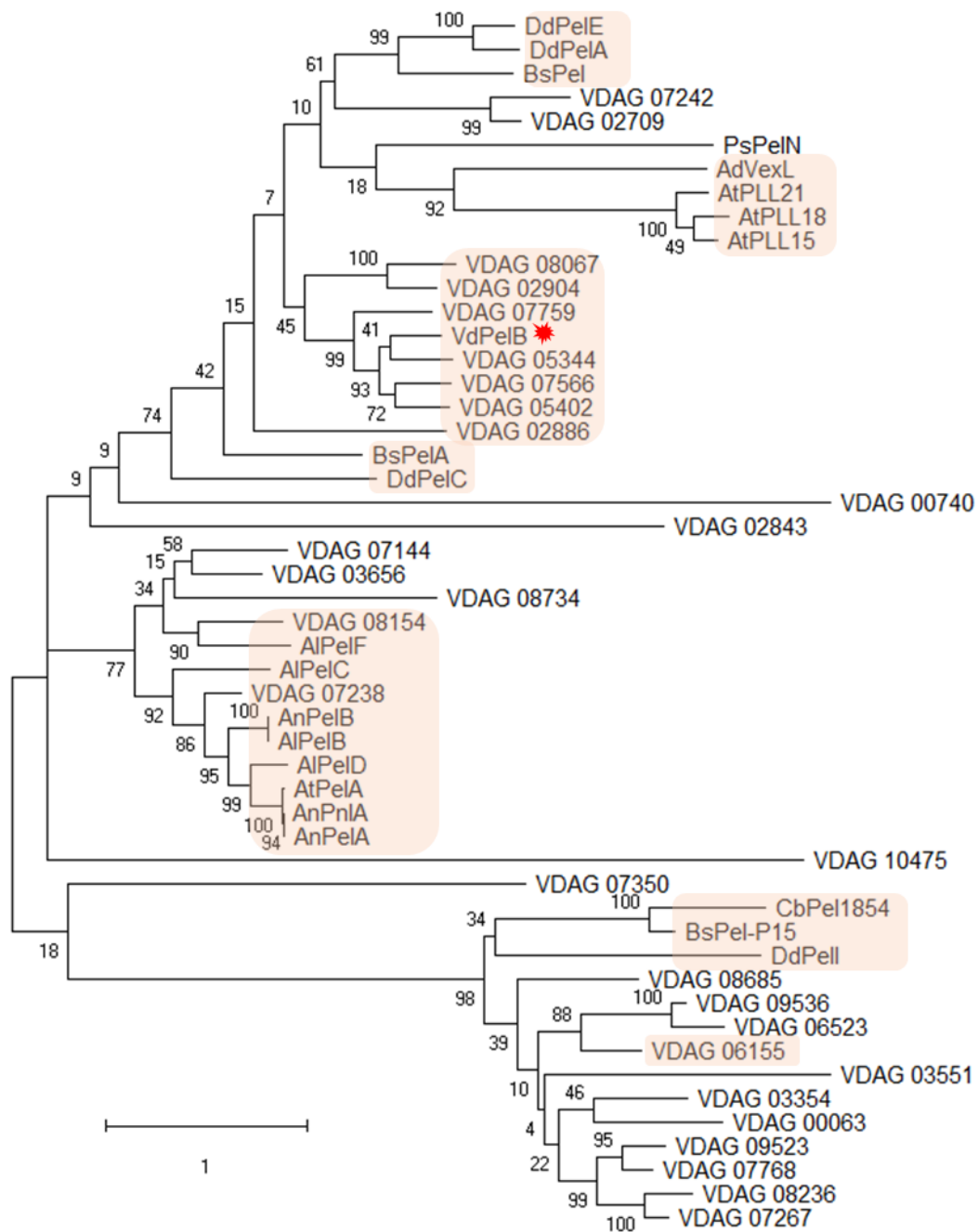
238 a Fourier grid spacing of 0.16 and a cubic B-spline interpolation level at 4. The simulations
239 were then performed on in-house machines, using GROMACS (Groningen Machine for
240 Chemical Simulations) version 2021.37. Each set of simulations were run for 150 ns each, at a
241 time step of 2 fs, with molecular dynamics trajectories written out every 10 ps. Simulations
242 were replicated 7 times for a total production run time of 1.05 μ s per complex. Replicates
243 differed with respect to the random particle velocity sets computed under the NVT ensemble.
244 For analysis, the first 50 ns of each production run were discarded as equilibration time. In-
245 house Python 3 scripts implemented using Jupyter notebooks [53] were used to carry out
246 analyses. Figures were created and rendered with Matplotlib [54] and VMD (Visual Molecular
247 Dynamics)[55].

248

249 3. Results and Discussion

250 3.1. Sequence and phylogeny analysis

251 In addition to 9 polygalacturonases (PGs) and 4 pectin methylesterases (PMEs), *V.*
252 *dahliae* encodes 30 putative endo-pectate lyases (EC 4.2.2.2), exo-pectate lyases (EC 4.2.2.9),
253 endo-pectin lyases (EC 4.2.2.10), belonging to PL1-PL3 and PL9 families, respectively [56].
254 For hierarchical clustering of *Verticilium*'s sequences with other PLLs, fifty-one amino acid
255 sequences encoding putative PLLs, belonging to bacteria, fungi and plants were aligned and a
256 phylogenetic tree was built. Different clades can be distinguished. (**Fig. 1**). *V. dahliae* PelB
257 (VdPelB, VDAG_04718) clustered with VDAG_05344 (59.68% sequence identity) with close
258 relations to VDAG_05402 (57.19% sequence identity) and VDAG_07566 (56.95% sequence
259 identity). VDAG_05402 and VDAG_05344, that are found in the protein secretome, have
260 orthologs in *V. alfalfa* (VDBG_07839 and VDBG_10041), which were shown to possess
261 putative lyase activity [36,57]. Plant PLLs from *A. thaliana* (AtPLL21, AtPLL15 and AtPLL18)
262 formed a separate clade with close connections to *A. denitrificans* (AdVexL), a PLL homologue
263 [58]. *D. dadanti* (DdPelI), *Bacillus Sp.* KSM-P15 (BsPel-P15) and *C. bescii* (CbPel1854)
264 formed separate clades similarly to *B. subtilis* and *D. dadantii* PLs (BsPel, DdPelA and
265 DdPelC). The clade corresponding to PNLs consisting of *A. tubingensis* PelA (AtPelA), *A.*
266 *niger* (AnPelA, AnPnlA and AnPelB), *A. luchuensis* AlPelB was closely related to
267 VDAG_07238 and VDAG_08154 which were indeed annotated as putative PNLs [18,56].
268 VDAG_06155 was previously named VdPel1 and previously characterized as a pectate lyase
269 [35].



