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2	Plant peptidoglycan precursor biosynthesis: Conservation
3	between moss chloroplasts and Gram negative bacteria
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19	
20	Short title:
21	Bacterial Peptidoglycan Precursors in Land Plants
22	
23	Abstract
24	An accumulation of evidence suggests that peptidoglycan, consistent with a bacterial cell wall, is synthesised around the
25	chloroplasts of many photosynthetic eukaryotes, from glaucophyte algae to land plants at least as evolved as pteridophyte

26 ferns, but the biosynthetic pathway has not been demonstrated. We employed mass spectrometry and enzymology in a

27 twofold approach to characterize the synthesis of peptidoglycan in chloroplasts of the moss Physcomitrium

28 (Physcomitrella) patens. To drive the accumulation of peptidoglycan pathway intermediates, P.patens was cultured with

29 the antibiotics phosphomycin, D-cycloserine and carbenicillin, which inhibit key peptidoglycan pathway proteins in 30 bacteria. Mass spectrometry of the TCA-extracted moss metabolome revealed elevated levels of five of the predicted 31 intermediates from UDP-Glc/Ac through to the UDP-Mur/Ac-D,L-diaminopimelate (DAP)-pentapeptide. 32 Most Gram negative bacteria, including cyanobacteria, incorporate meso-diaminopimelate (D,L-DAP) into the 33 third residue of the stem peptide of peptidoglycan, as opposed to L-lysine, typical of most Gram positive bacteria. To 34 establish the specificity of D,L-DAP incorporation into the P.patens precursors, we analysed the recombinant protein, 35 UDP-MurNAc-tripeptide ligase (MurE), from both P.patens and the cyanobacterium Anabaena sp. strain PCC 7120. 36 Both ligases incorporated D,L-DAP in almost complete preference to L-Lys, consistent with the mass spectrophotometric 37 data, with catalytic efficiencies similar to previously documented Gram negative bacterial MurE ligases. We discuss how 38 these data accord with the conservation of active site residues common to DL-DAP-incorporating bacterial MurE ligases

39 and of the probability of a horizontal gene transfer event within the plant peptidoglycan pathway.

40

### 41 Introduction

42 The endosymbiotic theory for the origin of photosynthetic eukaryotes proposes that an engulfed cyanobacterium evolved 43 into the first ancestors of chloroplasts (Dagan et al., 2013; Ponce-Toledo et al., 2017). As with bacteria, these organelles 44 (cyanelles) were surrounded by a peptidoglycan (or murein) wall (Scott et al., 1984). In bacteria, peptidoglycan covers 45 the organism in a mesh-like 'sacculus' confering resistance to osmotic stress, and a species-specific shape and size. 46 Although originally considered likely that peptidoglycan was lost from all photosynthetic organelles immediately after 47 the glaucophyte branch (Pfanzagl et al., 1996), there has been an accumulation of evidence including sensitivity of 48 chloroplast division to peptidoglycan-directed antibiotics, fluorescent labelling studies and gene knockout phenotypes to 49 indicate that many streptophytes, including the charophyte algae (Matsumoto et al., 2012; Takano et al., 2018) and some 50 bryophytes and pteridophytes (sister lineages to seed plants) (Takano and Takechi, 2010; Hirano et al., 2016), may have 51 chloroplasts that synthesize peptidoglycan. Furthermore, in gymnosperms (Lin et al., 2017) and also a diverse number of 52 eudicots (van Baren et al., 2016) all the critical genes for peptidoglycan synthesis have been identified, although a 53 potential penicillin binding protein (PBP) typically required for peptidoglycan cross-linking has not been confirmed in 54 eudicots.

The earliest evidence for peptidoglycan in embryophytes was uncovered when antibiotics affecting bacterial peptidoglycan synthesis in the bryophyte moss *P. patens* (Kasten and Reski, 1997; Katayama et al., 2003) and lycophytes and ferns (Izumi et al., 2008) were found to cause a decrease in chloroplast number with the formation of giant (macro)chloroplasts. Subsequently, genomics and *in silico* analyses confirmed the presence of all essential bacterial genes

59 for peptidoglycan biosynthesis (Rensing et al., 2008). These genes are nuclear-encoded, predominantly plastid-targeted 60 (Machida et al., 2006; Homi et al., 2009) and transcribed, as revealed by expressed sequence tags (ESTs). More recently, 61 a peptidoglycan layer surrounding P. patens chloroplasts has been visualized using a fluorescently labelled substrate 62 (Hirano et al., 2016) and electron microscopy (Sato et al., 2017). 63 Peptidoglycan in Gram negative bacteria has a repeating disaccharide backbone of  $\beta$ -(1,4) linked N-acetylglucosamine 64 (GlcNAc) and N-acetylmuramic acid (MurNAc) to which is appended a stem peptide comprising L-Ala, D-Glu, D,L-65 DAP, D-Ala--D-Ala. Variations in the amino acid residues have been identified and are consequent on either the 66 specificity of the Mur ligases (MurC-F) or later modifications in peptidoglycan biosynthesis. In Gram positive bacteria 67 MurE typically incorporates L-Lys as opposed to D,L-DAP, although Bacilli are a notable exception and several other 68 amino acids have been identified in this position (Schleifer and Kandler, 1972; Barreteau et al., 2008; Vollmer et al., 69 2008). The stem peptides of adjacent saccharide strands are crosslinked by transpeptidation to stabilize the mature 70 peptidoglycan (see biosynthetic pathway Figure 1).

71 Knock-out of P. patens homologs of bacterial peptidoglycan synthesis genes Ddl. MurA. MurE. MraY. MurJ or PBP1A. 72 results in a macrochloroplast phenotype, similar in appearance to antibiotic treatments that target their gene products, 73 while complementation with the intact genes restores the wild type number of about 50 typical chloroplasts per cell 74 (Machida et al., 2006; Homi et al., 2009; Hirano et al., 2016; Takahashi et al., 2016; Utsunomiya et al., 2020). Cross-75 species complementation using a *P.patens MurE* (*PpMurE*) knockout showed that *Anabaena MurE* (*AnMurE*) fused to 76 the plastid-targeting signal of *PpMurE* can also restore the wild type chloroplast phenotype (Garcia et al., 2008). In 77 contrast, the homologous Arabidopsis thaliana gene, AtMurE, failed to complement the PpMurE mutant (Garcia et al., 78 2008). Interestingly, MurE knockouts in both A. thaliana and Zea mays, appear bleached as opposed to having a 79 macrochloplast phenotype, are deficient in chloroplast thylakoids and lack many plastid RNA polymerase-regulated 80 chloroplast transcripts, indicating that angiosperm MurE has a primary function in plastid gene expression and biogenesis 81 rather than plastid division per se (Garcia et al., 2008; Williams-Carrier et al., 2014).

Although data suggestive of the formation of chloroplast peptidoglycan is available, no direct observation of the peptidoglycan precursors or the operation of the chloroplast peptidoglycan synthetic pathway has yet been made. Therefore, here, using pathway-inhibiting antibiotics to drive the accumulation of peptidoglycan intermediates, we establish that in a basal land plant, *P. patens*, the six *Mur* genes and *Ddl* actively synthesize all the main precursors of the peptide stem of peptidoglycan. Furthermore, we show that the pentapeptide building blocks are identical to those of most typical Gram negative bacteria, including the cyanobacteria, plus the Chlamydiae, the 'acid fast' *Mycobacterium* spp. and some Gram positive bacilli, where D,L-DAP is incorporated instead of L-Lys. Consistent with and supportive of this

89 observation, we show that in vitro the moss MurE ligase, PpMurE, incorporates D,L-DAP in strict preference to L-Lys as

90 the third amino acid within the stem peptide, as would be consistent with the cyanobacterial ancestral origin of the

91 chloroplast, and the enzyme kinetics of PpMurE are similar to cyanobacterial and other Gram negative D,L-DAP-

92 incorporating MurE ligases.

### 93 Materials and Methods

### 94 Plant Material

*P. patens* (Gransden strain, GrD13) was grown on modified KNOPS medium with 5 mM diammonium tartrate, to
 promote chloronemata formation (Schween et al., 2003). The medium was solidified with 0.85 % (w/v) plant agar

97 (Sigma) and overlaid with 9 cm cellophane discs (AA Packaging). Plants were grown in 90 mm diameter x 20 mm vented

98 tissue culture dishes sealed with Micropore (3M) surgical tape in a plant growth room at 21°C under continuous light

99 from Sylvania white F100W tubes at 65-100  $\mu$ mol.m<sup>-2</sup>.s<sup>-1</sup>. After being homogenised axenically in water in a 250 ml flask

100 using an IKA T18 digital Ultra Turrax homogeniser, for one to two 12 s bursts, *P. patens* protonemata were cultured as 2

101 ml aliquots per 25 ml solid KNOPS plus tartrate.

### 102 Confocal Microscopy of Antibiotic Treated *P. patens* Protonemata

103 Confocal single plane images and Z-series stacks were acquired on a Leica SP5 microscope, using a 63 x 1.4 Oil UV 104 immersion objective with the 405 nm and 496 nm laser lines and transmitted light, and photo multiplier tube spectral 105 detection adjusted for the chlorophyll emission (735-790 nm). Images were processed using the Fiji distribution 106 of ImageJ v2.0.0.

### 107 Trichloroacetic Acid (TCA) Extraction of Plant Metabolites

108 Antibiotics were added to KNOPS plus tartrate agar at 100  $\mu$ g ml<sup>-1</sup> carbenicillin, 100  $\mu$ g ml<sup>-1</sup> D-cycloserine or 200  $\mu$ g ml<sup>-1</sup> 109 <sup>1</sup> phosphomycin. After 15 days tissue was harvested, weighed and ground in liquid nitrogen using a pestle and mortar 110 before being frozen at -80°C. To extract TCA-soluble plant metabolites the tissue was ground again in 5 ml g<sup>-1</sup> of ice cold 111 10% (w/v) TCA (Fisons AR grade) before being mixed gently in 50 ml Falcon tubes on a rolling shaker for 30 min at 4°C 112 (Roten et al., 1991). Insoluble material was pelleted at 48,000 xg, 10 min, 2°C, the supernatant was retained and the pellet 113 reextracted twice more, first with 2.5 ml.g<sup>-1</sup> and then with 1.25 ml.g<sup>-1</sup> (of the original pellet weight) of ice cold 10% 114 (w/v) TCA. The pooled supernatants were extracted into an equal volume of diethyl ether, to remove TCA, by manually 115 shaking for 3 x 20 s in a separating funnel before recovering the lower, aqueous layer. The ether extraction of the aqueous 116 phase was repeated twice more. The pH of the combined lower phases was restored to pH 7-8 using 1 M NaOH and 117 residual ether was removed in vacuo at which point, the sample was lyophilised.

### 118 Purification of Muropeptide Precursors

119 The nucleotide precursors in the TCA-soluble metabolite extracts were first partially purified by size exclusion using a 120 Superdex Peptide 10/300GL column. The freeze-dried pellets were resuspended in deionised water, applied to the column 121 as a 500  $\mu$ l aliquot, eluted with deionised water and collected as 0.5 ml fractions at 0.5 ml min<sup>-1</sup>. The likely elution 122 volume of molecules of interest was established by elution of 20 nmols UDP-MurNAc-DAP-pentapeptide and 20 nmols

123 UDP-*N*-acetyl-glucosamine (Sigma) standards.

124 The A<sub>260</sub> of pooled Superdex Peptide fractions of 1-2 ml was used to determine the upper linit of the total concentration of

125 UDP species and an estimated 2, 10 or 20 nmols UDP species in 2 ml 10 mM ammonium acetate, pH 7.5, was loaded

126 onto a MonoQ 50/5 GL column equilibrated in the same buffer. Bound molecules were eluted with a 27 ml linear gradient

127 of 10 mM to 0.81M ammonium acetate (pH 7.5), at 0.7 ml.min<sup>-1</sup> and collected as 1ml fractions using an Äkta Pure where

128 the eluate absorbance was recorded at  $A_{230}$ ,  $A_{254}$  and  $A_{280}$ . Peaks with an absorbance ratio of usually 1:2  $A_{280}$ :  $A_{254}$  were

129 selected for freeze drying and mass spectrometry.

### 130 Mass Spectrometric Nano-spray Time of Flight Analysis of Peptidoglycan UDP-MurNAc Precursors

131 Identity of UDP-MurNAc precursors were confirmed by negative ion time of flight mass spectrometry using a Waters 132 Synapt G2Si quadrupole-time of flight instrument operating in resolution mode, equipped with a nanospray source 133 calibrated with an error of less than 1 ppm with sodium iodide over a 200-2500 m/z range (Catherwood et al., 134 2020). Samples, freeze dried three times to remove ammonium acetate, were diluted in LCMS grade 50% v/v 135 acetonitrile to between 1 µM and 5 µM. They were introduced into the instrument using Waters thin wall nanoflow 136 capillaries and up to 20 minutes of continuum data were collected at a capillary voltage of 2.0 kV, cone and source 137 offset voltages of 100 V and a source offset of 41 V, respectively. Source and desolvation temperatures were 80°C 138 and 150°C respectively, desolvation and purge gas flow rates were both 400 l.min<sup>-1</sup>. Scan time was 1 s with an 139 interscan time of 0.014 s. Scans were combined into centred mass spectra by Waters Mass Lynx software. Resolution 140 (m/z/half-height spectral peak width) was measured as 1 in 20,100.

