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Transcriptomic response in symptomless roots of clubroot infected kohlrabi mirrors resistant plants

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Abstract

Background: Clubroot disease is caused by *Plasmodiophora brassicae* (Phytomyxea, Rhizaria) and is one of the economically most important diseases of brassica crops. The formation of the typical hypertrophied roots is accompanied by altered metabolism and hormone homeostasis of infected plants. Not all roots of an infected plant show the same phenotypic changes: while some roots remain uninfected, others develop galls of diverse sizes. Aim of this study was to analyse and compare the intra-plant heterogeneity of *P. brassicae,* root galls and symptomless roots of the same host plants (*Brassica oleracea* var. *gongylodes)* collected from a commercial field in Austria using transcriptome analyses.
Results: Symptomless roots did show transcriptomic traits that had previously described for resistant plants: Genes involved in host cell wall metabolism or salicylic acid (SA) mediated defence response were up-regulated in symptomless roots, while being down-regulated in gall tissues. Transcriptomes between symptomless roots and gall tissue were markedly different, with those differences being in accordance with visible physiological differences between the two tissues. On the pathogen side, a secreted SA methyl transferase (PbBSMT) was one of the highest expressed genes in gall tissues.
Conclusions: Infected and uninfected roots of the same clubroot infected plant showed transcriptomic differences which were previously only observed between clubroot resistant and susceptible hosts. We provide further evidence for the biological relevance of PbBSMT which on the one hand likely causes a decrease of SA in the galls, while the PbBSMT produced Methyl-SA potentially leads to increased pathogen tolerance in uninfected roots. The here described intra-plant heterogeneity was unexpected and highlights the need for targeted analyses of clubroot interaction using cell, tissue type, organ specific probing to identify traits that prevent the formation of clubroot disease.

Keywords: Clubroot, Host-pathogen interaction, *Plasmodiophora brassicae*, *Brassica oleracea*, root transcriptome, protist

List of abbreviations

ABA: Abscisic acid; BR: Brassinosteroids; C: Control; CDS: Coding sequence; CK: Cytokinin; COG: Clusters of Orthologous Groups; DEG: Differentially expressed gene; dpi: days post inoculation; ET: Ethylene; FDR: False discovery rate; FPKM: Fragments per kilobase per million; HR: hypersensitive response; IsoPct: Isoform percentage; JA: Jasmonic acid; MeSA: Methyl-salicylate; NCBI: National Center for Biotechnology Information; OG: Older root gall; ORF: Open reading frame; PbraAT: Austrian *P. brassicae* field population; PR: Pathogenesis related; RNA: Ribonucleic acid; SA: Salicylic acid; SAM: S-adenosylmethionine; SL: Symptomless root; TAIR: The Arabidopsis Information Resource; YG: Younger root gall.

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54

55 **Background**

56 Clubroot disease is one of the most important diseases of Brassica crops worldwide
57 accounting for approximately 10% loss in *Brassica* vegetable, fodder, and oilseed crops
58 (Dixon, 2009). Clubroot is caused by *Plasmodiophora brassicae*, an obligate biotrophic
59 protist, taxonomically belonging to Phytomyxea within the eukaryotic super-group Rhizaria
60 (Bulman & Braselton, 2014, Neuhauser *et al.*, 2014). This soil borne pathogen has a complex
61 life cycle: zoospores infect root hairs where primary plasmodia form. These plasmodia
62 develop into secondary zoospores, which are released into the soil and re-infect the root
63 cortex where secondary plasmodia develop (Kageyama & Asano, 2009). The secondary
64 plasmodia mature into resting spores, which are released into the soil. In infected host tissue
65 division and elongation of cells is triggered upon infection, which leads to hypertrophies of
66 infected roots resulting in the typical root galls or clubroots (Fig. 1).