270

271 **Fig. 1. Phylogenetic analysis of *V. dahliae* pectate lyase VdPelB with selected PLLs**

272 Phylogenetic tree representing *V. dahliae* VdPelB (VDAG_04718, G2X3Y1, red star) amino acid sequence in
 273 comparison with PLLs from *Verticillium* [VDAG_00740 (G2WQU8), VDAG_02904 (G2WXC5), VDAG_05344
 274 (G2X628), VDAG_07242 (G2XBA4), VDAG_07759 (G2XC77), VDAG_02709 (G2WWT0), VDAG_02843
 275 (G2WX64), VDAG_02886 (G2WXA7), VDAG_03656 (G2X1P5), VDAG_05402 (G2X597), VDAG_07144
 276 (G2X9U9), VDAG_07238 (G2XBA0), VDAG_07566 (G2XBY8), VDAG_08067 (G2XD35), VDAG_08154
 277 (G2XDC2), VDAG_08734 (G2XF02), VDAG_10475 (G2XJZ3), VDAG_03354 (G2WZB2), VDAG_03551
 278 (G2WZV9), VDAG_07267 (G2XBC9), VDAG_07768 (G2XC86), VDAG_08236 (G2XDK4), VDAG_06155
 279 (G2X8L4), VDAG_06523 (G2X7R5), VDAG_08685 (G2XEV0), VDAG_09523 (G2XH91), VDAG_09536
 280 (G2XHA4), VDAG_00063 (G2WR80), VDAG_07350 (G2XGG7)], *Arabidopsis thaliana* [AtPLL15
 281 (At5g63180), AtPLL18 (At3g27400), AtPLL21 (At5g48900)], *Dickeya dadanti* [DdPelA (P0C1A2), DdPelC

282 (P11073), DdPelE (P04960), DdPelI (O50325)], *Bacillus subtilis* [BsPel (P39116)], *Bacillus Sp.* KSM-P15
283 [BsPel-P15 (Q9RHW0)], *Bacillus sp.* N16-5 [BsPelA (D0VP31)], *Aspergillus niger* AnPelA [(Q01172), AnPelB
284 (Q00205), AnPn1A (A2R3I1)], *Achromobacter denitrificans* [AdVexL (A0A160EBC2)], *Aspergillus tubingensis*
285 [AtPelA (A0A100IK89)], *Aspergillus luchuensis* [AlPelB (G7Y0I4)], *Acidovorax citrulli* [AcPel343, (A1TSQ3)],
286 *Paenibacillus sp.* 0602 [PsPelN (W8CR80)], *Caldicellulosiruptor bescii* [CbPel1854 (B9MKT4)]. Maximum
287 likelihood tree was constructed with 1000 bootstrap replicates. Most important clades are indicated in orange
288 squares while VdPelB is marked by red star. Amino acids sequences were retrieved from Uniprot and TAIR.

289 **3.2. Expression and purification of VdPelB**

290 Since the expression was performed using the pPICZ α B vector with the yeast alpha-factor
291 directing VdPelB secretion in the culture media, the protein could be easily recovered and
292 purified. The protein is composed of 343 amino acids, including the 6xHIS tag at the C-terminus
293 used for affinity chromatography purification. After purification, VdPelB purity was assessed
294 using SDS-PAGE with an apparent molecular mass of ~38 kDa (**Fig. S1**), higher than what was
295 predicted on the basis of the amino acid sequence (33.8 kDa). However, this shift is likely to
296 correspond to the two tags that are fused to the protein (His and C-myc) and to the presence of
297 19 putative O-glycosylation sites, as predicted by NetOGlyc 4.0 Server.

298 **3.3. VdPelB has a right-handed parallel β -helix fold**

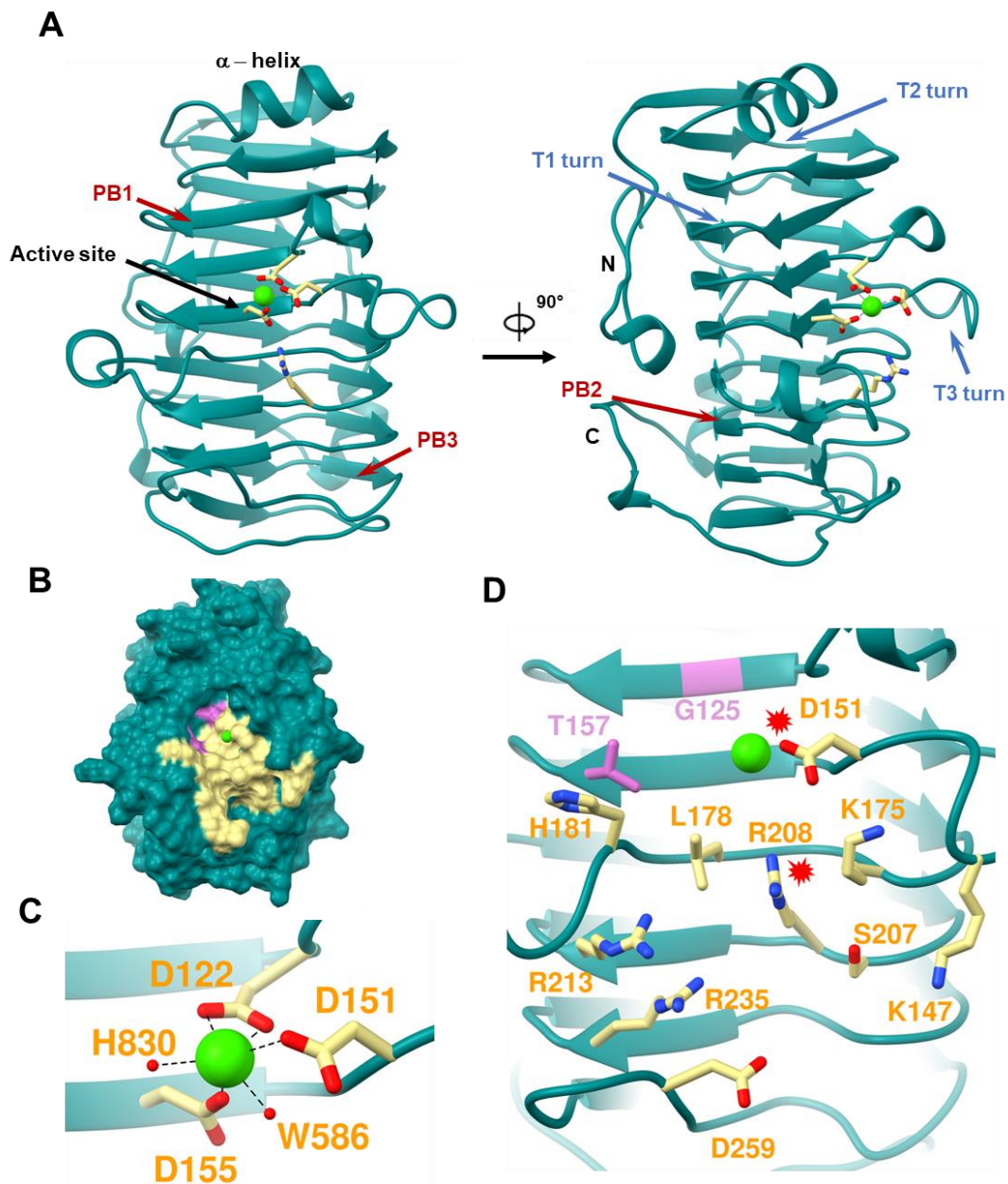
299 Pectate lyase VdPelB was crystallized and its 3D structure was determined using X-ray
300 diffraction. VdPelB crystallized in monoclinic P 1 2₁ 1 asymmetric unit. Four data sets,
301 collected from the same crystal at 1.2 Å resolution, were integrated, scaled and merged. There
302 are two molecules in the asymmetric units: chains A and B are highly similar with a C α root
303 mean square deviation (rmsd) value of 0.227 Å (**Fig. S2A**). The VdPelB structure consists of
304 298 amino acids with 18 at the N-terminus and 27 amino acids at the C-terminus that were not
305 resolved because of poor electron densities, while overall electron densities were well defined.
306 While no N-glycosylation sites could be revealed on the VdPelB structure, six O-glycosylation
307 sites carrying mannose are visible for each molecule: T22, T44, T45, T46, S48 and T54, in
308 accordance with the shift in size previously observed (**Fig. S2A**). During data acquisition no
309 heating of the crystal was observed, as shown by low B factors and good occupancies (**Fig. S3A**
310 **and B**). The final models' geometry, processing and refinement statistics are summarized
311 (**Table 1**). The VdPelB's structure has been deposited in the Protein Data Bank as entry 7BBV.