#### 141 Construction of Heterologous Expression Plasmids

142 PpMurE (derived from Pp3c23 15810V3.2) and AnMurE (derived from Anabaena sp. strain PCC 7120 MurE 143 WP 010995832.1 Q8YWF0|MURE NOSS1) were inserted into the vector pPROEX HTa (Addgene) in order to be 144 expressed in frame with an amino terminal, TEV protease-cleavable, hexa-histidine (His6) tag. The MurE sequences were 145 PCR amplified from their respective cDNAs (Machida et al., 2006; Garcia et al., 2008) in pTFH22.4 using the primers 146 PpMurE L63 Forward (TTTGCGACATGTTGAAAATGGGGGTTTGGGGGATTCGAAATTGACGGATCG) and 147 **PpMurE** Reverse (AAACGCGCGGCCGCTTATTTCTAAGTCGCAAAGCCTCCCGACATTCCTC) and 148 Anabaena PCC7120 MurE Forward

and

### 149 (TTTGCGGGTCTCTCATGAAATTGCGGGGAATTACTAGCGACAGTAGACAGTG)

150 Anabaena\_PCC7120\_MurE\_Reverse

151 (AAACGCGCGGCCGCTTATAATTTTTCTCTTTCTGTCAAAGCGGCGCGTGCG). The amplified region for 152 PpMurE started at leucine 63, effectively deleting the chloroplast transit peptide at the cleavage site predicted by the 153 ChloroP1.1 Prediction server (Emanuelsson, 1999) and introducing a unique Nco1-compatible Pci1 site around the novel 154 ATG and a Not1 site immediately 3' to the stop codon. A TAA stop codon was substituted for the native TGA. The 155 AnMurE primers amplified the cDNA and novel Bsa1 and Not1 sites were introduced 5' and 3' to the ATG start and TAA 156 stop codons, respectively. The former was sited to create a Ncol-compatible 5' cohesive end. The vector pPROEX HTa 157 was restricted with Nco1 and Not1 and gel purified before being ligated to Pci1-Not1 restricted PpMurE L63 or Bsa1-158 Not1 restricted AnMurE PCR fragments that had been cleaned up with a PCR clean up kit (Qiagen). Coding sequences 159 were confirmed by Sanger sequencing (Eurofins).

#### 160 Expression of *PpMurE\_L63* and *AnMurE* and Protein Purification

161 For protein purification Escherichia coli strains were tested for optimal expression: BL21 DE3 (Thermofisher) was 162 selected for PpMurE L63 pPROEX and BL21(DE3), with the chaperone plasmid pG-KJE8 (Takara Bio Inc.), was 163 selected for AnMurE pPROEX. These were grown in L-Broth plus 0.2% v/v glucose, 100 µg ml<sup>-1</sup> ampicillin and 35 µg 164 ml<sup>-1</sup> chloramphenicol at 37°C to an A<sub>600</sub> of 0.6 when PpMurE protein expression was induced with 0.5 mM IPTG and 165 AnMurE expression was induced by 0.5 mM IPTG with 1.5 mg.ml<sup>-1</sup> arabinose and 8 ng.ml<sup>-1</sup> tetracycline to induce pG-166 KJE8 chaperones. Over-expressing cells were then grown overnight at 19°C. Bacteria were harvested by centrifugation at 167 5600 x g, 15 min at 4°C and resuspended in Buffer A: 50 mM HEPES-NaOH, 0.5 M NaCl, 10 mM imidazole and 10% 168 v/v glycerol (pH 7.5) containing EDTA-free protease inhibitor tablets, as recommended by the supplier (Pierce), and 2.5 169 mg ml<sup>-1</sup> lysozyme, with gentle mixing for 30 min at 4°C. Lysis was by sonication on ice for 10 x 15 s bursts at 70%, 170 interspersed by 1-2 min cooling on ice. Insoluble material was pelleted at 50,000 xg for 30 min at 4°C and the supernatant 171 loaded directly onto a 5 ml His Trap HP (GE Healthcare) at 2 ml.min<sup>-1</sup> and washed with 50 ml Buffer A at 4 m.min<sup>-1</sup> at 172 4°C. Bound material was eluted with an 100 ml linear gradient to 100% Buffer B: 50 mM HEPES-NaOH, 0.5 M NaCl, 173 5% w/w glycerol and 0.5 M imidazole (pH 7.5) at 4 ml.min<sup>-1</sup>. Selected peak fractions were pooled and concentrated in 174 either 30 or 50 kDa MWCO Vivaspin concentrators (GE Healthcare), for AnMurE or PpMurE L63, respectively, at 175 2,800 xg at 4°C. Proteins were further purified by size exclusion chromatography on Superdex G200 XK26 (GE Life 176 Sciences) pre-equilibrated and eluted with 50 mM HEPES-NaOH, 150 mM NaCl (pH 7.5) and purity of the eluted MurE 177 proteins was established by SDS-PAGE (Supplemental Figure S3). Pooled peak fractions were dialysed against DB2: 30

- 178 mM HEPES-NaOH, 1 mM MgCl<sub>2</sub>, 50 mM NaCl, 50% v/v glycerol with 0.2 mM PMSF, 1 μM leupeptin, 1 μM pepstatin,
- 179 3 mM dithiothreitol (pH 7.6) overnight at 4°C, before storage at -20°C and -80°C.

### 180 TEV Protease-Cleaved Protein Preparation

- 181 Bacteria were expressed as above, harvested and lysed using a cell disruptor and the proteins first purified on 5ml His
- 182 Trap HP columns, using Buffer A and B (pH 8.0) as above, except that 100 mM Tris replaced 50 mM HEPES and Buffer
- 183 A included 2% v/v glycerol, 10 mg.l<sup>-1</sup> DNase1 (DN25) and 1 mM DTT. Pooled fractions were exchanged into a buffer of
- 184 50 mM PIPES, 100 mM NH<sub>4</sub>SO<sub>4</sub>, 200 mM KCl, 20 mM MgCl<sub>2</sub>, 1 mM DTT, 30 mM imidazole, 2% v/v glycerol (pH
- 185 7.7). using a stack of four 5ml HiTrap Desalting columns (Pharmacia). Peak fractions were incubated for 48h at 4°C in
- 186 the ratio 1 mg TEV protease: 50 mg protein before reverse His-tag purification, collecting the column flow through.
- 187 Samples were concentrated using 50 kDa concentrators as above.

188 Streptococcus pneumoniae MurE and Pseudomonas aeruginosa MurF were over-expressed and purified exactly as
 189 described (Blewett et al., 2004; Majce et al., 2013).

### 190 Mur Ligase Activity Assays

191 The assays employed a continuous spectrophotometric method following ATP consumption at 37°C in a Cary 100 192 UV/Vis double beam spectrophotometer. Mur ligase catalysed ADP release, coupled to NADH oxidation by pyruvate 193 kinase and lactate dehydrogenase, led to stoichiometric consumption of NADH measured by a fall in the  $A_{340}$ . Assay 194 volumes were 0.2 ml and contained 50 mM PIPES, 10 mM MgCl<sub>2</sub> adjusted to pH 6.7 for AnMurE or 50 mM Tricine, 10 195 mM MgCl<sub>2</sub> adjusted to pH 8.7 for PpMurE L63, 1 mM dithiothreitol, 0.2 mM NADH, 2 mM phosphoenol pyruvate, 196 1mM ATP, 50 mM.min<sup>-1</sup> pyruvate kinase and 50 mM.min<sup>-1</sup> lactate dehydrogenase (as assayed by the manufacturer, 197 Sigma). Ligases were diluted prior to assay as required in 50 mM HEPES pH 7.7, 50 mM KCl, 1 mM MgCl<sub>2</sub> 3 mM 198 DTT, 50% v/v glycerol, 0.2 mM PMSF. Concentrations of UDP-MurNAc dipeptide, Mur ligase and amino acid 199 substrates were as described in the text or table legends. Control rates were collected usually in the absence of the amino 200 acid, or UDP-MurNAc-dipeptide as specified, and the activity of the enzyme was initiated by addition of the missing 201 component. Mur ligase initial rates were recorded as mols ADP.mol Mur ligase<sup>-1</sup>.s<sup>-1</sup> (ADP/s) within the linear range of 202 the time course of the assay.

203

204

#### 205 Results

### 206 Identification of antibiotics with the most profound effect on *P. patens* chloroplast division

207 P.patens was grown on a variety of antibiotics that impact peptidoglycan biosynthesis in bacteria in order to select those 208 best able to cause an accumulation of peptidoglycan intermediates in the moss, so that they might be more readily 209 detected. Of the antibiotics tested the three that appeared most specific at inhibiting peptidoglycan synthesis, as measured 210 by a widespread macrochloroplast phenotype with least effect on chlorophyll intensity, were phosphomycin (500 µg.ml<sup>-</sup> 211 <sup>1</sup>), a PEP analog that inhibits MurA by alkylating an active site cysteine residue (Figure 1<sup>1</sup>), the β-lactam ampicillin (100 212  $\mu$ g.ml<sup>-1</sup>), which binds covalently to the active site serine of PBPs (Figure 1<sup>7</sup>), and D-cycloserine (20  $\mu$ g.ml<sup>-1</sup>), with at least 213 two enzyme targets in peptidoglycan biosynthesis, DDL (Figure  $1^2$ ) and alanine racemase (Figure 2 B, D and G). Even at 214 higher concentrations, where growth rate was impaired, the protonemata were green indicating chlorophyll synthesis and 215 therefore chloroplast function was not significantly impaired. The impact of antibiotics that had either a more profound 216 and pleiotropic effect or that had little impact on phenotype are described in Supplemental Text S1 and include 217 vancomycin, bacitracin, murgocil and A22 (Figure 2 C, E, F and H).

# 218 The TCA-extracted metabolome contains peptidoglycan precursors in *P. patens*

219 *P. patens* was grown separately on the three most specific and effective antibiotics, phosphomycin (400  $\mu$ g.ml<sup>-1</sup>), D-220 cycloserine (100  $\mu$ g.ml<sup>-1</sup>), and carbenicillin (100  $\mu$ g.ml<sup>-1</sup>) to facilitate accumulation of different peptidoglycan precursor 221 molecules (Figure 1<sup>1, 2 and 7</sup>). After size exclusion and anion exchange chromatography to purify UDP-linked 222 intermediates from the TCA-extracted metabolome, mass spectrophotometric analysis identified precursors common to 223 most Gram negative bacterial cell wall syntheses (Table 1 and Figure 3 C, identified precursors numbered 1-5). Precursor 224 molecules were detected only in the earlier fractions from the Superdex Peptide column (Figure 3 B), as expected from 225 the elution profiles of UDP-GlcNAc and UDPMurNAc-pentapeptide standards (not shown).

The identification of UDP-MurNAc-Ala-Glu-D,L-DAP in three of the samples as well as the D,L-DAP pentapeptide (Table 1 and Figure 3 C, numbers 4 and 5), together with the inability to identify UDP-MurNAc-Ala-Glu-Lys or UDP-MurNAc-Lys-pentapeptide suggested that *in vivo*, PpMurE specifically incorporated DL-DAP in the third position of stem peptide. By comparison, when the plant was grown on phosphomycin (Figure 1<sup>1</sup>), anticipated to block synthesis of UDP-MurNAc, only the UDP-GlcNAc precursor was identified (Table 1 and Figure 3 C, number 1). Interestingly, this metabolite was not detected in the samples treated with the other antibiotics.

232 Similarly, the MurC and D products, UDP-MurNAc-Ala and UDP-MurNAc-Ala-Glu were detected in the D-cycloserine-

- 233 grown extract consistent with the accumulation of precursors up to the UDP-MurNAc-tripeptide MurF substrate (Figure 3
- 234 C, numbers 2 and 3). From the MonoQ anion exchange chromatograms (Figure 3, C) and the mass spectral data

235 (Supplemental Figure S2) we can conclude that use of the different antibiotics proved to be an effective way to ensure

236 most of the intermediates were detected, confirming the utility of this method for the purpose.

## 237 *P. patens* MurE incorporates DL-DAP into the peptidoglycan stem peptide

238 To account for the composition of the *P.patens* peptidoglycan stem peptide, we analysed the activity and substrate 239 specificity of the MurE ligase protein product of its murE gene, with the predicted 62 residue chloroplast transit peptide 240 sequence deleted (PpMurE L63). The enzyme was compared with the cyanobacterial Anabaena MurE ligase. Analysis of 241 the ability of both AnMurE and PpMurE L63 to utilise D,L-DAP D,D-DAP L,L-DAP and L-Lys revealed that both 242 MurE enzymes were catalytically active in the aminoacylation of UDP-MurNAc-dipeptide, Removal of the His tag by 243 TEV protease cleavage did not enhance the efficiency of either enzyme (Figure 4, B and Supplemental Figure S4) and 244 significantly, both proteins favoured D,L-DAP as a substrate over the other DAP diastereoisomers (Figure 4, A). 245 Noticeable was the slow rate of turnover of D,D-DAP by PpMurE L63, in particular, possibly indicative of a weak 246 stereo-selectivity for the L- over the D stereocentre of DAP utilised by the enzyme when at high concentrations. 247 Significantly, neither enzyme incorporated L-Lys. As a control, lysylation of UDP-Mur/Ac-Ala-Glu was also assayed 248 with the L-Lys specific Streptococcus pneumoniae Pn16 MurE (Blewett et al., 2004) and resulted in a rate (vo) of 1.94 249 ADP.s<sup>-1</sup> at 150 µM L-Lys, with the same UDP-Mur/Ac-dipeptide and ATP concentrations as the other assays (data not

250 shown).