67

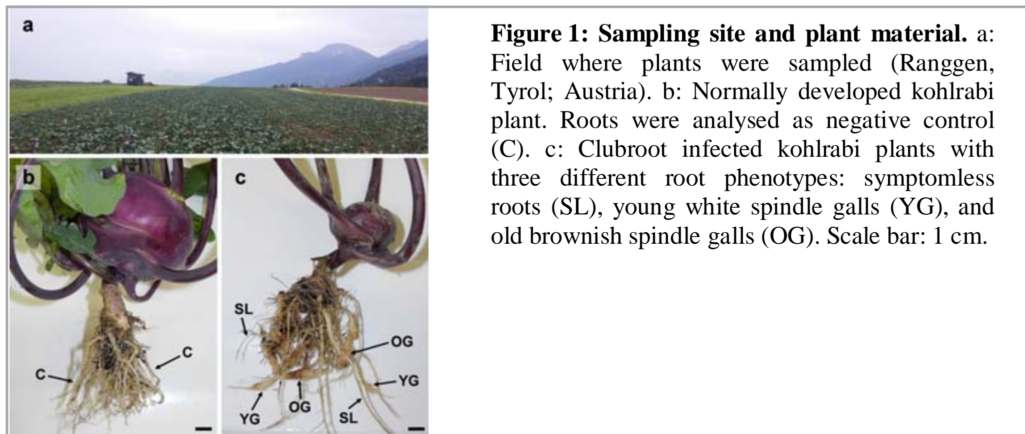

Figure 1: Sampling site and plant material. a: Field where plants were sampled (Ranggen, Tyrol; Austria). b: Normally developed kohlrabi plant. Roots were analysed as negative control (C). c: Clubroot infected kohlrabi plants with three different root phenotypes: symptomless roots (SL), young white spindle galls (YG), and old brownish spindle galls (OG). Scale bar: 1 cm.

68
69

70 *P. brassicae* can only be grown and studied in co-culture with its host. This has hampered
71 both, targeted and large scale studies on the molecular basis of *P. brassicae* and the
72 interaction with its host (Schwelm *et al.*, 2018). Because of the economic importance of
73 clubroot disease, numerous studies analysed specific aspects of the biology, physiology and
74 molecular biology of the interaction from the plant side to better understand and control the
75 disease. The first of these experimental studies were based on the
76 *Arabidopsis/Plasmodiophora* pathosystem (e.g. Siemens *et al.*, 2006, Devos *et al.*, 2006).
77 During the last years not only an increasing number of *Brassica* (host) genomes became
78 available (Wang *et al.*, 2011, Chalhoub *et al.*, 2014, Liu *et al.*, 2014, Cheng *et al.*, 2016), but
79 also several *P. brassicae* genomes (Schwelm *et al.*, 2015, Rolfe *et al.*, 2016, Bi *et al.*, 2016,
80 Daval *et al.*, 2018) permitting new research approaches, including targeted transcriptome
81 studies. There are analyses of (plant) transcriptomes of whole roots of clubroot infected
82 compared with uninfected plants (Agarwal *et al.*, 2011, Chen *et al.*, 2016a, Malinowski *et al.*,
83 2016, Zhao *et al.*, 2017), of host varieties susceptible and tolerant to clubroot (Jubault *et al.*,
84 2008, Chen et al., 2016a), or the host response to different *P. brassicae* isolates (Siemens et
85 al., 2006, Agarwal et al., 2011, Lovelock *et al.*, 2013, Zhang *et al.*, 2016).

86 Plants infected with *P. brassicae* show marked physiological changes including cell wall
87 biosynthesis, plant hormone metabolism and plant defence related processes. Expansin genes,
88 involved in plant cell expansion and elongation (Cosgrove, 2005), were up-regulated in
89 *P. brassicae* infected roots (Siemens et al., 2006, Irani *et al.*, 2018, Agarwal et al., 2011). In
90 *P. brassicae* infected roots enzymatic activity of Xyloglucan endo
91 Transglucosylase/Hydrolases (XTHs) increases (Devos *et al.*, 2005), while an early response
92 to *P. brassicae* infection was up-regulation of the phenylpropanoid pathway that provides
93 lignin precursors (Zhao et al., 2017). With progression of clubroot development, the
94 lignification of clubroot tissue was reduced (Deora *et al.*, 2013) and genes involved in