312 **Table 1. Data collection, processing and refinement for VdPelB**

Characteristics	VdPeIB
Data collection	
Diffraction source	PROXIMA2
Wavelength (Å)	0.980
Temperature (°C)	-100.15
Detector	DECTRIS EIGER X 9M
Crystal-to-detector distance (mm)	115.02
Rotation range per image (°)	0.1
Total rotation range (°)	360
Crystal data	
Space group	P1 21 1
<i>a, b, c</i> (Å)	60.89, 59.69, 93.87
α, β, γ (°)	90.00, 96.35, 90.00
Subunits per asymmetric unit	2
Data statistics	
Resolution range (Å)	60.52-1.2 (1.243 - 1.2)
Total No. of reflection	8536161 (112943)
No. of unique reflection	207497 (19983)
No. of reflections, test set	10362 (1003)
R_{merge} (%)	0.1036 (4.709)
Completeness (%)	99.30 (96.11)
$\langle I/\sigma(I) \rangle$	18.03 (0.22)
Multiplicity	41.1 (5.6)
$CC_{1/2}$ (%)	1 (0.116)
Refinement	
$R_{\text{crys}}/R_{\text{free}}$ (%)	17.3 / 19.7
Average B – factor (Å ²)	33.18
No. of non-H atoms	
Protein	9097
Ion	2
Ligand	234
Water	1052
Total	10385
R.m.s. deviations	
Bonds (Å)	0.013
Angles (°)	1.39
Ramachandran plot	
Most favoured (%)	95.1
Allowed (%)	4.90
Outlier (%)	-

314 The VdPelB has a right-handed parallel β -helix fold which is common in pectinases
315 [59]. The β -helix is formed by three parallel β -sheets - PB1, PB2 and PB3 which contain 7, 10
316 and 8 β -strands, respectively. Turns connecting the PB1-PB2, PB2-PB3 and PB3-PB1 β -sheets
317 are named T1-turns, T2-turns and T3-turns, respectively, according to Yoder and Jurnak (**Fig.**
318 **2A, S4A and B**) [60]. T1-turns consist of 2-14 amino acids and builds the loop around the
319 active site on the C-terminus. T2 turns mostly consist of 2 amino acids with Asn being one of
320 the predominant amino acid, forming an N-ladder with the exception of an N245T mutation in
321 VdPelB [15,20]. Pectate lyase VdPelB has a α -helix on N-terminus end that shields the
322 hydrophobic core and is commonly conserved in PLs and PGs [15,61], while the C-terminus
323 end is also protected by tail-like structure carrying one α -helix. Interestingly N- and C- terminus
324 tails pack against PB2 (**Fig. 2A**). There are only two Cys (C25 and C137) that do not form a
325 disulphide bridge.

326 Sequence and structural alignments show that VdPelB belong to the PL1 family. The
327 VdPelB shares the highest structural similarity with BsPelA (PDB: 3VMV), with 30.06%
328 sequence identity and C α rmsd of 1.202 Å. The second-best structural alignment was with
329 DdPelC (PDB: 1AIR) with 24.20% identity and C α rmsd of 1.453 Å [43,62]. Both of these
330 structures lack the long T3 loop described in *A.niger* pectate lyase (AnPelA, PDB: 1IDJ, **Fig.**
331 **S2B and S5**) [15]. The putative active site is positioned between the T3 and T2 loops (**Fig. 2A**
332 **and B**).



333

334 **Fig. 2. Structure determination of VdPelB**

335 A) Ribbon diagram of VdPelB crystalized in P1 21 1 space group. VdPelB is a right-handed parallel β helical
 336 structure consisting of β strands (red arrows) and turns (blue arrows). VdPelB active site's amino acids are yellow-
 337 colored while Ca atom is green. B) Surface representation of VdPelB binding groove. C) Active site of VdPelB
 338 highlighting conserved amino acids and atoms interacting with Ca. D) Structure of VdPelB binding groove
 339 highlighting amino acids involved in the interaction (yellow) and amino acids not of previously characterized in
 340 PLs (plum). Red stars indicate amino acids from the active site.