That the assay followed the aminoacylation of UDP-MurNAc-dipeptide by D,L-DAP to yield D,L-DAP tripeptide was confirmed by the ability of the assay product to be utilised as a substrate by *Pseudomonas aeruginosa* MurF (PaMurF). This was achieved in the same coupled assay by adding PaMurF at t=0, initiating the MurE ligase reaction with D,L-DAP and then the MurF ligase with D-Ala-D-Ala as the second substrate once the MurE reaction had reached completion to yield the UDP-MurNAc-pentapeptide (Supplemental Figure S5).

### 256 pH and Temperature Optima of *P. patens* and *Anabaena* MurE

257 Prior to kinetic investigation of the properties of PpMurE the pH optimum was determined, with that of the 258 cyanobacterial AnMurE, by comparing rate of ADP generated (vo) at pH 5.7-9.7 at approximately saturating 259 concentrations of its substrates (Supplemental Figure S6, A-D). Neither of the coupled enzymes in the MurE/ADP release 260 assay was a major factor affecting rate over the pH range studied as evidenced by the independence of the measured 261 MurE rate from coupling enzyme concentration. Additionally, the similarity of activities of the MurE proteins in 262 different buffers allowed us to discount the impact of buffers over the pH range under consideration (Supplemental Figure 263 S6, B and D). Assuming saturation with substrates and the only variable responsible for a change in enzyme activity was 264 pH range we fitted vo data vs pH to an equation that follows the relationship of activity versus pH. From these data it was

265 concluded that the optimum for AnMurE is 7.5 and that for PpMurE\_L63 is approximately pH 7.5-8.5. The data fit for for 266 PpMurE\_L63 ( $R^2 = 0.94$  and 0.89, for 1 and 2 x coupling enzymes, respectively) is better than that for AnMurE ( $R^2 =$ 267 0.78) indicating that the assumption that other variables (kinetic constants and substrate ionization) are not influenced by

268 pH may be less true for AnMurE.

#### 269 *P. patens* MurE has similar kinetic properties to cyanobacterial MurE

270 The two enzymes AnMurE and PpMurE L63 were assayed to calculate their kinetic efficiency for the preferred substrate 271 D,L-DAP. From the tabulated data PpMurE L63 was more sensitive to substrate inhibition from D,L-DAP than AnMurE, 272 as indicated by its greater R<sup>2</sup> value for fit of data to the kinetics of substrate inhibition compared to those for standard 273 Michaelis Menten kinetics (Figure 4, B and the two fitted curves in Supplemental Figure S4, C and D). However, the 274 Kcat<sup>App</sup>,KM<sup>App</sup> ratio for the plant enzyme were similar to the cyanobacterial one, the most marked difference being the lower 275 D,L-DAP KM<sup>App</sup> value, indicative that the plant enzyme may operate at lower substrate concentrations in vivo. These 276 figures were compared with reported data for other MurE activities (Supplemental Figure S7) and reveal that the plant 277 and cyanobacterial MurE are at least as catalytically active, as indicated by the Kcat<sup>App</sup>/KM<sup>App</sup> ratio, as the bacterial 278 homologs. It was apparent that removal of the His tag by TEV protease cleavage did not enhance the efficiency of either 279 enzyme (Figure 4, B and Supplemental Figure S4, B and D).

#### 280 Conservation of amino acid residues common to DL-DAP-incorporating MurE ligases

281 BLASTP searches and ClustalW (EMBL-EBI) alignment indicated that the closest bacterial homolog to PpMurE is the 282 MurE of Gemmatomonidates bacterium (50.0% homology), which is a photoheterotrophic Gram negative bacterium in a 283 phylum quite distal to the cyanobacteria (Zeng et al., 2014). The next closest is the Gram positive Bacillus fortis (43.0%), 284 which would be anticipated to incorporate D,L-DAP (Barreteau et al., 2008). Both are considerably more closely related than the cyanobacterial AnMurE (37.8%), determined in this paper to be D,L-DAP incorporating, E.coli MurE<sup>D,L-DAP</sup> 285 (34.9%) and Mycobacterium tuberculosis MurE<sup>D,L-DAP</sup> (34.7%). The L-Lys incorporating enzymes, all from Gram 286 negative species, share still less homology: Thermatoga maritima MurE<sup>L-Lys</sup> (33.0%), Streptococcus pneumoniae MurE 287 288 (30.1%) and *Staphylococcus aureus* MurE<sup>L-Lys</sup> (26.6%). Likewise, the neighbour-joining phylogram computed in Jalview 289 (Supplemental Figure S8) placed AnMurE as more distantly related than Gemmatimonadetes to plant MurE, as 290 represented by PpMurE and the algal streptophytes Mesotaenium endlicherianum MurE and Coleochaete scutata MurE. 291 M. endlicherianum (66.2%) represents a late charophyte ancestor within the Zygnemophyceae which are predicted to be 292 on a branch point preceding embryophyte evolution (Donoghue and Paps, 2020), whereas MurE from Klebsormidium 293 nitens (41.8%) and C. scutata (51.3%) in the Klebsomidiophyceae and Coleochaetaceae, respectively, and also within 294 the charophyte algae, are on more divergent branches.

To relate homology to functionality, PpMurE was aligned in Clustal Omega (EMBL-EBI) with homologs of both L-Lysand DL-DAP-incorporating MurE ligases (Supplemental Figure S9). Many amino acid residues are conserved not only between MurE from bacterial and early plant species but also across the Mur ligase family (as indicated by asterisks on Supplemental Figure S9). Mur ligases comprise three domains: an N-terminal Rossmann-fold domain responsible for binding the UDP-MurNAc substrate; a central ATP-binding domain and a C-terminal domain associated with binding the incoming amino acid. Most of the amino acids conserved between the different Mur ligases lie within the central ATPbinding domain, those in the N- and C-termini generally do not co-localise with the known substrate binding residues.

Amino acids of published importance for ATP binding (species abbreviation subscripted); the P-loop within TGTXGKT<sup>Sa</sup>, E220<sup>Mt</sup>, D356<sup>Sa</sup>, N347<sup>Mt</sup>, R377<sup>Mt</sup> and R392<sup>Mt</sup> are conserved in the plant enzymes *M. endlicherianum* MurE and PpMurE, as well as a lysine, K219<sup>Sa</sup>, carbamylated in MurD for positioning the MgATP complex for the generation of a transient UDP-MurNAc-phosphodi-peptide intermediate (Dementin et al., 2001). K360<sup>Sa</sup> and Y343<sup>Mt</sup> have undergone conservative changes. Similarly, residues that bind UDP-MurNAc, S28<sup>Ec</sup>, HQA45<sup>Ec</sup>, NTT158<sup>Ec</sup>, E198<sup>Mt</sup>, S184<sup>Ec</sup>, OXR192<sup>Ec</sup> and H248<sup>Mt</sup> are no less conserved in plants than they are between bacteria.

308 Although most of the UDP-MurNAc-tripeptide interactions are within the MurE central domain, those made in relation to 309 the appended amino acid, D,L-DAP or L-Lys, are within the C-terminal domain. All of the identified bacterial MurE 310 residues that interact with D.L-DAP are highly conserved in plant MurE proteins. More specifically, with reference to E. 311 coli MurE and M. tuberculosis MurE, it is possible to distinguish those that interact with either the D- or L-stereocentre 312 carboxylates of D,L-DAP : G464<sup>Ec</sup>, E468<sup>Ec</sup>, D413<sup>Ec</sup> and N414<sup>Ec</sup>, which bond to the D-stereocentre, R389<sup>Ec</sup>, which bonds 313 with the L-stereocentre, and especially R416<sup>Ec</sup>, which interacts with both the L- and D-centre carboxylates. Of these 314 R389<sup>Ec</sup>, N414<sup>Ec</sup>, R416<sup>Ec</sup>, G464<sup>Ec</sup> and E468<sup>Ec</sup> are less consistently present in MurE ligases from Gram positive bacteria 315 that incorporate L-Lys, a decarboxylated derivative of D,L-DAP, which has only been reported to interact with the 316 R383<sup>Sa</sup>, D406<sup>Sa</sup> and E460<sup>Sa</sup> residues (Ruane et al., 2013). Similarly, the pattern of charged residues in the C-terminal 317 domain of the basal streptophyte MurE (those highlighted red or purple in Supplemental Figure S9) would indicate a 318 binding cleft for the amino acid substrate that is more basic and resembles that of the Gram negative MurE ligases. 319 Together these data are in complete accord with our kinetic findings that D,L-DAP is the preferred substrate in plants and 320 AnMurE, rather than L-Lys. As would be anticipated from the phylogeny, the more closely related G. bacterium MurE 321 aligns strongly with the Gram negative DL-DAP incorporating enzymes, and includes the DNPR motif, which confers 322 specificity for the D-stereocentre carboxyl and amino groups of D,L-DAP, indicating that this phylum is most likely to 323 incorporate DL-DAP.

324

#### 325 Discussion

### 326 *P. patens* peptidoglycan is synthesized from a UDP-MurNAc-D,L-DAP-pentapeptide

327 Growth of P. patens on the antibiotics phosphomycin, D-cycloserine and ampicillin facilitated the detection, by mass 328 spectrophotometric analysis of the TCA-extracted metabolome, of peptidoglycan intermediates up to UDP-MurNAc-Ala-329 Glu-DAP-Ala-Ala in the moss. These data enable us to conclude that the identical basic building blocks for the Gram 330 negative bacterial cell wall are found in basal embryophytes. With evidence for knock-out phenotypes for P.patens 331 homologs of bacterial MraY, MurJ and PBP1A and the presence of mRNA for MurG (Machida et al., 2006; Homi et al., 332 2009; Utsunomiya et al., 2020) it would be expected that the D,L-DAP-containing pentapeptide within the stroma is lipid-333 linked then flipped across the chloroplast inner envelope membrane and polymerised into peptidoglycan to form a 334 'sacculus' bounding the organelle, as indicated from fluorescent-labelling using a D-Ala-D-Ala analogue (Hirano et al., 335 2016). By analogy with bacteria and from the predicted transit peptides of the peptidoglycan-maturing proteins it is 336 anticipated that the peptidoglycan will lie between the inner and outer membranes of the chloroplast, although this has yet 337 to be determined (Figure 1).

### 338 PpMurE appends D,L-DAP to UDPMurNAc-Ala-Glu

From our data, it is evident that the moss MurE ligase, with the transit peptide omitted, PpMurE\_L63, can efficiently append D,L-DAP to UDP-MurNAc-L-Ala-D-Glu *in vitro*, as can the cyanobacterial enzyme from *Anabaena* sp. strain PCC 7120, AnMurE. This is in accordance with the D,L-DAP content of peptidoglycan in the cyanobacteria *Synechococcus* sp. and *Synechocystis* sp. (Jurgens et al., 1983; Woitzik, 1988) and is inconsistent with the observation that *Anabaena cylindrica* may incorporate L-Lys (Hoiczyk and Hansel, 2000). Our *in vitro* MurE enzymological data also complement the mass spectrometric analysis of the antibiotic-grown *P. patens* which identified UDP-MurNAc-D,L-DAP intermediates as being present *in vivo* in the TCA-extracted metabolome.

346 That UDP-MurNAc-L-Ala-D-Glu is an efficient substrate for PpMurE L63 is significant in that there is no obvious 347 homolog in most green plants for glutamate racemase (MurI), exceptions include the glaucophyte alga Cyanophora 348 paradoxa (Contig25539), the charophyte alga K. nitens (GAQ85716.1) but not M. endlicherianum, a zygenematophycean 349 alga proposed to be closest to the embryophyte branch point. Here, this function may be replaced by a D-alanine amino 350 transferase (DAAA), of which there are two genes having weak homology to Bacillus subtilis DAAA in both P. patens 351 and M. endlicherianum (Phytozome v.13 P. patens: Pp3c6 5420 (15.7%), Pp3c16 17790 (14.7%) and OneKP M. 352 endlicherianum: WDCW scaffolds 2009723 (17.6%) and 2007189 (16.5%)). Alternatively P. patens diaminopimelate 353 epimerase (DapF), like Chlamydial DapF, may possess the dual specificity required to racemase L-Glu to D-Glu in 354 addition to its epimerization of L,L-DAP to D,L-DAP (De Benedetti et al., 2014).