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95 lignification processes were down-regulated (Cao *et al.*, 2008). On the other hand cell wall
96 thickening and lignification was suggested to limit the spread of the pathogen in tolerant
97 *B. oleracea* (Donald *et al.*, 2008) and *B. rapa* (Takahashi *et al.*, 2001).
98 The development of clubroot symptoms is accompanied by changes of plant hormone
99 homeostasis (Siemens et al., 2006, Ludwig-Müller *et al.*, 2009, Malinowski et al., 2016).
100 During clubroot development auxins mediate host cell divisions and elongation. Auxins
101 increase over time during clubroot development and are accumulated in *P. brassicae* infected
102 tissues in a sink like manner (Ludwig-Müller et al., 2009). Also genes belonging to the auxin
103 conjugating GH3 protein family are regulated differentially during clubroot development
104 (Jahn *et al.*, 2013) with one GH3 protein gene (PbGH3; CEP01995.1) identified in the
105 *P. brassicae* genome (Schwelm et al., 2015). Cytokinins (CKs) increase initially, but
106 decreases again with the onset of gall formation (Malinowski et al., 2016). At the same time
107 *P. brassicae* plasmodia produce minute amounts of CKs (Müller & Hilgenberg, 1986).
108 Therefore, CKs play a crucial role in disease development not only through their regulation of
109 cell division but also through their interference in the sugar metabolism and invertase
110 production, which might be crucial for the nutrition of *P. brassicae* (Siemens *et al.*, 2011).
111 Stress- and defence related phytohormones like salicylic acid (SA), jasmonic acid (JA),
112 brassinosteroids (BR), and ethylene (ET) and their regulatory pathways also change in
113 response to pathogen infection (Kazan & Lyons, 2014). The accumulation of SA plays a key
114 role in plant defence against biotrophic pathogens, often resulting in a localized
115 hypersensitive response and induction of pathogenesis-related (PR) genes. Systemic acquired
116 resistance (SAR) is a form of induced resistance that is activated by SA throughout a plant
117 after being exposed to elicitors from microbes or chemical stimuli (Klessig *et al.*, 2018). High
118 endogenous levels of SA and exogenous SA reduced the infection of the host by *P. brassicae*
119 (Lovelock et al., 2013, Lovelock *et al.*, 2016) and in tolerant hosts SA related genes are
120 induced upon infection (Siemens *et al.*, 2009, Agarwal et al., 2011, Zhang et al., 2016). The
121 SAR-deficient *npr1-1* and SA-deficient isochorismate synthase 1 (ICS1) *sid2 Arabidopsis*
122 mutants showed an increased susceptibility to *P. brassicae*, whereas the *bik-1* mutant, with
123 elevated SA levels, was more resistant (Chen *et al.*, 2016b). Pathogenesis-related (PR)
124 defence proteins are induced by SAR and higher expressed in resistant than in susceptible
125 *B. rapa* and *Arabidopsis* species (Chen et al., 2016a, Jubault *et al.*, 2013, Jia *et al.*, 2017). Via
126 a secreted methyltransferase (PbBSMT; AFK13134.1), *P. brassicae* might counteract the
127 plant SA-defence. This SABATH-like methyltransferase has been shown to convert SA to
128 methyl-salicylate (MeSA) *in vitro* (Ludwig-Müller *et al.*, 2015). The proposed function *in*
129 *planta* is the removal of SA in local infected tissue as MeSA is volatile. *Arabidopsis* mutants
130 expressing the PbBMST gene showed a higher susceptibility towards *P. brassicae* (Bulman *et*
131 *al.*, 2018).
132 The *P. brassicae* PbGH3 was also able to conjugate JA with amino acids *in vitro*
133 (Schwelm et al., 2015). In general, JA is associated with resistance against necrotrophic
134 microbes (Pieterse *et al.*, 2012, Fu & Dong, 2013). In *A. thaliana* Col-0 several JA-responsive
135 genes were induced in infected root tissues and JA accumulates in galls (Siemens et al., 2006,
136 Gravot *et al.*, 2012). *Jasmonate resistant 1* (*jar1*) mutants, impaired in JA-Ile accumulation,
137 showed a higher susceptibility to *P. brassicae* (Agarwal et al., 2011, Gravot et al., 2012).
138 Thus, JA responses contributed to a basal resistance against some strains of *P. brassicae* in
139 *A. thaliana* Col-0 (Gravot et al., 2012). But in partially resistant *Arabidopsis* Bur-0 only weak
140 JA responses compared with the susceptible Col-0 were found (Lemarié *et al.*, 2015).
141 Generally, clubroot susceptible hosts show a high level of JA response, whereas it is reduced
142 in resistant hosts (Jubault et al., 2008, Chen et al., 2016a). Those differences might be due to
143 if aliphatic or aromatic glucosinolate production is induced by JA in the particular host (Xu *et*
144 *al.*, 2018).
145 Aim of this study was to generate the first data set of root tissue specific transcriptomic
146 response of individual plants during clubroot development. Usually, clubroot infected plants
147 do not develop symptoms uniformly on all root parts with some roots showing strong
148 symptoms and others not showing symptoms at all (Fig. 1). We collected samples of kohlrabi
149 (*Brassica oleracea* var. *gongylodes*) infected with *P. brassicae* (PbraAT) from a field in

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150 Austria. We compared young and old clubroots and symptomless roots of the same infected
151 plants and a control plant. We analysed similarities and differences of their transcriptomic
152 profile focussing on cell wall metabolism, hormone metabolism, and defence response.
153