341 **3.4. Active site harbours Ca^{2+} that is involved in catalysis**

342 The VdPelB active site is well conserved, harbouring strictly conserved acidic and basic
 343 amino acids that are required for Ca^{2+} binding. Previously reported structures showed that two
 344 Asp (D122 and D155) and one Arg (R208) in VdPelB, are conserved, while D151 can be

345 mutated to Glu, or Arg in PNLs (**Fig. 2C and D**, Mayans et al., 1997). Other conserved amino
346 acids in VdPelB include K175 and R213, with K175 being responsible for binding the carboxyl
347 oxygen while R213 hydrogen bonds to C-2 and C-3 of GalA (**Fig. 2D**) [29,63]. Mutating any
348 of these amino acids leads to decreased enzyme activity [64]. In VdPelB, Ca²⁺ ion is directly
349 coordinated by D122, two carboxyl oxygen, D151, D155 and two water molecules (W568 and
350 W830, **Fig. 2C**). In addition, mutation of D122T (VdPelB numbering) in BsPelA, is responsible
351 for reduced affinity for Ca²⁺ [43]. In the catalytic mechanism, Ca²⁺ is directly involved in
352 acidification of the proton absorption from C5 and elimination of group from C4, generating an
353 unsaturated product. R208 act as a base, similarly to the hydrolysis in the reaction mechanism
354 of the GH28 family [65,66].

355 **3.5. Structural analysis of VdPelB suggests a PL activity mediated by peculiar** 356 **specificities**

357 The VdPelB binding groove comprises a number of basic and acidic amino acids
358 including K147, D151, K175 L178, H181, S207, R208, R213 and R235 and D259 (**Fig. 2C**
359 **and D**), that have previously been shown to be characteristics of PLs. This would suggest an
360 enzyme activity on low DM pectins as amino acids positioned at the binding groove were
361 indeed shown to differ between PNL and PL [15,21,24,29]. These amino acids are indeed
362 mutated in Arg, Trp, Tyr, Gln and Gly in PNLs, which, by reducing their charge, would favour
363 higher affinity for highly methylesterified pectins [15,29]. In VdPelB, as the T3 loop is missing,
364 there are no equivalent to PNL specific W66, W81, W85, W151 amino acids (**Fig. S2B**, AnPelA
365 numbering) and, moreover, W212 and W215 are replaced by K175 and L178 in VdPelB. In
366 BsPelA and DdPelC, these amino acids are replaced by K177/K190 and L180/L193,
367 highlighting the high conservation of amino acids in VdPelB/BsPelA/DdPelC and subsequently
368 in PLs. When a DP4 ligand from DdPelC crystal structure is superimposed to VdPelB, hydrogen
369 bonds and van der Waals interactions are visible with the above-mentioned amino acids (**Fig.**
370 **S6**) [15,21,29,43].

371 Interestingly, despite this rather conserved PL-related binding groove, VdPelB harbors,
372 in the vicinity of the active site, G125 and T157 that are not present in the well characterized
373 DdPelC [62]. At these positions, DdPelC, which was shown to be a *bona fide* PL with a high
374 activity on polygalacturonic acid and alkaline pH with Ca²⁺-dependency, harbours Arg and Lys
375 [15,63,67]. In that respect, the presence of G125 and T157 in VdPelB is similar to that identified
376 in *Bacillus sp.* Pel-22, Pel-66, and Pel-90 and *Bacillus sp.* which showed activity on both PGA
377 and high methylesterified pectins (**Fig. 2B and D**) [22,68,69]. The DdPelC amino acids being

378 overall positively charged, could explain the binding preference towards non-methylesterified,
379 negatively charged substrates in the vicinity of the active site. In contrast, considering the size
380 of Gly and Thr, they would sterically accommodate the increased size of methylesterified
381 substrate. G125 and T157 could therefore account for a potential dual activity of VdPelB.
382 Moreover, in previously characterized PLs and PNLs there is the presence of a small amino
383 acids, Ser or Ala that replace H181. While H181 interacts directly with the substrate these
384 amino acids do not provide this interaction instead the primary Ca^{2+} in the active site induce a
385 substrate conformation that could be recognized by PLs [43]. Finally, L178 is positioned in-
386 between the catalytic Ca^{2+} and R208 and is involved in substrate binding making it a perfect
387 candidate to assess its importance (**Fig. 2D**).

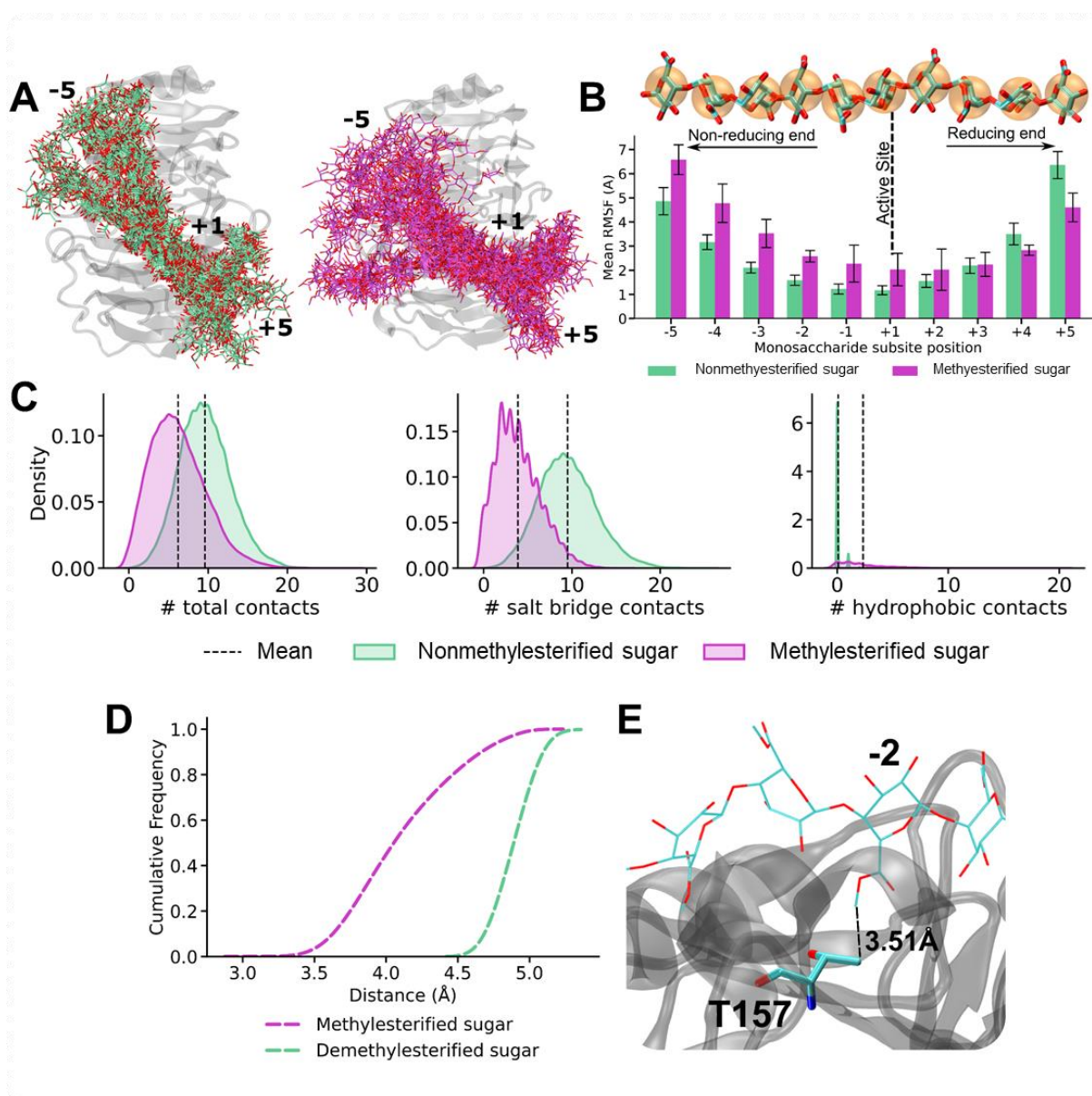
388 **3.6. Molecular dynamic simulations show higher dynamics of VdPelB in complex with** 389 **methylesterified substrates**