#### 355 Substrate Preference of AnMurE and PpMurE

356 The high degree of specificity of both AnMurE and PpMurE L63 for D,L-DAP, over the alternatives L,L-DAP, D,D-357 DAP and L-Lys, is consistent with other D,L-DAP-incorporating enzymes assayed in vitro, including E. coli MurE, M. 358 tuberculosis MurE and Chlamydia trachomatis MurE, for which L-Lys is either a very poor substrate or is not accepted 359 at all (Supplemental Table S7). Similarly, the L-Lys-incorporating S. aureus MurE does not incorporate D,L-DAP in 360 vitro. Not all MurE ligases are as selective, Thermotoga maritima MurE incorporates L-Lys and D-Lys in almost equal 361 amounts in vivo (Huber, 1986) and can efficiently incorporate D,L-DAP in vitro (Boniface et al., 2006). In this regard it is 362 notable that T. maritima MurE possesses a DDPR motif, which includes the arginine residue of the consensus DNPR of 363 D,L-DAP-incorporating enzymes which hydrogen bonds to and stabilises D,L-DAP, consequently the almost complete 364 absence of D,L-DAP in T. maritima peptidoglycan has been attributed to its low intracellular concentration. This almost 365 absolute specificity of most MurE ligases is indicative of a requirement that the stem peptide be composed of the correct 366 amino acids to facilitate optimal transpeptidation (Vollmer et al., 2008).

### 367 **PpMurE is a slow but efficient MurE ligase**

368 Kinetic analyses of PpMurE L63 demonstrated an enzymatic efficiency similar to bacterial MurE homologs, as estimated 369 by comparison of  $K_{eat}^{App}/K_M^{App}$  (Supplemental Table S7). Further comparisons with other D,L-DAP-incorporating 370 enzymes, and in particular those of the obligate intracellular pathogens C. trachomatis and M. tuberculosus, revealed the 371 plant MurE to have a similarly low  $K_M$  for the amino acid substrate relative to the L-Lys-incorporating enzymes. This 372 may reflect either (or both) a lower abundance of D,L-DAP or the potential toxicity of the D,L-diamino acid, particularly 373 in a eucaryotic cell (Kolukisaoglu and Suarez, 2017). A higher K<sub>M</sub> for L-Lys-incorporating MurE ligases has been 374 attributed to the much greater abundance of this amino acid in bacteria (Mengin-Lecreulx et al., 1982; Ruane et al., 2013). 375 The availability of the D.L-DAP substrate in plants, as in cyanobacteria and Chlamydiae, is not in question as the 376 biosynthesis of L-Lys is catalysed by DAP decarboxylase (LysA) from D,L-DAP which is ultimately derived from 377 aspartate (Hudson et al., 2006).

Comparison of the PpMurE\_L63 K<sub>cat</sub><sup>App</sup> with the bacterial enzymes reveals the rate of turnover to be quite low, possibly reflecting the apparent low density of peptidoglycan surrounding the chloroplast and a concomitant slower rate of synthesis compared to rapidly dividing, free-living bacteria. Moreover, the plant enzyme has UDP-MurNAc-Ala-Glu kinetics best fitted to a substrate inhibition model, possibly to ensure that peptidoglycan synthesis proceeds at a rate insufficient to consume the majority of available prenyl phosphates that are otherwise required for other pathways.

383 It is important to mention that the *P. patens* genome encodes two MurE homologs (PpMurE1: Pp3c23\_15810, studied in

this paper, and PpMurE2: Pp3c24\_18820) which have 72.2% amino acid identity to each other over the conventional

385 bacterial MurE ligase domains and 48.4% identity overall. PpMurE2 primarily differs from PpMurE1 in encoding a long, 386 relatively unstructured extension at the amino terminus and a short carboxy terminal extension (expanded description in 387 Supplemental Figure S10). Although the DNPR motif and other amino acids associated with D,L-DAP binding are 388 retained in PpMurE2, knock out mutations of PpMurE1 alone results in a comprehensive macrochloroplast phenotype 389 (Machida et al., 2006; Garcia et al., 2008), consistent with the hypothesis that this protein is sufficient for peptidoglycan 390 synthesis in the moss. Moreover, preliminary in vitro experiments indicate that intact PpMurE2 does not function as a 391 MurE ligase (data not shown) and we would suggest that both the amino and carboxy terminal extensions have been 392 acquired during streptophyte evolution to participate in novel interactions thereby facilitating an alternative function for 393 MurE within the chloroplast transcription and translation apparatus.

In contrast to *P. patens* (and the Polypodiidae ferns, Supplemental Figure S10), many in the same and closely related phylla encode a single *MurE* homolog with both the amino and carboxy terminal extensions and aligning more closely to PpMurE2, yet these proteins would be anticipated to function as MurE ligases. We propose the shorter MurE in *P. patens* and the Polypodiidae ferns represents a de-evolution of streptophyte MurE to more closely resemble its bacterial counterpart. It has yet to be determined at what point in streptophyte evolution the function of MurE changed and whether in any plants it remains a bifunctional protein capable of both MurE ligase activity and interaction with chloroplast RNA polymerase in chloroplast transcription.

That basal embryophyte MurE has evolved a new role essential to plastid photomorphogenesis in seed plants indicates an exaptation from its original function in peptidoglycan biosynthesis and plastid division (Williams-Carrier et al., 2014). This raises the intriguing question why important residues of the D,L-DAP-binding motif are retained, in similar proximity to the ATPase domain, in these proteins. We would speculate that the novel function of the MurE-like proteins in seed plants could have evolved consequent on the two whole gene duplication events which occurred in an ancestral moss, as opposed to in the liverworts or hornworts (Lang et al., 2018).

### 407 Predicted streptophyte peptidoglycan structure from peptidoglycan gene homologies

The moss 'sacculus', like that of Chlamydiae, has been recalcitrant not only to visualisation by electron microscopy but also to common extraction protocols, making analysis of the mature polymer a future goal. The moss chloroplast envelope membranes were found to be closely appended with little dense intervening material (Takano and Takechi, 2010; Matsumoto et al., 2012; Sato et al., 2017), likewise in Chlamydiae the apparent deficit of a bounding sacculus lead to the term the 'chlamydial anomaly' (Packiam et al., 2015). This is in marked contrast to most cyanobacteria where the cell wall is highly cross-linked and forms a broad, electron dense layer (Hoiczyk and Hansel, 2000). Intermediate between these extremes is the earliest side branch in plant evolution, the glaucophyte algae, where the cyanelles comprise

415 a peptidoglycan layer that has been more tractable to visualisation and analysis (Pfanzagl et al., 1996; Higuchi et al., 416

2016).

417 It would appear that progressive transition of a bacterium from free-living to endosymbiont or pathogen and thence to an 418 integrated organelle is associated with a reduction in substance of the sacculus. Presumably there are not the same 419 osmotic constraints and risks of dehydration within the host cell and the vestigial peptidoglycan may function primarily 420 or exclusively for the purpose of assembly of the division apparatus. Additionally, it may be that for cyanobacterial 421 evolution into a cyanelle and subsequently a plastid that a finer, net-like cell wall would be a prerequisite if extensive 422 exchange of larger molecules, including lipids and proteins, were to occur. Supportive of this suggestion is the fact that 423 most of the bacterial PBPs which cross-link the lipid-linked GlcNAc-MurNAc-pentapeptide precursor, have been 424 identified as having no predicted product from RNA-seq data (data not shown). Currently the only reported exception is a 425 PBP1A homolog, the transpeptidase and transglycosylase functions of which have an almost complete knock out 426 phenotype (Machida et al., 2006; Takahashi et al., 2016).

427 We also propose that streptophyte peptidoglycan must differ in its mature form by being uniquely modified to distinguish 428 it from the peptidoglycan of potential plant pathogens. The P. patens genome encodes a battery of proteins that include 429 peptidoglycan-binding and LysM domains and which frequently but not invariably include cell export signals (data not 430 shown). Many of these proteins will be part of the defences of the plant cell which are activated on detection of fungal 431 and bacterial cell wall material. To evade the host cell defences it is anticipated that an endosymbiont, obligate pathogen 432 or evolving organelle must protect its peptidoglycan from the host defences, conceivably by modification of the peptide 433 stem (Wolfert et al., 2007) or the Glc/NAc-Mur/NAc backbone (Davis and Weiser, 2011). Predictions as to what those 434 modifications might be in streptophytes are hampered by the fact that the ancestry of the modifying enzymes is not 435 necessarily cyanobacterial. We have reported here the closer homology of PpMurE to MurE in the Gemmatimonadetes 436 phylum and we can further include P. patens PBP1A, MurF, MurD, MurG and Ddl as most closely related to homologs 437 within the same Fibrobacteres-Chlorobi-Bacteroidetes group of Gram negative bacteria (data not shown). The diverse 438 origins of several peptidoglycan biosynthesis-related proteins have previously been reported (Sato and Takano, 2017). 439 Therefore, it appears highly probable that a horizontal gene transfer event of a distinct Gram negative peptidoglycan-440 related gene cluster must have occurred early in the plant lineage. Hence we conjecture a simultaneous transfer of 441 peptidoglycan-modifying genes could have occurred that would introduce novel modifications to the mature polymer, 442 distinct from any in cyanobacteria. This is not without precedent, as the divergent glaucophyte algae were found to 443 append N-acetyl-putrescine to the second residue in the stem peptide (Pfanzagl et al., 1996).

444	Here we have determined that chloroplast peptidoglycan in the streptophyte, P. patens, is constructed from typical Gram
445	negative UDP-MurNAc-D,L-DAP-pentapeptide peptidoglycan precursor. However, we propose that the final
446	polymerised structure derived from this building block differs from its cyanobacterial progenitor by being both less
447	highly polymerised and, to distinguish it from plant pathogens and thereby evade the plant immune response,
448	significantly modified.
449	
450	Supplemental Data
451	Supplemental Text S1. Effects of antibiotics on <i>P. patens</i>

- 452 Supplemental Figure S2. Negative ion nanospray TOF mass spectra of TCA-extracted peptidoglycan intermediates
- 453 Supplemental Figure S3. PAGE gel of AnMurE and PpMurE L63 after gel filtration
- 454 Supplemental Figure S4. D,L-DAP substrate curves for AnMurE and PpMurE\_L63
- 455 Supplemental Figure S5. Assay data demonstrating PaMurF utilises the product of AnMurE and PpMurE\_L63
- 456 Supplemental Text S6. Activities of AnMurE and PpMurE\_L63 with pH and buffer
- 457 Supplemental Table S7. Comparison of AnMurE and PpMurE\_L63 kinetics with published data for other MurE ligases
- 458 Supplemental Figure S8. Neighbour joining phylogram of MurE of Gram-negative bacteri and early plant species
- 459 Supplemental Figure S9. Clustal Omega multiple sequence alignment of MurE homologs
- 460 Supplemental Figure S10. Phylogram of evolutionary relationship of both PpMurE proteins to selected MurE homologs
- 461

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- 470
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**Figure 1** Schematic of the fundamental cytoplasmic and periplasmic enzyme steps in peptidoglycan (murein) biosynthesis. Enzymes: MurA-J, murein synthases A-J; Ddl, D-Ala—D-Ala ligase; MraY, phospho-N-acetylmuramoyl-pentapeptide-transferase and PBP, transglycosylase and transpeptidase activities of penicillin-binding proteins. Superscript numbers indicate targets for the following antibiotics: 1, phosphomycin, 2, D-cycloserine, 3, pacidamycin, 4, tunicamycin, 5, murgocil, 6, bacitracin, 7, penicillins and 8, vancomycin. The cytoplasmic Mur Proteins MurA and MurB catalyze the formation of UDP-*N*-acetylmuramic acid (UDP-MurNAc), Mur ligases (MurC, D, E and F) sequentially append amino acids to form UDP-MurNAc-pentapeptide. The transmembrane protein MraY attaches MurNAc-pentapeptide to C55-P to yield C55-PP-MurNAc-pentapeptide (lipid I) and MurG Glc/NAc transferase creates C55-PP-MurNAc-(pentapeptide)-Glc/NAc (lipid

II). Finally, the disaccharide pentapeptide monomer is flipped into the periplasm, polymerized by the transglycosylase activities of penicillin-binding-proteins (PBPs), or functionally related shape, elongation, division and sporulation (SEDS) proteins, and the peptides are 4-3 cross-linked to pre-existing peptidoglycan by the transpeptidase activities of PBPs or 3-3 cross-linked by L,D-transpeptidases. C55-PP is then subject to pyrophosphatase activity and C55-P recycled.

**Figure 2** Confocal microscope images showing the effects of antibiotics on *P. patens* chloronemata. Chlorophyll autofluorescence (red) reveals macrochloroplasts consequent on growth on phosphomycin, D-cycloserine, vancomycin, bacitracin, ampicillin and A22. A. untreated, B. phosphomycin (500 µg.ml<sup>-1</sup>), C. vancomycin (25 µg.ml<sup>-1</sup>), D. D-cycloserine (20 µg.ml<sup>-1</sup>, two images), E. bacitracin (100 µg.ml<sup>-1</sup>), F. murgocil (10 µg.ml<sup>-1</sup>), G. ampicillin (100 µg.ml<sup>-1</sup>), H. A22 (2.5 µg.ml<sup>-1</sup>) and I. A22 (10 µg.ml<sup>-1</sup>). Sequential fluorescence and transmitted light images, from a Leica SP5 with 63 x oil immersion lens, were processed using LAS AF lite to optimise intensity and combined as hyperstacks using Fiji on Image J. Scale bars 10µm.

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**Figure 3** Size exclusion (Superdex Peptide, A and B) and ion exchange (MonoQ, C) chromatography elution profiles at A254 of the TCA-extracted metabolome from *P. patens* grown on KNOPS with and without antibiotics. A. Superdex Peptide traces for the four treatments; KNOPS alone, or KNOPS with phosphomycin (400 µg.ml<sup>-1</sup>), D-cycloserine (100 µg.ml<sup>-1</sup>) or carbenicillin (100 µg.ml<sup>-1</sup>). (For carbenicillin the A254 was divided by two). B. Enlargement of the earlier fractions, where the intermediates were anticipated to elute (as determined by controls). C. MonoQ traces of pooled Superdex Peptide fractions of 2-20 nmoles of UDP species (from B2-C12). Boxed numbers represent fractions positively identified as intermediates (see Table 1) and asterisks indicate peaks with no recognised component.

Peptidoglycan Intermediate	Number	Con	Growth	Superdex	Species of	Expected	Nanospray
	on	ducti	Medium	Peptide	Inter-	mZ	TOF value
bioRxiv preprint doi: https://doi.org/10.110	1/MonoQ1.0	5. <b>475</b> 093;	this version	p <b>aste</b> d Janua	lynnie 20) afe The o	opyright holde	r <b>consisterap</b> rint
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d		f' chr <sup>a</sup> ) <sup>-D</sup>	-INC-IND 4.0	Fractions	icense.		expected
UDP-GIcNAc	1	18.3	KNOPS +	C3-C4 F15	(m-1)/1	606.0738	606.0814
o II			Phos <sub>400</sub>		(m+Na+-1)/1	628.0557	628.0628
					(m-2)/2	302.5330	302.5352
UDP-MurNAc-L-Ala	2	29.41	KNOPS +	C3-C4 F19	(m-2)/2	374.0621	374.0696
HN HN		33.77	D-Cycl0100	C3-C4 F20	(m-1)/1	749.1320	749.1476 749.1488
					(m+Na+-1)/1	771.1139	771.1294 771.1281
					(m+2Na+-1)/1	793.0959	793.1107 793.1127
ACHN // ÖÖ					(m+3Na+-1)/1	815.0778	815.0909 815.0963
					(m+Na+-2)/2	385.0531	385.0607 385.0611
UDP-MurNAc- <i>L</i> -Ala- <i>D-</i> γ Glu	3	44.78 50.97	KNOPS + D-cyclo <sub>100</sub>	C3-C4 F23 C3-4 F24-	(m-2)/2	438.5833	438.5928 438.5935
HIN HIN				25	(m+Na+-2)/2	449.5744	449.5839 449.5858
					(m+2Na+-2)/2	460.5653	460.5750 460.5716
COOH O AcHN // O OH	3	41.73	KNOPS	C7-C8 F23	(m-2)/2	438.583	438.5916
0			+Cb100		(m+Na+-2)/2	449.5744	449.5829
					(m+2Na+-2)/2	460.5653	460.5743
					(m-3)/3	292.0530	292.0575
UDP-MurNAc- <i>L-</i> Ala- <i>D-</i> γ Glu- <i>meso-</i> DAP	4	29.6 32.74	KNOPS alone	C7-C8 F18- 19	(m-2)/2	524.6258	524.6289 524.6289
но о				C7-C8 F20	(m+Na+-2)/2	535.6168	- 535.6196
					(m+2Na+-2)/2	546.6077	- 546.6108
					(m-3)/3	349.4146	- 349.4155
COON C CACHN // NO CH	4	33.77	KNOPS +	C3-C4 F20	(m-2)/2	524.6258	524.6377
			D-cyclo <sub>100</sub>		(m+Na+-2)/2	535.6168	535.6290
					(m+2Na+-2)/2	546.6077	546.6197
	4	20.69	KNOPS		(m+3Na+-2)/2	557.5987	501.0108
	4	29.08 32.83	+Cb100	C7-C8 F20	(111-2)/2	524.0258	524.6317
					(m+Na+-2)/2	535.6168	535.6230 535.6234
					(m+2Na+-2)/2	546.6077	546.6139 546.6144
					(m+3Na+-2)/2	557.5987	- 557.6004
					(m-3)/3	349.4146	349.4173 349.4178
UDP-MurNAc-L-Ala-D-v Glu-meso-	5	29.6	KNOPS	C7-C8 F18-	(m-2)/2	595.6629	595.6646
DAP-D-Ala-D-Ala			alone	19	(m+Na+-2)/2	606.6539	606.6553
HO O I					(m-3)/3	396.7726	396.7740
	5	29.68	KNOPS	C7-C8 F19	(m-2)/2	595.6629	595.6693
			+Cb100		(m+Na+-2)/2	606.6539	606.6602
HOOC N N N N N N N N N N N N N N N N N N					(m+2Na+-2)/2	617.6446	617.6509
' о соон о о <sub>'Асни</sub> ́ // ` он					(m-3)/3	396.7726	396.7763
				l	1		

**Table 1** UDP-linked intermediates in peptidoglycan biosynthesis as detected by mass spectrometry of the *P. patens* TCA-extracted metabolome, with expected mass:charge (mZ) ratios and actual TOF nanospray values as listed. (Figures in italics represent where a species was detected in more than one fraction). *P. patens* was grown on KNOPS medium with or without antibiotics, including Phos<sub>400</sub> (phosphomycin 400  $\mu$ g.ml<sup>-1</sup>), D-cyclo<sub>100</sub> (D-cycloserine 100  $\mu$ g.ml<sup>-1</sup>) and Cb<sub>100</sub> (carbenicillin 100  $\mu$ g.ml<sup>-1</sup>). Superdex Peptide (C) and MonoQ fractions (F) where the different species were identified are listed with their peak conductivities on MonoQ, as detailed in Figure 3. The negative ion nanospray TOF mass spectra from which the data are derived are in Supplemental Figure S2.



D,L-DAP	Mic	chaelis Me	enten		Substrate	Inhibition		
Enzyme	<i>К</i> м µМ	Vmax s <sup>-1</sup>	R <sup>2</sup>	<i>К</i> м µМ	Vmax s <sup>-1</sup>	R <sup>2</sup>	<i>K</i> i μM	<i>k</i> cat/ <i>К</i> м s <sup>-1</sup> . mM <sup>-1</sup>
AnMurE+His	56.9	6.75	0.979	67.2	7.58	0.979	1480	119
							3.06e+	
AnMurE-His	39.4	4.34	0.988	39.4	4.34	0.988	125	110
								220
PpMurE_L63+His	8.16	1.79	0.903	15.2	2.41	0.950	340	159
								76.0
PpMurE_L63-His	8.62	0.657	0.926	13.0	0.793	0.948	447	61.0

**Figure 4** Substrate specificity and kinetics of AnMurE and PpMurE \_L63. A, Activity of AnMurE and PpMurE \_L63 with 150 µM D,L-DAP, L-Lys, D,D-DAP or L,L-DAP. Assays included 375 µM UDPMurNAc-dipeptide and 100 or 300 nM AnMurE+His or PpMurE \_L63+His in 50 mM Hepes pH 7.6, respectively. Results (v<sub>0</sub>) are presented as ADP.s<sup>-1</sup> (mols ADP.mol Mur ligase<sup>-1</sup>.s<sup>-1</sup>). Error bars are 95% confidence intervals of 5 or more rates from up to 8 replicate experiments. Students t test indicate D,D-DAP rates for both enzymes are significantly greater than those for either L,L-DAP or L-Lys. B. Michaelis Menten and substrate inhibition values for KM (µM), Vmax (ADP.s<sup>-1</sup>) and R<sup>2</sup> (coefficient for data fit to either model), as computed by Prism, for both enzymes with and without His tags. All constants are 'apparent', obtained at fixed concentrations of the other two substrates. Kcat derives from Vmax in mols ADP.mol Mur ligase<sup>-1</sup>.s<sup>-1</sup>. Kcat/KM values are for Michaelis Menten kinetics for AnMurE and for substrate inhibition (bold) and Michaelis Menten kinetics (italics) for PpMurE\_L63. (D,L-DAP substrate curves are in Supplemental Figure S4).

#### Supplemental Text S1 Effects of antibiotics on P. patens

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A22 hydrochloride, a smaller molecule at 271.6 kDa, was tested at 2.5 and 10 µg.ml-1 and was likewise found to result in macrochloroplast formation in some but not most cells, although at higher concentrations its impact was more pleiotropic and chloroplasts were considerably bleached (Figure 2, H and I). A22 inhibits MreB, an actin homolog and cytoskeletal protein that controls bacterial width in rod-shaped bacteria by spatiotemporal regulation of peptidoglycan synthesis. Since there is not an evident MreB homolog in the moss (Ozdemir et al., 2018), any effect of A22 may be consequent on a less specific effect on chloroplast heat shock proteins having homology to MreB, especially HSP70 (Gao and Gao, 2011).

Another antibiotic clearly pleiotropic in its effect was tunicamycin (0.2, 1.0 and 5.0 µg.ml-1), a glycoprotein that inhibits the transfer of phospho-MurNAc-pentapeptide to the lipid carrier undecaprenyl pyrophosphate by MraY (Figure 1<sup>4</sup>). At concentrations equal to or above 1 µg.ml-1 it caused chloroplast malformation, slow growth and apoptosis (data not shown). This could be attributed to its effect on the maturation of glycoproteins in the endoplasmic reticulum since, in eucaryotes, tunicamycin also blocks the transfer of UDP-GlcNAc to dolichol phosphate.

Pacidamycins 1 and 5, cationic peptides with homology to the bacteriophage ØX174 lysis protein Arg-Trp-x-x-Trp motif, believed to bind the cytoplasmic surface of MraY and thereby inhibiting it (Figure 1<sup>3</sup>) (Rodolis et al., 2014; Bugg and Kerr, 2019), had little effect on either growth rate or chloroplast division (data not shown). Likewise, Murgocil, a 448Da steroid-like molecule, which inhibits peptidoglycan synthesis in *Staphylococcus aureus* and is predicted to bind in the Murg active site blocking UDP-GlcNAc access (Figure 1<sup>5</sup>), when tested at 1, 5 and 25 µg.ml-1 was found to have little effect on protonemata phenotype (Figure 2, F).

The effect of the three antibiotics, phosphomycin, D-cycloserine and ampicillin (Figure 3 B,D and G), subsequently selected for investigating the accumulation of peptidoglycan intermediates is detailed in the text of the paper.

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**Supplemental Figure S3** PAGE gel of AnMurE and PpMurE\_L63 after gel filtration. Lane 1, protein size marker, 2, AnMurE (predicted mass 56.57 kDa) and 3, PpMurE\_L63 (predicted mass 62.59 kDa).



**Supplemental Figure 4** D,L-DAP substrate curves for AnMurE (A,B) and PpMurE\_L63 (C,D). A and C, substrate curves for AnMurE and PpMurE with His tags, at 50 nM and 146 nM, respectively. B and D, substrate curves for AnMurE and PpMurE\_L63 after His tags have been cleaved by TEV protease, at 76 nM and 328 nM respectively. Assays were with 1 mM UDP-MurNAc-dipeptide in 50 mM PIPES pH 6.7 (AnMurE) or 50 mM Tricine pH 8.7 (PpMurE\_L63). Rates (v<sub>0</sub>) in ADP.s<sup>-1</sup> are mols ADP.mol Mur ligase<sup>-1</sup>.s<sup>-1</sup>. Data show Michaelis Menten curves superimposed on those for substrate inhibition and indicate best fit to Michaelis Menten kinetics for AnMurE and to substrate inhibition for PpMurE\_L63 (R<sup>2</sup> values for Michaelis Menten and substrate inhibition are in Figure 4, B).



**Supplemental Figure S5** Assay data demonstrating PaMurF utilises the product of AnMurE and PpMurE\_L63. A, AnMurE and B, PpMurE\_L63. Change in NADH absorbance at A<sub>340</sub> is coupled to the release of ADP by the MurE and MurF ligases on addition of their substrates D,L-DAP and D-Ala--D-Ala, respectively. Assays included 492 nM PaMurF in 50 mM Hepes, pH 7.6, 375 µM UDP-MurNAc-dipeptide and A, 100 AnMurE+His or B, 300 nM PpMurE\_L63+His.



**Supplemental Figure S6** Activities of AnMurE and PpMurE\_L63 with pH and buffer. Data are presented in two ways: A and C, with coloured symbols to indicate buffers (see legend) and B and D, with non-linear fit to estimate pH optima. Assays for A and B, An-MurE+His and C and D, PpMurE\_L63+His, respectively, were in 50 mM buffers in the pH range 5.7-9.7. For PpMurE\_L63 C, symbols with black outlines and D, square symbols represent assays with the coupling enzymes lactate dehydrogenase (LDH) and pyruvate kinase (PK) at double the normal concentration (see materials and method), to confirm these were not limiting. Assays included 260  $\mu$ M UDPMurNAc-dipeptide and 150  $\mu$ M D,L-DAP. If we make the assumption that the only variable responsible for a change in enzyme activity over the pH range tested is the change in [H+] we can derive an equation that follows the relationship of activity *versus* pH (E) where Ka<sub>1</sub> and Ka<sub>2</sub> are dissociation constants of ionizable groups responsible for the ascending and descending limbs of the pH profile. Data indicate that the pH optima for AnMurE and PpMurE L63 are 7.5 and 7.5-8.5 respectively.