390 To determine how the structure of VdPelB and the observed differences in amino-acidic
391 composition might influence the affinity with differently methylated substrates, we performed
392 MD simulations on VdPelB in complex with either a non-methylesterified or fully
393 methylesterified deca-saccharides, which are able to occupy the entire binding groove (**Fig. 3**).
394 MD simulations show substantially differential dynamic profiles for oligosaccharides with and
395 without methylesterification. Expectedly, the dynamics is lowest in proximity of the catalytic
396 subsite (+1) and increases consistently towards both the reducing and non-reducing ends of the
397 substrate: with the highest dynamics found at the non-reducing end (**Fig. 3A**).

398 A quantitative estimation of the substrate dynamics was obtained by monitoring the root
399 square mean fluctuations (RMSF) of each sugar residue and shows that a polygalacturonate
400 substrate associates more stably in the subsites of the binding groove placed towards the sugar's
401 reducing end (subsites -1 to -5), with differences between methylesterified and non-
402 methylesterified substrates approaching the obtained standard deviation. In some cases, the
403 dynamics reversed for non-methylesterified sugars, with a higher RMSF for de-methylesterified
404 sugars towards the saccharide's reducing end (**Fig. 3B**). Moreover, and in line with an observed
405 positively charged binding groove, non-methylesterified substrates retain a higher number of
406 contacts than methylesterified sugars, with salt-bridges contributing the most to the observed
407 differences (**Fig. 3C**).

408 We then additionally and specifically focused on the analysis of the interactions made by
409 T157, which is nested with the -2 subsite of the binding groove. Simulations sample consistently

410 higher contacts formation between a methylesterified sugar docked in subsite -2 and T157, with
 411 distances shifted to lower values when compared to a non-methylesterified sugar (**Fig. 3D**),
 412 even the RMSF of non-methylesterified monosaccharides docked in subsite -2 experience on
 413 average, significantly lower dynamics. Altogether, the formation of a larger number of contacts
 414 between methylesterified saccharides docked in the -2 subsite suggests an active role of T157
 415 in the binding of methylesterified chains: with the butanoic moiety of T157 engaging in
 416 hydrophobic interactions with the methyl-ester presented by methylesterified sugar units (**Fig.**
 417 **3E**). While T157 is seen to have an active role in engaging with the substrate's hydrophobic
 418 moieties, an active role of G125, also in the same subsite, was not observed but it is plausible
 419 that the minimal size of G125 would increase the accommodability of methylesterified sugars
 420 in that position.



421

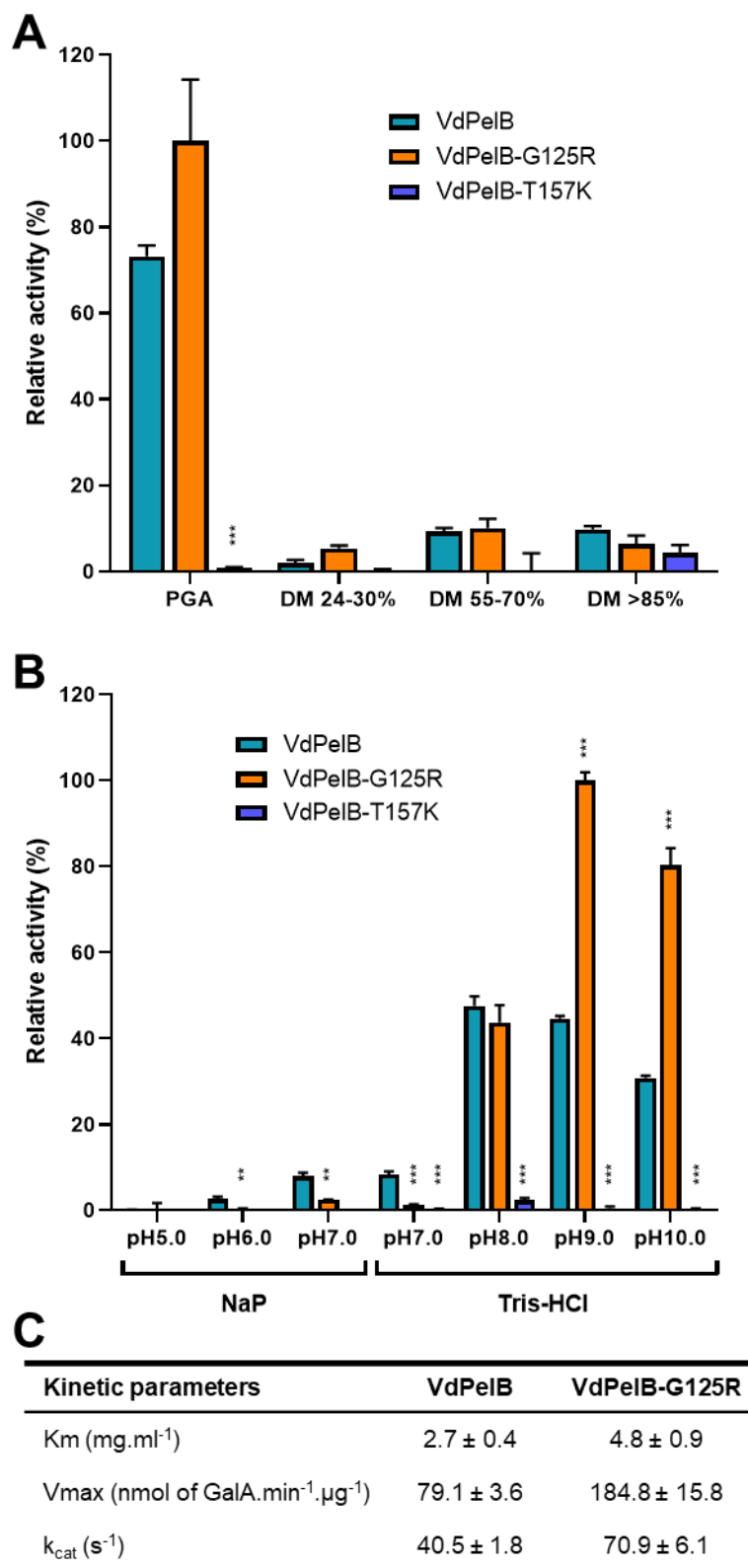
422 **Fig. 3. VdPelB substrate dynamics in complex with fully de-methylesterified and methylesterified complex**