		DAF/L-Lys/	
This paper	6.75	56.9	119
	4.34	39.4	110
	2.41	15.2	159
	0.793	13.0	61.0
(Paradis-Bleau et al., 2009)	22.2	140	160*
(Patin et al., 2009)	0.233	23.0	10.1
(Munshi et al., 2013)	1.2	69.0	17.4
(Patin et al., 2010)	1.24*	40.0	31.1
(Patin et al., 2010)	4.79*	550	8.71
(Ruane et al., 2013)	4.83	550	8.79
(Boniface et al.,	24.6*	2800	10.3
2006)	4.38*	4800	0.912
	This paper (Paradis-Bleau et al., 2009) (Patin et al., 2009) (Munshi et al., 2013) (Patin et al., 2010) (Patin et al., 2010) (Ruane et al., 2013) (Boniface et al., 2006)	This paper      6.75        4.34      2.41        0.793      (Paradis-Bleau et al., 2009)        (Patin et al., 2009)      0.233        (Munshi et al., 2009)      0.233        (Munshi et al., 2010)      1.24*        (Patin et al., 2010)      1.24*        (Patin et al., 2010)      4.79*        (Ruane et al., 2013)      4.83        (Boniface et al., 2013)      4.38*	DAPIC-Lys)        This paper      6.75      56.9        4.34      39.4      2.41        2.41      15.2        0.793      13.0        (Paradis-Bleau et al., 2009)      22.2        (Patin et al., 2009)      0.233      23.0        (Munshi et al., 2009)      0.233      23.0        (Patin et al., 2010)      1.24*      40.0        (Patin et al., 2010)      4.79*      550        (Ruane et al., 2013)      4.83      550        (Boniface et al., 213)      4.83      4800

**Supplemental Table S7** Comparison of AnMurE and PpMurE\_L63 kinetics with published data for other MurE ligases. PaMurE<sup>D,L-DAP</sup> *Pseudomonas aeruginosa*, CtMurE<sup>D,L-DAP</sup> *Chlamydia trachomatis*, MtMurE<sup>D,L-DAP</sup> *Mycobacterium tuberculosis*, EcMurE<sup>D,L-DAP</sup> *Escherichia coli*, SaMurE<sup>L-Lys</sup> *Staphylococcus aureus* and TmMurE<sup>L-Lys</sup> *Thermotoga maritima*. Asterisks indicate where data were extrapolated from the published values.

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**Supplemental Figure S8** Neighbour joining phylogram of MurE of Gram negative bacteria and early plant species, computed using percentage identity in Jalview. Ec *E. coli* (strain K12), An *Anabaena nostoc* PCC7120, Mt *Mycobacterium tuberculosum*, Pp1 *P. patens* (Pp3c24\_18820V3.2 v3.3 from Phytozome), Me *Mesotaenium endlicherianum* (WDCW from Onekp CNGBDB), Cs Coleochaete scutata (VQBJ from Onekp), Gb *Gemmatimonadetes bacterium*. All sequences are from the Uniprot or NCBI databases unless stated otherwise.