423 A) Ensembles of non-methylesterified (green, left panel) and methylesterified (pink, right panel) HG
424 decasaccharide at every 100 frames for the simulated VdPelB complexes. Substrate within the enzymes' binding
425 grooves are labelled from -5 (HG-non-reducing end) to +5 (HG-reducing end) B) RMSF of non-methylesterified
426 (green) and methylesterified (pink) HG bound across the binding groove of VdPelB. Numbers indicate the beta
427 sheet position from the active site. C) Analysis of the contacts between VdPelB and non-methylesterified (green)
428 and methylesterified substrate (pink). D) Distance between T157K residue and the substrate residues plotted as
429 cumulative frequency. E) T157 hydrophobic interaction with methyl-ester of methylesterified substrate.

430 **3.7. Biochemical characterization of VdPelB**

431 We first determined the activity of VdPelB by following the release of 4,5-unsaturated
432 bonds which can be detected at 235 nm using UV spectrophotometer. The VdPelB activity was
433 first tested for its dependency towards Ca^{2+} . Using a standard PL assay, with PGA as a substrate,
434 an increase in the VdPelB activity was measured in presence of calcium. In contrast in presence
435 of EDTA, used as a chelating agent, no activity was detected, confirming the calcium-dependent
436 activity of the enzyme (**Fig. S7A**) [15]. Activity measured in absence of added CaCl_2 reflects
437 the presence of calcium from the culture media that is bound to VdPelB during production, and
438 previously identified in the 3D structure. We tested the effects of increasing CaCl_2
439 concentrations and showed that the maximum activity was already reached when using as low
440 as 0.125 μM (**Fig. S7B**). To test the substrate-dependence of VdPelB, four substrates of
441 increasing degrees of methyl-esterification were used. The VdPelB showed the highest activity
442 on PGA, with less than 10% of the maximum activity measured on the three others substrates
443 (**Fig. 4A**). This shows that, as inferred from above-mentioned structural and dynamical data,
444 The VdPelB act mainly as a PL although it can still show residual activity on high DM pectins.
445 Considering this, PGA was used as substrate to test the pH-dependence of the enzyme's activity
446 in sodium acetate and Tris-HCl buffers (**Fig. 4A**). Pectate lyase VdPelB was most active at pH
447 8, with only a slight decrease in activity at pH 9 (93%). In contrast, the relative activity at pH
448 5-7 was close to null. The pH optimum for VdPelB was the same as *B. fuckeliana* Pel (pH 8)
449 [70], close to that reported for *D. dadantii* PelN (pH 7.4) [71], but was lower to that measured
450 for *B. clausii* Pel (pH 10.5) [72]. In contrast, the pH optimum was higher compared to five PNLs
451 from *Aspergillus* sp. AaPelA (pH 6.1), AtPelA (pH 4.5), AtPelA (pH 6.4), AtPelD (pH 4.3)
452 [18] and *A. parasiticus* Pel (pH 4) [19]. The optimum temperature assay showed that VdPelB
453 was most active at 35°C (**Fig. S8**). The VdPelB appeared less heat-tolerant as compared to
454 thermophilic PLLs reported from *Bacillus* sp. RN1 90°C [73], *B. clausii* Pel, 70°C [72], *B.*
455 *subtilis* Pel168, 50°C [74]. However, its optimum temperature is in the range of that measured

456 for *X. campestris* Pel [75] and cold-active Pel1 from *M. eurypsychrophila* [76]. The lack of
 457 disulphide bridges previously shown in the structure could be responsible for the lower stability
 458 of the enzyme at high temperatures, in comparison with previously characterized PLs [77].



459

460 **Fig. 4. Biochemical characterization of VdPelB**

461 A) Substrate-dependence of VdPelB, G125R and T157K. The activities were measured after 12 min of incubation
462 with PGA, pectins DM 24-30%, DM 55-70%, DM>85% with addition of Ca²⁺ at 35°C. B) pH-dependence of
463 VdPelB, G125R and T157K activity. The activities were measured after 12 min of incubation with PGA in sodium
464 phosphate (NaP) and Tris-HCl buffer at 35°C. Values correspond to means ± SD of three replicates. Welch t-test
465 comparing native with mutant VdPelB forms was used for statistical analysis. P value ***<0.001 and **<0.01. C)
466 Determination of Km, Vmax and kcat for ADPG2 and PGLR. Activity was assessed using various concentrations
467 of PGA at 35°C and pH8.0 (VdPelB) and pH9.0 (VdPelB-G125R).