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Pp1/1-606	101 LNEARVSPLST	EGDLDVEITGIQQDSBLVAPGDLFV	CVKGLKSDGHQFAIQAIEKGAVAIISLM	EVSLTEGL-KAAVIVE	DTSVILSALAG 190
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5a/1-494 Tm/1-490		TGTNGKTTTTMMIYHMITSIGEBGS	VITTAVKBUGNS- YYDDITTPDAITU	SAMKENBEGGGKEEALEVSSE	ALVOOBVEGVE 192
Mt/1-535	137 TVYGHPSERLTVIGI	TGTSGKTTTTYLVEAGLRAAGRVAG	LIGTIGIRVGGAD-LPSALTTPEAPTLQ	AMLAAMVERGVDTVVMEVSSH	ALALGRVDGTR 235
Me/1-531	102 A FYGHPSQSLTVVGI	<mark>T G T N G K T T T S Y</mark> L V R S I <mark>Y</mark> D A M <mark>G</mark> L K <mark>T</mark> G	LL <mark>GT</mark> IAYSI <mark>GS</mark> KQ-QEAT <mark>HTTPD</mark> AINVQ	K LMA SMVHQ <mark>RC</mark> DAC I M <mark>EV</mark> SSH	ALAL <mark>GR</mark> CTRVE 200
Pp1/1-606	191 VIYGHPSKKLSVVGI	TGTNGKTTTSYLLQSLYEAMGLQVG	LLGTIQYYIGGKNKLEADHTTPEALNLQ	NLMA SMVQNGT EVCIMEV SSH	GLVLGRCEDIE 290
EC/1-495 An/1-496	100 AFYGYPGOKLKLVGV	TGTNGKTTTTHLIEFELTKAKLSTA	LMGTLYTRWPGFE- OTATHTTPFAVELO	OO LAQAVNAGC E EGVMEV SSE	ALAOGBVLGCP 198
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Sa/1-494 Tm/1-490	193 EDVGLETNISBOHLD	FHGTMEAYGHAKSLLFSQLGEDL		KITEGTSK-NADYBIGNIEVS	
Mt/1-535	236 FAVGAETNI SEDHLD	- EHPSMADYEEAKASLEDPDSALB			
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Me/1-531	201 FDVAVFTNLTRDHMD	FHATPEEYRDAKAQLFQRMVDPA	RHR KVVNLDDPAADFFVDQGHPD- VP	TVT <mark>Y</mark> GLEREDADVYPLEVSLS	SLFE TELVVR 293
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Me/1-531 Pp/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535 Me/1-531 Pp/1/1-606 Ec/1-495 An/1-496 Sp/1-481	201 FDVAVFTNLTRDHMD 201 FDVAVFTNLTRDHMD 199 FAVSVFTNLSRDHLD 199 FAVSVFTNLSRDHLD 199 FEVGVFSNLTQDHLD 285 TPFG-TYPVKBPVG 285 TPFG-TYPVKBPVG 294 TPFG-LLKVFTRAIG 327 DPAGVGHHIGIRLPG 294 TPKG-PLEISGLLG 299 SSWG-DGEIESHLMG 299 SSWG-DVEISSLLG 299 SSWG-DVEISSLLG 299 SSWG-DVEISSLLG 299 SSWG-DVEISGLLUN 8383 <sup>6</sup> 278 NKGESFHKDFGLLUN	FHAT PEEY RDAMAOL FORMVDPA FHAT PEEY RDAMAOL FORMVDPA FHKT EEEY RDAMAOL FAMVDP YHGDMEHY EAAKMLLY SE H H - YHSDMEDY FAAKALLF SPEY L * YXXXN347 <sup>Mt</sup> FS00 <sup>30</sup> NFNO ENAVAAGLACLR LGASLEDIK K FNI SNIMAAMIAVWSK GT SLETII DFNAY NAAAAIAALHO LGY DFKDLA RYNVANCLVALAILDT VGV SPEOAY RHNVY NILAAIAU CT AVGADLEDIV AFNV SNILAAIAVGIAVGADLEDIV AFNV SNILAAIAVGIAVGADLEDIV AFNV SNLLLAAVGAVLHGLNLGLIA ** * * Y D406 <sup>50</sup> DNXR416 <sup>60</sup> DNXR416 <sup>60</sup>	RHR KVVN LDD PAD FFV0G HD - VP RQR KVVN LDD PAD FFV0G RWLAK LPDAVA (GQ - A I NAD EVG RWLAK LPDAVA KGR - A I NAD TYGOR LIKALSPKVW KGR - A I NAD TYGOR LIKALSPKVW KGR - A I NAD TYGOR LIKALSPKVW KGR - A I NAD TYGOR LIKALSPKVW KGI AAT - RVP GR EVLTQ KNGAKVF KGI AAT - RVP GR EVLTQ KNGAKVF KGI AAT - RVP GR EVLTQ KNGAKVF KGI AAT - RVP GR EVLTQ KNGAKVVV VG I EVDAVP GR EL IDE GOG FLALVV KGI EAVDAVP GR EL IDE GOG FLALVV KGI EAVDAVP GR EL IDE EQA FAVIV KGI EAVDAVP GR EL IDE EQA FAVIV KI AARLOP VCGR WEY FTA - PGK FTVVV NAL FEFPGV GM WERVOINP DODISVIV * Streptophyte Carboxy-Terminal Loo	TY TY GLEREDADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS WTFGMGDKSADVY PLAVKLS WTFGMGDKSADVY PLAVKLS WTFGMGDKSADVY PLAVKLS WTFGMGDKSADVY PLAVKLS VALLSA	CK 1ALVLGSTG 377 CK 1ALVLGSTG 377 CK 1ALVLGSTG 377 CK 1ALVLGSTG 377 CK 1ALVLGSTG 375 FR LAVVFGAGG 375 FR LAVVFGAGG 375 FR LAVVFGAGG 388 CK 11TLVGCGG 380 CK 11TLVGCGG 388 CK 11TLVGCGG 388 CK 11TLVGCGG 387 C464 <sup>4</sup> <sup>c</sup> C464 <sup>c</sup> C46 <sup>c</sup> C4 <sup>c</sup> C46 <sup>c</sup> C46 <sup>c</sup> C46 <sup>c</sup> C4 <sup>c</sup>
Me/1-531 Pp/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535 Me/1-531 Pp/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490	201 FDVAVFTNLTRDHMD 291 FDVAVFTNLTRDHMD 199 FAVSVFTNLSRDHLD 199 FAVSVFTNLSRDHLD 199 FEVGVFSNLTODHLD 285 TPFG-TYPVKSPY0 277 TPDG-LLKVFTRAIG 277 TPDG-LLKVFTRAIG 277 TPCG-VFUSSGLG 297 TPKG-PLEISSGLG 294 TPKG-PLEISSGLG 299 SSWG-DGEIESHLMG 299 TPEG-NVSFRSPLVG 280 TPEG-NVSFRSPLVG 280 TPEG-SNVSFRSPLVG 280 TPEG-NVSFRSPLVG 276 NKGESHEKDFGLLLN 389 550 376 NKGESHEKDFGLLLN 387 ERDLTKTPEMGEVAS	FHAT PEEY RDAMAOL FORMVD PA FHKT PEEY RDAMAOL FORMVD PA FHKT PEEY RDAMAOL FAMVD PA YHGDMEHY EAA KALL FAKWVD PE - YHSDMEDY FAA KALL FSP EY L * YXXXN347 <sup>Mt</sup> F300 <sup>364</sup> NFNQ ENAVAAGLACL RLGASLEDIK K FN I SN MAAMIAVWSK GT SLETI DFNAY NAAAAIAAL HOLGY DFK DLA RYN VANCL VALAILDT VG VSP EOAV RHNY SN ILAAIAVGIAV GADLEDIO RHNY NILLAAIAVGIAV GADLEDIO AFNV SN ILLAAIAVGIAV GADLEDIO AFNV SN ILLAATLLALGY PLADLL QYNLENLLAAVGAVLHLGUNLGLIA * * * Y Y DA06 <sup>56</sup> DNR416 <sup>666</sup> DNR416 <sup>666</sup> DNR416 <sup>666</sup> ONF COVFLTAD DPNY EDPMAIADE - RADVY VIFT PDNPANDDPKMLTAE	RHR KVVN LDDPAAD FFVDQGHD - VP RQR KVVN LDDPAAD FFVDQGHD - VP (GQ AIINAD EVGRRWLAK LFDAVAV KGR - AIINAD EVGRRWLAK LFDAVAV KGR - AIINAD EVGRRWLAK LFDAVAV KGR - AIINAD EVGRRWLAK LFDAVAV KGR - AIINAD EVGRRWLAK LFDAVAV KGIAAT - RVPGREVLTQ KNGAKVFI KAVEN LFVEGRLEVLDP SLPIDLII SSLET FTGVEGR FEVVRGAKK IGUVV KGIEVDAVPGRCELIDE GOGFLALV KGIEVDAVPGRCELIDE GOFFAVIV KTAARLOPVCGRWEVFTA - PGKPTVVV XTAARLOPVCGRWEVFTA - PGKPTVVV KTAARLOPVCGRWEVTA - PGKPTVVV Streptophyte Carboxy-Terminal Loo SYNNH- LAKGATHQ	TY TY GLEREDADVYPLAVKL VYTFGMGDKSADVYPLAVKL SWEDHINENCHGRWKLAIEV SWEDHINENCHGRWKLAIEV SWEDHINENCHGRWKLAIEV D392 <sup>MI</sup> D356 <sup>5</sup> *K360 <sup>5</sup> DYAHNGDSLKKLINVVETHOT DYAHTADGNKLIDAVPFVK DFAHSPDALEKLLDVVETHOT DYAHTPDALGELLDVFECGF DYAHTPDALGKLOAALHOA DYAHTPDALEKLOAALHO DYAHTPDALEKLOAALHO DYAHTPDALEKLOAALHO SKIL PVEKTADROEAIKAAMAINNY SKIL PVEKTADROEAIKAAMAINNY SKIL	C I A L V L G I T A 20 V E - SEV L V R 383 V E - NG V T G T H 288 C I A L V L G I T H 288 C I A L V L G I T H 288 C I A L V L G I T H 288 C I A L V L G I T H 288 C I A L V L G I T H 288 C I A L V L G I T H 288 C I A L V L G I T H 288 C I A L V L G I T G 377 C I T F L X V F G A G G 475 C I T V V F G A G C 422 T H I T V V F G A G C 422 T H I T V V G C G G 387 C I A L V F G C G 387 C I A L V F G C G 387 C I A L V F G C G 387 C I A L V F G C G 387 C I A L V F G C G C 387 C I A L V F G C G C 387 C I A L V F G C G C 387 C I A L V F G C G C 387 C I A L V F G C G C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C 387 C I A L V F G C G C C C 387 C I A L V F G C G C C C C 37 C I A L V F G C C C C C C C C C C C C C C C C C C
Me/1-531 Pp/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535 Me/1-531 Pp11-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535	201      FDVAVFTNLTRDHMD        291      FDVAVFTNLTRDHMD        291      FDVAVFTNLTRDHMD        199      FAASVFTNLTRDHMD        199      FAASVFTNLTRDHMD        199      FACSVFTNLTRDHMD        199      FEVGVFSNLTDDHLD        281      FPGC        277      TPGG        270      FAGVGHHIG        294      FPKG        294      TPKG        294      TPKG        294      TPKG        294      TPKG        295      SWG-DGEISRLLG        289      TPEG-NVSFRSPLVG        289      TPEG-NVSFRSPLVG        289      TPEG-NVSFRSPLVG        378      NKGESREKDFGLLLN        389      FFDLTKTPEMGFVAS        330      NDGKRAPMMSEVAS        330      PDCKRAPMGFIAA	FHAT PEEY RDAKAOL FORWUD PA FHKI PEEY RDAKAOL FORWUD PA FHKI PEEY RDAKAOL FAKWUD PA - YHGOMEHY EAAKALL FAKWUD PA - YHSOMEDY FAAKALL FSP EY L * YXXXN347 <sup>MI</sup> F300 <sup>56</sup> NFNQ ENAVAAGLACL RLGASLEDIK K FN ISNIMAAMI AVWSKGT SLETI I DFNAY NAAAAI AALHQLGY DFKDL K KAN YAYAU RHNY SNILAAIAYGI AVGADLEDIQ RHNY NILLALAUTLAL GYD PKDLA AFNY SNILAAIAYGI AVGADLEDIQ RHNY NILLALAITLALGY PLADLL QYNLENLLAAVGAVLHLGLNLQLIA ** * YY D406 <sup>56</sup> DNXR416 <sup>66</sup> DNXR416 <sup>66</sup> DNXR416 <sup>66</sup> CHP EIQVFLTADOPNY EDPMAIADE KLADY - VIFTPDNPANDPKMLTABE KLADY - VIFTDDPRGDDP EQIMED	RHR K V VN L DD P AAD F F V DQ GH P - VP RQ R K V VN L DD P NY SY F V SQ GNOD - VP (G Q A I I NAD E V G R WL AK LP DAVA V K R - A I I NAD E V G R WL AK LP DAVA V K G I AA - R VP G R VL AK LP DAVA V K G I AA - R VP G R E V L TQ KN G AK V F I AV EN L E P VEG L E V L D SL P I D L I I SS LET F T G V E G F E V V R G AK K I G L NV V PG L RE I - R VP G R LO I D R SQ F L A V I V K G I E E V D V P G C E L I D E E Q F F A V I V K G I E E V D AV P G C E L I D E E Q F F A V I V K G I E E V D AV P G C E L I D E E Q F F A V I V K T A AR L Q P V G R ME V F T A - P G K F T V V N AI P E F P G V P G M E R V Q I N P D Q D I SV I V * * Streptophyte Carboxy-Terminal Loo I SS Y I N H- LAKGA T HQ. LI K G I D K	TY YG LER EDADVYP LEVSLS VYT FGMGDK SADVYP LEVSLS VYT FGMGDK SADVYP LAVK S SWEDH INPNCHGRWLKAILEVN SYSVNDSSADLWMSDLS D392 <sup>MI</sup> D356 <sup>5</sup> K360 <sup>5</sup> DYAHK GD SLKKLINVVETHOT DYAHK GD SLKKLINVVETHOT DYAHK PDALGALLDTVRECG DYAHK PDALGALLDTVRECG DYAHTPDALGALLAVVRECG DYAHTPDALGALLAVRECG DYAHTPDALGALLAVRECG DYAHTPDALEKALQAARLHCA SKILADRQEALKAAMAIINH NY LEFDDRAEGIKHAIDIAE PVLKIADRQEALKAAMAIINH NY LEFDRAEGIKHAIDIAE	L F E - T E L V F 293 L V E - S E V L V R 293 L V E - S E V L V R 293 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V L V R 393 L V E - S E V E V R 393 L V E - S E V E V R 393 L V E - S E V E V R 393 L V E - S E V E V R 393 L V E - S E V E V R 393 L V E V E S E V E V E S E V E V E S E V E V
Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535 Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-496 Mt/1-535 Me/1-531	201 FDVAVFTNLTRDHMD 291 FDVAVFTNLTRDHMD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 285 TPFG-TYPVK BPYVG 277 TFPG-LLKVFTRAIG 270 FDFG-LLKVFTRAIG 270 FDFG-LLKVFTRAIG 289 TPFG-NVSFRBLLG 289 TPFG-NVSFRBLVG 289 TPFG-NVSFRBLVG 289 TPFG-NVSFRBLVG 289 TPFG-NVSFRBLVG 289 TPFG-NVSFRBLVG 289 TPFG-NVSFRBLVG 280 TFFG-NVSFRBLVG 280 TFFGFKBFMMSEVAS	FHAT PEEY RDAKAOL FORWUD PA FHKI PEEY RDAKAOL FORWUD PA FHKI PEEY RDAKAOL FAKWUD PA - YHGOMEHY EAA WUL YSE-H H - YHSOMEDY FAA KALL FSP EY L * YXXXN347 <sup>MI</sup> F300 <sup>56</sup> NFNQ ENAVAAGLACL RLGASLED IK KFN ISN IMAAMI AVWSKGT SLETI I DFNAY NAAAAI AALHOL GYDP KDLA RHNY SN ILAAIAVGI AVGADLED IQ RHNY SN ILAAIAVGI AVGADLED IQ AFNY SN ILAAIAVGI AVGADLED IQ AFNY SN ILAAIAVGI AVGADLED IQ AFNY SN ILAAIAVGI AVGADLED IQ QH PE IQ VFLT ADDNY EDPMAIAD E - RADY- VI FT PDN PANDD PKMLIAE KLADV - VI LT DDP FGEDP EQ IMED QADL - VV VTD DNP RD EDT AI RRE DK SD I- TILT SDNP RN EDACE II DU	RHR KVVN LDD PAAD FFVQGHD - VP RQR KVVN LDD PAAD FFVQGHD - VP (GQ - A I NADD EVGRRWLAKLPDAVAV KGR - AI NADD EVGRRWLAKLPDAVAV KGR - AI NADD EVGRRWLAKLFDAVAV KGR - AI NADD EVGRWLAKLFDAVAV KGR - AI NADD EVGRWLYGR - SLP V ROMAN - RVPGHEVLTQ KNGAKVFI SSLET FTGVEGR FEVVRGAKKIGLNVV FGLREI - RVPGHEQIDR - SQGFLAVV KGI EEVDAVPGRCELIDE - EQAFAVIV KGI EEVDAVPGRCELIDE - EQAFAVIV KGI EEVDAVPGRCELIDE - GOTFAVLV KTAARLOPVCGRWEVTA - PGKFTVVV NAIPEFPGVPGRMERVOINPDQDISVIV * Streptophyte Carboxy-Terminal Loo ISSY NH- LAKGATHQ	THY GLER EDADVYPLAVKE VTFGMGDKSADVYPLAVKE SMEDHINPNCHGRWLKALEVN SMEDHINPNCHGRWLKALEVN SMEDSADUMS-DLS D32 <sup>MI</sup> D356 <sup>3</sup> × (360 <sup>5</sup> ) DYAHNGDSLKKLINVVETHOT DYAHTADGMNKLIDAVOPFYK DFAHSPDALEKLLKNYKISC DYAHKPALRSVLTLAHP DYAHTPDALGRLLDTVRECG DYAHTPDALGRLLDTVRECG DYAHTPDALEKALOARLHCA DYAHTPDALEKALOARLHCA DYAHTPDLENLLKAARPFI * * pSCTL PVEKIADRQEAIKAAMAILNH NYIEFDDRAEGIKHAIDIAE- PYUKIADRQEAIKAAMAILNH NYIEFDDRAEGIKHAIDIAE- PYUKIADRQEAIKAAMAILNH NYIEFDDRAEGIKHAIDIAE- PYUKIADRQEAIKAAMAILNH	GK IA LVL GST G 377 COK LIFLVG K 458 CK LKV FGA GG 452 THITVVG CG 390 K IITVLGC GG 387 C4644c C444c C444c C446c
Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Mt/1-535 Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-496 Mt/1-535 Me/1-531 Pp1/1-606	201 FDVAVFTNLTRDHMD 291 FDVAVFTNLTRDHMD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 285 TPFG-TYPVKBPYVG 287 TPFG-TYPVKBPYVG 290 FPFG-LLKVFTRAIG 327 DPAGVGHHIGIRLPG 294 TPKG-PLEISGLLG 328 TPGG-DVEISSRLG 299 SSWG-DGEIESHLMG 299 SSWG-DGEIESHLWG 299 SSWG-DGEIESHLWG 299 SSWG-DGEIESHLWG 299 SSWG-DGEIESHLWG 299 SSWG-DGEIESHLWG 299 SSWG-DGEIESHLWG 299 SSWG-DGEIESHLWG 200 TKTPEMGEVAC 376 NSDECKENMSEVAS 423 DRDPGKRAPMGKIAT 481 DRDKGKEPIMGKIAT	FHAT PEEY RDAMAOL FORMVDPA FHAT PEEY RDAMAOL FORMVDPA FHKT EE EY RDAMAOL FAMVDP YHGOMEHY EAAKALLE FAKWVDP YHSOMEDY FAAKALLE SP EY L * YXXXXX347 <sup>MI</sup> F300 <sup>44</sup> NFNQ ENAVAAGLACLRLGASLEDIK K FNI SNIMAAAIAALHOL GYDPK BLA RYNVANCLVALAILDT VG VSP EGAY RHNVYNILLAAVGAVLHOL VG VSP EGAY CNNXR416 <sup>EC</sup> OHP EI OVFLTAD OP NYE DPMAIADE - RADY - VILTD DP ROEDP FAI ARE KLADV - VILTD DP ROEDP FAI ARE KLADV - VILTD DP ROEDP FAI ARE KLADV - VILTSDNP RNEDACE II DD DK SDV - CIITSDNP RT EKPLOII	RHR KVVN LDD PAAD FFVDQGHED - VP RQR KVVN LDD PAAD FFVDQGHED - VP (GQ AI I NADD EVGRRWLAK LFDAVAV KGR - AI I NADD TYGOR LIKAL SP EKVW * TR77M KGIAAT - RVPGMEVLTQ KNGAKVFI KAVENLEPVEGHEVLDP - SLPIDLI SSLET FTGVEGFEVVRGAKKIGLNVVV PGLREI - RVPGRLEQIDR - GOG FLALV KGIEAVDAVPGRCELIDE - GOG FLALV KGIEAVDAVPGRCELIDE - GOT FAVLV KGIEAVDAVPGRCELIDE - KIENVVV KIAARLOPVCGMEVFTA - PGKPTVVV NAIP EFPGVPGMERVOINPDQDISVIV KTAARLOPVCGMEVFTA - KIENVVV KIAARUPVCGMEVFTA - GOT FAVLV KIAARUPVCGMEMEVFTA - FAVLV KIAARUPVCMEMEVFTA - FAVLV	TY TY GEREDADVY PLAVKES VYTFGMGDKSADVY PLAVKES VYTFGMGDKSADVY PLAVKES VTFGMGDKSADVY PLAVKES VTFGMGDKSADVY PLAVKES VTFGMGDKSADVY PLAVKES D392 <sup>MI</sup> D356 <sup>5</sup> + K360 <sup>5</sup> DYAHNGDSLKKLINV VETHOT DYAHT ADGMNKLIDAVG FYK DYAHT PDALEKLKVSKIS DYAHT PDALEKLKVSKIS DYAHT PDALEKLKVSKIS DYAHT PDALEKLAVSKIS DYAHT PDALEKLANSKIS DYAHT PDALEKLANSKIS VANT PDALEKLANSKIS VANT PDALEKLANSKIS VANT PDALEKLANSKIS VYAHT PDALEKLANSKIS VYAHT PDALEKALOASHLGA DYAHT PDALEKALOASHLGA VYAHT PDALEKALOASHLGA VYUEFDBREAI KAAMAITNH NYIEFDBREAI KAAMAITNH VYUEFDBREAI KAAMAITNH VYUEFDBREAI HAVAWAR ENTFVHDCRDIAV HAAVAWAGE ENTFVHDCRDIAV HAAVAWAGE	C F E - T E L V R 293      L F E - T E L V R 293      L V E - S EV L V R 383      M D S G AT IR FS 291      SY E N S V T G T I R S 291      SY E N S V T G T I R S 291      SY E N S V T G T I R S 291      SY E N S V T G T I R S 291      SY E N S V T G T I R S 291      SY I V Y G A G G 375      R I A V V F G A G 375      R I I T V G C G G 390      S H I I T V G C G G 381      G M I C V F G C G 387      G 464 <sup>25</sup> I E D A V I I A G K G 458      R G D S V I A S K G 454      P G D T V L A S K G 454      P G D V V I A G K G 578      F G A V V A G K G 488      E G D A V V I A G K G 574      P G D V V I A G K G 574
Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535 Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-496	201 FDVAVFTNLTRDHMD 291 FDVAVFTNLTRDHMD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 285 TPFG-TYPVKBPVG 287 TPFG-TYPVKBPVG 294 TPKG-PLEISGLLG 297 DPAGVGHHGIRLPG 294 TPKG-PLEISGLLG 289 TPKG-DVEISKLLG 289 TPEG-NVSERSPLVG 289 SSWG-DGEIESHLMG 289 TPEG-NVSERSPLVG 376 NSDEGKRPMMSEVAS 378 NSDEGKRPMMSEVAS 378 NSDEGKRPMMSEVAS 391 EDRGKRPIMAGIAT 431 EDRGKRPIMAGIAT	FHAT PEEY RDAMAOL FORWUD PA FHAT PEEY RDAMAOL FORWUD PA FHKT EE EY RDAMAOL FORWUD PA YHGOMEHY EAAKALLE FAKWUD PA YHGOMEDY FAAKALLE SP EY L * YXXXXX347 <sup>ME</sup> FS00 <sup>44</sup> N FNQ ENAVAAGLACLRLGASLEDIK K FNI SNIMAAAIAALHOL GYD PK KDLA RYNVANCLVALAILDT VG VSP EOAY RHNVYNILLAAVGAULEDIV AFNY SNILLAAIAVGIAVGADLEDIV AFNY SNILLAAVGAULEGINLGLLA QYNLENLLAAVGAULHLGLNLGLLA * * * * Y D406 <sup>45</sup> DNXR446 <sup>EE</sup> OHPE TOVFLTADOPNYEDPMAIADE - RADY - VILTTDDPR GEDP EQIMED QLADL - VVVTDDNPR NEDACEIIDD KSD1 - TITSDNPRTEKPLOIDINE	RHR KVVN LDD PAD FFVDQGHD - VP RQR KVVN LDD PAD FFVDQGHD - VP (GQ AI I NAD EVGR RWLAK LPDAVAV KGR - AI I NAD TYGOR LIKALSP EKVW * RGI AAT - RVPG MEVLTQ KNGAKVFI KAVENLEPVEG LEVLDP - SLPIDLI SLET FTGVEG FEVVRGAKKIGLNVVV PGLREI - RVPG LEQIDR - GOG FLALV KGI EAVDAVPG CELIDE - COAFAVIV KGI EAVDAVPG CELIDE - COAFAVIV KI AARLOP CGM EV FTA - PG K FT VVV NAIPEF CY CAMBERVOINPDQ ISVIV KI AARLOP COM EQUCAVE CO CY PP LKNGH KAGV CWM EQUCAVE CO CY PP LKNGH KAGV CWM EQUCAVE CO CY PP LKNGH KAGI FDT	TY TY GLEREDADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDSLKKLINVY ETHOT DYAHNGDSLKKLINVY ETHOT DYAHNGDSLKKLINVY ETHOT DYAHTPDALEKLLKNYKLS DYAHKPEALRSVITTLANPE DYAHTPDALEKLLKNYKLS DYAHTPDALEKLLKNYKLS DYAHTPDALEKLLKARPFIF * * PVEKIADROEALKAAMAITNH VYTFFDBAEGIKHAIDIAE PVLVLFDREALEKALMAITNH VYTFFDBREALETALTIAN- QVLFFNREALETALTIAN- QVLFDREALENAVMAGE HAKVMEGBAEANYKAA	C F E - T E L V R 293      L F E - T E L V R 293      L V E - S EV L V R 383      M D S G AT I R F S 291      M D S G AT I R F S 291      SY E N S V T G T I R H 298      GK I A L V L G S T G 377      C K I A L V L G S T G 377      C K I A L V V F G A G G 375      R L A V V F G A G 422      T R I I T V V G C G G 480      V K C V F G C G 387      G A M C V F G C G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V F G C G G 387      G A M C V A G K G 458      R G D S V V I A S K G 454      P G D V V I A S K G 575      S O V V I A G K G 578      E O A V V I A G K G 488      E O A V V I A G K G 477      P G D V V I A G K G 486      E D A V V I A G K G 486
Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Mt/1-535 Me/1-531 Pp1/1-606 Ec/1-495 An/1-496 Sp/1-481 Sa/1-494 Tm/1-490 Mt/1-535 Me/1-531 Pp1/1-606 Ec/1-495 An/1-496	201 FDVAVFTNLTRDHMD 291 FDVAVFTNLTRDHMD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 199 FAXVFTNLSRDHLD 285 TFFG-TYPVKBPYVG 277 TFPG-TYPVKBPYVG 294 TPKG-PLEISGLLG 297 DFAGVGHHIGIRLPG 298 TFKG-DVEISSRLG 299 TFKG-DVEISSRLG 299 TFG-NVSFRSPLVG 299 TFG-	FHAT PEEY RDAMAOL FORWUD PA FHAT PEEY RDAMAOL FORWUD PA FHKT EE EY RDAMAOL FORWUD PA YHGOMEHY EAAKALLE FAKWUD PA YHGOMEDY FAAKALLE SP EY L * YXXXXX347 <sup>MI</sup> F300 <sup>44</sup> NFNQ ENAVAAGLACLRLGASLEDIK K FNI SNIMAAMIAWSK GT SLETII DFNAY TAAAAIAALHOL GYD PK KDLA RYNVANCLVALAILDT VG VSP EGAY RHNVYNILLAAAIAALHOLGYD PK KDLA RYNVANCLVALAILDT VG VSP EGAY RHNVYNILLAAVGIAVGADLEDIU AFNY SNILLAAIAUCIA LGYPLADLL QYNLENLLAAVGAVLHLGLNLGLLA * * * * Y D406 <sup>45</sup> DNXR446 <sup>65</sup> OHPEIOVFLTAD OP NY EDPMAIADE - RADY. VI FT PONPANDDP KMLTAE KLADV. VI LTD DP RGEDP EGIMED QLADL - VVVT DDNP RD EDPTAIRE KLADV. VI LTSDNP RNEDACEIIDD KSDU - TILTSDNP RNEDACEIIDD DKSDV - CI IT SDNP RT EEP AIIND ELADL. AFVT SDNP RT EDP CR IIND	RHR KVVN LDD PAD FYDQGHD - VP RQR - KVVN LDD PAD FYDQGHD - VP (GQ - A I NAD EYGR RWLAK LPDAVAV KGR - A I NAD EYGR RWLAK LPDAVAV KGR - A I NAD FYGR RWLAK LPDAVAV KGR - A I NAD FYGR LVLTQ - KNGAK VF KGI AAT - RVPG MEVLTQ KNGAK VF KGI AAT - RVPG MEVLTQ KNGAK VF KGI FAL EP VEG LPV LPQ SQ F LALV KGI FAL AD VPG CEL DE SQ F LALV KGI FAL AV VG CEL DE SQ F LALV KGI FAL AV VG CEL DE SQ F LALV KGI FAL AV VG CEL DE SQ F LALV KGI FALAVAV GG CEL DE SQ F LALV KGI FALAV AV GG CEL DE SQ F LALV KGI FALAVAV GG CEL DE SQ F LALV KGI FALAV SG CEL DE SQ F LAV KGI FALAV SG CEL DE SQ F LAV KGI FALAV SG CEL DE SQ F LAV KGI FALAV SG CEL DE SQ F LL KGI FALAV SG CEL SG SY PF LKNGH KGI FALAV SG CEL SSY FLL FNGH KAGY SW SMEGY CKWEED SSY FLL FNGH LAGI FDT	TY TY GLEREDADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS VYTFGMGDKSADVY PLAVKLS D392 <sup>MI</sup> D356 <sup>5</sup> +K360 <sup>5</sup> DYAHNGDSLKKLINV VETHOT DYAHT ADGMNKLDAVGFYK DYAHT PDALEKLKNY KISS DYAHK PEALRSVLTTLAHPL DYAHT PDALEKLKNY KISS DYAHT PDALEKLKNY KISS DYAHT PDALEKLALGAALHCA DYAHT PDALEKLAGAALHCA DYAHT PDALEKLAGAALHCA DYAHT PDALEKLAGAALHCA DYAHT PDALEKLAGAALTNH VEKIADROEALKAAMAITNH VIEFDBAEGIKHAIDIAE PVEKIADROEALKAAMAITNH VIEFDBAEGIKHAALIAH VIEFDBAEGIKHAALIAA QVEFVHOREALETALTIAN- QVEFVHOREALETALTIAN- QVEFVHOREALETALTIAN- QVEFVHOREALETALTANAG	C F C - C E V F 203 L F E - T E V F 203 L V E - S E V L V F 383 M D S A T I F F S 291 M D S A T I F F S 291 M D S A T I F F S 291 M D S A T I F F S 291 M D S A T I F S 291 M D S A S 291 M D S 291 M
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**Supplemental Figure S9** Clustal Omega (EMBL-EBI)(Madeira et al., 2019) multiple sequence alignment of MurE homologs displayed using Jalview (Waterhouse et al., 2009) with Clustalx designated colours: Sp *Streptococcus pneumoniae*, Sa *Staphylococcus aureus*, Mt *Mycobacterium tuberculosum*, Pp1 *P. patens* (Pp3c24\_18820V3.2 v3.3 from Phytozome), Tm *Thermotoga maritima*, Me *Mesotaenium endlicherianum* (WDCW from Onekp CNGBDB), Ec *E. coli* (strain K12), An *Anabaena nostoc* PCC7120. All sequences are from the Uniprot or NCBI databases unless stated otherwise. Green arrows indicate ChloroP predicted cleavage site for PpMurE and red arrows the domain hinge points (Smith, 2010). Black arrows indicate residues with a reported role in MtMurE catalysis (Basavannacharya et al 2010). Letter labels indicate numbered residues with published ligand interractions: <sup>Ec</sup> for EcMurE (Gordon et al., 2001), <sup>Mt</sup> for MtMurE (Basavannacharya et al., 2010; Maitra et al., 2019) and <sup>Sa</sup> for SaMurE (Ruane et al., 2013) with colours indicating binding to UDP (blue), MurNAc sugar (green), ATP or ADP (mauve) and DL-DAP (orange) ligands. Blue asterisks indicate residues common to the Mur ligase family, which includes folylpolyglutamate synthetase, cyanophycin synthetase and the capB enzyme from Bacillales (Gordon et al., 2001; Smith, 2010) and pink asterisks indicate residues common to MurC, D, E and F ligases (Basavanacharya et al., 2010). Two streptophyte-specific features are identified by black boxes and the DNPR consensus by a red box.