468 **3.8. Mutation of specific amino-acids affects VdPelB activity**

469 Considering the structure of VdPelB and our hypothesis related to the role of some
470 amino acids in determining the mode of action of VdPelB, we generated mutated forms of the
471 enzyme for five amino acids that likely to be involved in the catalytic mechanism and/or
472 substrate binding: G125R, D151R, T157K, L178K and H181A. Enzymes were produced in *P.*
473 *pastoris* and purified (**Fig. S1**). If the importance of some of these amino acids (i.e. D151 and
474 H181) in the catalytic mechanism was previously shown for others PL [43,78], our study
475 highlights the key role of some novel amino acids in the catalytic mechanism. The activities of
476 mutants were tested at iso-quantities of wild-type enzyme. Surprisingly, the G125R mutant was
477 25% more active on PGA compared to the native enzyme and a shift in the optimum pH was
478 observed (**Fig. 4A**). While native enzyme was most active at pH8.0 with slight decrease in
479 activity at pH9.0, the activity of G125R mutant was approximately doubled at pH9.0 and
480 pH10.0 (**Fig. 4B**). Both enzymes kept the same activity at pH8.0. Moreover, using PGA as a
481 substrate, at 35°C, VdPelB and VdPelB-G125R differ slightly in their Km (2.7 versus 4.8
482 mg.ml⁻¹), Vmax (79.1 versus 184.8 nmol of GalA.min⁻¹.µg⁻¹) and kcat (40.5 versus 70.9 s⁻¹,
483 **Fig. 4C**). The substitution of Gly with Arg, present in a number of previously characterized
484 PL could facilitate the interaction with the substrate and alkalinization of the active site thanks
485 to its physio-chemical properties [62]. Despite not knowing the exact rotamer orientation, the
486 presence of Arg with high pK_a allowing strong hydrogen bonding and complex electrostatic
487 interactions within the active site, could change the optimal pH by acting on the protonation
488 state of amino acids involved in catalysis, as previously reported for a number of different
489 enzymes [65,79–82]. The activity of T157K was closed to null when tested on PGA and
490 different pHs. The introduction of a Lys, a chemically different and larger amino acids is likely
491 to introduce steric clashes notably with H181 and L178 that are important for the activity. The
492 H181A mutant lost much of its activity as there are less interaction/recognition with the
493 substrate. No activity for D151R and L178K mutants were observed in line with the fact that
494 D151 is an active site amino acid that binds the Ca²⁺. Mutation of this amino-acid is negatively

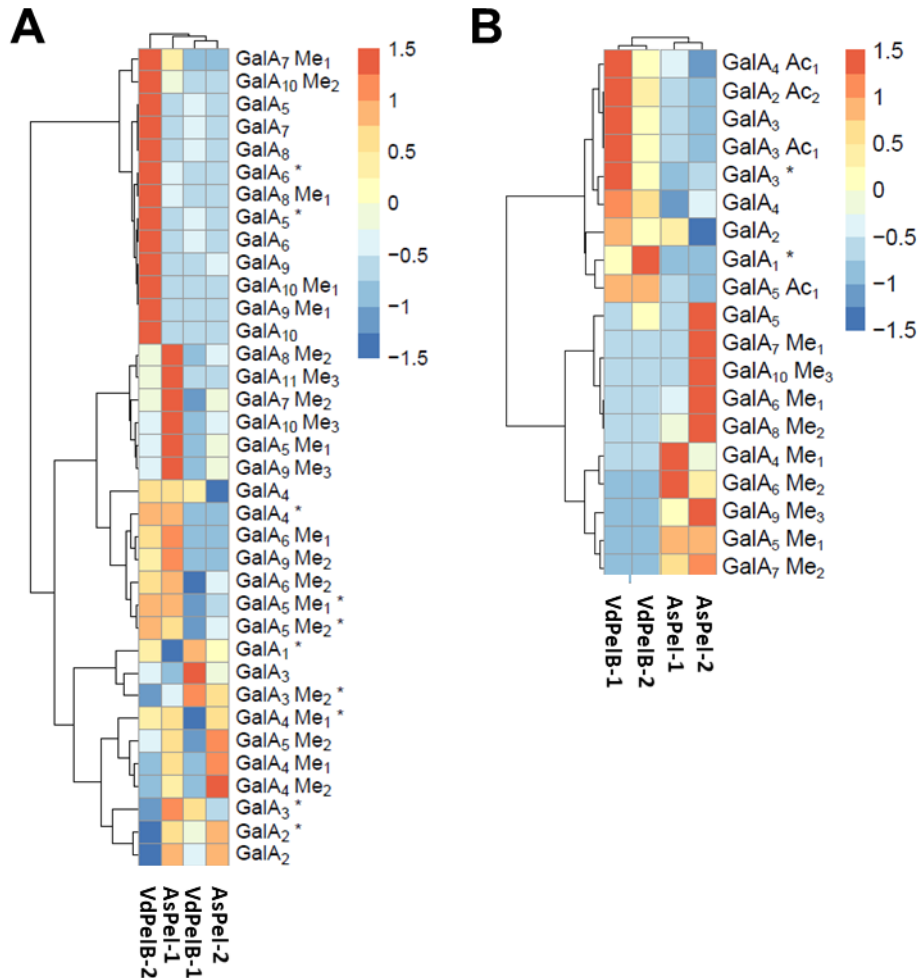
495 impairing the functioning of the enzyme (**Fig. S7A and B**) [43,78]. We can hypothesize that
496 L178K mutation positioned in between Ca²⁺, R208 and the substrate, induces specific substrate
497 conformation that diminishes the direct interaction between the enzyme catalytic centre and the
498 substrate, which translates to loss of activity (**Fig. S9**).

499 **3.9. Identification of the OGs released by VdPelB from commercial and cell wall** 500 **pectins**

501 To further understand the specificity of VdPelB on different substrates, we performed
502 LC-ESI-MS/MS to determine the profiles of digestion products (OGs) and to compare with that
503 of commercially available *Aspergillus sp.* Pel (AsPel, **Fig. S10**). To be fully comparable,
504 digestions were realized, for each substrate, at iso-activities for the two enzymes. On the basis
505 of digestion profiles, we identified 48 OGs and created a dedicated library that was used for
506 identification and integration of peaks (**Table S3**). MS² fragmentation allowed determining the
507 structure of some of the OGs (**Fig. S11 and S12**). The OGs released by either of the enzymes
508 mainly corresponded to 4,5-unsaturated OGs, which is in accordance with β-eliminating action
509 of PLLs. When using pectins DM 20-34% and at low enzyme's concentration (VdPelB-2),
510 VdPelB mainly released non-methylesterified OGs of high DP (GalA₅, GalA₆, GalA₇, GalA₈,
511 GalA₉, GalA₁₀) that were subsequently hydrolysed when using more concentrated VdPelB
512 (VdPelB-1, **Fig. 5A**). These digestion products strikingly differed to that generated by AsPel,
513 that are methylesterified OGs of higher DP (GalA₄Me₁, GalA₄Me₂, GalA₅Me₁, GalA₆Me₂,
514 GalA₁₁Me₃...), thus showing distinct enzymatic specificities. Altogether, these first results
515 unequivocally shows both VdPelB and AsPel act as endo-PLs, but they differ in their
516 processivity [9]. When using sugar beet pectins, that are known to be highly acetylated (DM
517 42%, DA 31%), VdPelB released acetylated OGs (GalA₂Ac₂, GalA₃Ac₁, GalA₄Ac₁,
518 GalA₅Ac₁), while AsPel showed much lower activity and relative abundance of these OGs (**Fig.**
519 **5B**). Previous reports have shown that differences exist between PNL, in particular with regards
520 to acetyl substitutions [18]. In contrast, AsPel released mainly methylesterified OGs (GalA₆Me₁,
521 GalA₈Me₁, GalA₁₀Me₃...).

522

523



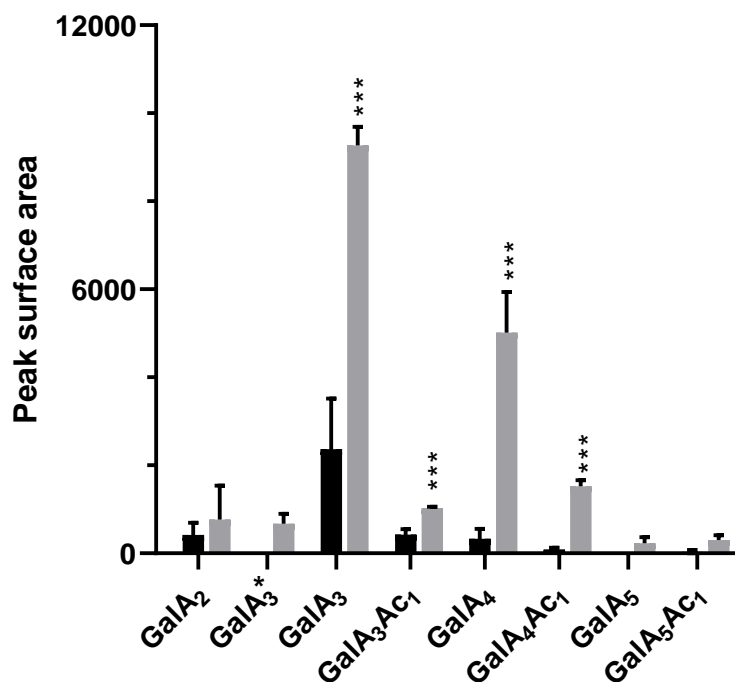
524

525 **Fig. 5. Analysis of OGs produced by the action of VdPelB and AsPeI on pectins of various degrees of**
 526 **methylesterification and acetylation**

527 OGs were separated by SEC and analysed by MS/MS. A) Pectins DM 24-30%. B) Sugar beet pectins (DM 42%
 528 DA 31%). Substrates were digested overnight at 40°C and pH8.0 using isoactivities of VdPelB and AsPeI. Enzyme
 529 concentrations are stated in Table S2. Subscript numbers indicate the DP, DM and DA. * indicate non-unsaturated
 530 OGs. Values correspond to means of three replicates.

531 *V. dahliae* and closely related fungus from the same genus are known flax pathogens,
 532 where they use their enzymatic arsenal, that includes pectin degrading enzymes, for penetrating
 533 the host cell leading to infection [30]. To assess the potential role of VdPelB in flax
 534 pathogenicity we digested root cell walls from two flax cultivars, Evea (Verticillium-partially
 535 resistant), and Violin (Verticillium-susceptible), and we compared the OGs released. On root
 536 cell walls, VdPelB released mainly unsaturated OGs up to DP5 (**Fig. 6**). From similar starting
 537 root material, the OG total peak area detected was five times lower for Evea compared to Violin,
 538 suggesting that it is less susceptible to digestion by VdPelB. OGs released by VdPelB were
 539 mainly non-methylesterified but could be acetylated (GalA₂, GalA₃, GalA₃Ac₁, GalA₄,

540 GalA₄Ac₁ GalA₅, GalA₅Ac₁), and the abundance of GalA₃ and GalA₄ was five and fifteen times
 541 higher in Violin, respectively. These data together with that obtained from sugar beet pectins
 542 strongly suggests that VdPelB preference is for non-methylesterified and acetylated substrates.
 543 Our data suggest that cell wall structure differ between the two cultivars and that VdPelB could
 544 determine *Verticillium* pathogenicity thanks to a better degradation of the cell wall pectins of
 545 sensitive cultivars [37]. Similarly, VdPel1 was previously identified as virulence factor, where
 546 the deletion of this gene decreased virulence in tobacco, as compared with the wild-type
 547 *Verticillium* [35].



548

549 **Fig. 6. Analysis of OGs released by VdPelB from flax roots.**

550 The VdPelB was incubated overnight with roots from Évéa (spring flax, partially resistant to *Verticillium* wilt,
 551 black) and Violin (winter flax, more susceptible to *Verticillium* wilt, grey). Values correspond to means \pm SD of
 552 three replicates. Welch t-test comparing Eevea with Violin was used for statistical analysis. P value ***<0.001.
 553 Subscript numbers indicate the DP, DM and DA.

554

555 4. Conclusion

556 We have characterized, by multidisciplinary approaches, a novel pectinolytic enzyme
 557 from *V.dahliae*, VdPelB, that belongs to the PLL family. The protein was crystallised and its
 558 3D structure determined at a high resolution. Pectate lyase VdPelB showed a conserved

559 structure, with parallel β -sheet topology for PLs and the active site harboured three conserved
560 Asp coordinating Ca^{2+} and Arg involved in the β -elimination mechanism. The binding groove
561 of VdPelB reveals conserved amino acids that are characteristic of PLs, with MD simulations
562 confirming the lower dynamics/higher affinity of the enzyme towards non-methylesterified
563 pectins. As inferred from structural and dynamical analyses, VdPelB showed high activity on
564 non-methylesterified substrates, with a maximum activity at pH8.0 and 35°C. The analysis of
565 the structure led the identification, in the VdPelB, of peculiar amino acids that are normally
566 present in PNL. In particular, G125R mutant increased activity on PGA and switch in pH
567 optimum from 8.0 to 9.0 related to amino acids protonation. The analysis of the digestion
568 products showed that VdPelB act as an endo enzyme and that it can release a large diversity of
569 OGs with a preference for non-methylesterified and acetylated products. The OGs generated by
570 VdPelB from pectins extracted from *Verticillium*-partially tolerant and *Verticillium*-sensitive
571 flax cultivars showed that the enzyme could be a determinant of pathogenicity, since these OGs
572 differ as a function of pectins' structure. Thus, our study now paves the way for generating,
573 using dedicated enzymes, OG pools that could be used for protecting plants against
574 phytopathogens.

575

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584 **Author's contribution**

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587 **Bouckaert**: Data curation, Investigation, Methodology. **Olivier Habrylo**: Formal analysis,
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589 Methodology. **Jean-Xavier Fontaine**: Data curation, Formal analysis, Investigation,
590 Methodology. **Adrien Lemaire**: Investigation, Methodology. **Aline Voxeur**: Investigation,
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593 - review & editing. **Jérôme Pelloux**: Funding acquisition, Conceptualization, Writing - review
594 & editing. **Fabien Sénéchal**: Conceptualization, Writing - review & editing.

595 **Conflicts of interest**

596 There are no conflicts of interest.

597

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