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Supplemental Figure S10 Evolutionary relationship of both PpMurE proteins to selected MurE homologs. P. patens, as well as many ferns in the Polypodiidae, encodes two MurE homologs: PpMurE1 and PpMurE2, labelled in green. Different taxonomic groups are boxed to highlight the relationship of the P. patens proteins to bacterial, algal and streptophyte phylla. Sequences, except P. patens, were sourced from the ONEKP database and selected to represent each group (Leebens-Mack et al 2019, Carpenter et al 2019). The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992) and computed using MegaX software (Kumar et al 2018). The evolutionary distances, are in the units of the number of amino acid substitutions per site. PpMurE1 and the shorter Polypodiidae fern 'MurE1' homologs are evolutionarily closer to charophyte algae than land plants. PpMurE2 is closer to most marchantiophytes and other bryophytes, which lack a second MurE homolog, whereas the longer Polypodiidae 'MurE2' are closer to the Acrogymnsopermae (conifers).

PpMurE2 primarily differs from PpMurE1 in comprising a long, relatively unstructured extension at the amino terminus and a short carboxy terminal extension. The former is considerably longer than a conventional transit peptide (290 residues longer than typical bacterial homologs, compared to 94 residues for PpMurE1) and is common to most seed plant MurE-like proteins, as well as some streptophyte algal and bryophyte MurE homologs. The extended amino terminus is typically proline-rich in the amino terminal residues, being more glycine-rich in lower orders, and, in the later residues, more conserved within different plant divisions. The carboxy terminal extension (24 residues in PpMurE2 beyond a consensus streptophyte DDREECREAL motif in PpMurE1 (Supplemental Figure S9) is more highly conserved, with a consensus sequence (DDREECREALQXVDXLHXAGIDTFESPWRXPESX) that is common to most streptophyte MurE homologs, although streptophyte algae lack the terminal PESX. However, where there are two distinct MurE homologs, as there are for P. patens and some ferns, this carboxy terminal extension is typically absent from the shorter MurE homologs and these proteins appear to have de-evolved to more closely resemble their bacterial counterparts. The retention of a DNPR motif is common not only to the non-seed plants but also most seed plant MurE homologs, with the similarly charged DNPK also being common, and the Poaceae and a few Pinaceae being notable exceptions (DNPA and DNSR, respectively). In contrast to P. patens and the Polypodiidae ferns, many in the same and closely related phylla, including the Acrogymnospermae (Lin et al., 2017) do not have two candidate MurE homologs yet they encode most of the peptidoglycan synthesis enzymes.

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# **Parsed Citations**

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