154 **Methods**

155 **Sampling**

156 Kohlrabi "purple Vienna" plants with clubroots and without visible root infections were
157 collected from a *P. brassicae* infested field in Ranggen (Tyrol, Austria;) in August 2016.
158 Root samples from symptomless roots (SL), young white spindle galls with waxy appearance
159 (YG) and old brownish spindle galls (OG) (Fig. 1) were taken in triplicates from three
160 individual clubroot infected plants. Only one plant without apparent infection could be
161 identified in the vegetable plot, which was additionally sampled as uninfected control (C)
162 (Fig. 1). Galls and roots were thoroughly washed with tap water, before samples were taken
163 (categories C, SL, YG, and OG), and transferred to RNA later (Ambion, Austin, TX, USA)
164 where they were stored until RNA extraction.
165

166 **RNA extraction and sequencing**

167 The outer layer of the root galls was trimmed-off and the trimmed galls and symptomless
168 roots were snap-frozen in liquid nitrogen and transferred to 1.5 mL tubes containing RNase
169 free zirconia beads (0.5 mm and 2 mm in diameter). Samples were homogenized using a
170 FastPrep (MP Biomedicals, Santa Ana, CA, USA) for 40 s at 6 m s^{-1} followed by manual
171 grinding with pistils after repeated snap-freezing. Total RNA was extracted using the Qiagen
172 RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's
173 instructions, but with an additional 80% ethanol column wash prior elution. RNA quantity
174 and quality were determined using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo
175 Alto, CA, USA). Additional RNA quality assessment, polyA selection (SENSE mRNA-Seq
176 Library Prep Kit; Lexogen, Vienna, Austria), library construction (10 libraries; 1x C, 3x SL,
177 3x YG, and 3x OG) and sequencing was performed at the VBCF NGS Unit (Vienna, Austria).
178 Sequencing was performed with on the Illumina HiSeq 2500 platform (Illumina, San Diego,
179 CA, USA) with a strand specific paired end library (2x 125 bp) using v4 chemistry.
180

181 **Bioinformatics**

182 Raw reads were quality checked using FastQC (Andrews, 2010). Illumina adapters were
183 removed and good quality reads were kept using Trimmomatic v0.36 (sliding window 5 bp;
184 average quality score > 20) (Bolger *et al.*, 2014). Only reads with a minimum length of 75 bp
185 were processed further after a repeated FastQC check to confirm quality improvement. From
186 the uninfected control library, 75% of the reads were randomly picked three times to generate
187 pseudo-triplicates. Transcripts were *de novo* assembled using Trinity v2.2 (Grabherr *et al.*,
188 2011) with strand specific library type (RF) and jaccard clip options. Expression estimation
189 was performed using Trinity embedded RSEM (Li & Dewey, 2011) keeping only transcripts
190 with more than at least one fragment per kilobase per million (FPKM > 1) and an isoform
191 percentage (IsoPct) > 1%.

192 The assembled transcripts were blasted using BlastN (Altschul *et al.*, 1990) against the
193 coding sequences (CDS) of *B. oleracea* (Liu et al., 2014) and a custom database containing
194 the CDS of the *P. brassicae* isolates e3 (Schwelm et al., 2015) and PT3 (Rolfe et al., 2016) to
195 identify, if the transcript derived from the pathogen or host (E-value < 10^{-5}). Transcripts with
196 blast hits in both reference databases were analysed manually to identify their origin
197 according to sequence identity and E-value. Transcripts with no hit in any reference were
198 blasted (BlastP) against National Center for Biotechnology Information (NCBI) non
199 redundant protein database and manually assigned to the corresponding species or discarded
200 for further analysis. Transcripts with a best hit to a Brassicaceae reference sequence were
201 assigned to the host transcriptome. Transcripts with hits to *P. brassicae* sequences were
202 assigned to the pathogen transcriptome. Open reading frames (ORFs) were predicted using
203 TransDecoder v3.0.1 (Haas & Papanicolaou, 2017). Only the longest ORF per transcript was

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204 used for further analysis. Translated peptide sequences were annotated using the KEGG
205 (Kyoto Encyclopedia of Genes and Genomes) Automatic Annotation Server (KAAS) (Moriya
206 *et al.*, 2007) and eggNOG-mapper v0.99.3 (Huerta-Cepas *et al.*, 2016). Kohlrabi genes were
207 additionally annotated using Mercator (Lohse *et al.*, 2014) with default settings. Mercator
208 categories were used to bin predicted genes into groups. Putative secreted proteins of
209 *P. brassicae* were predicted with Phobius v1.01 (Käll *et al.*, 2004) and SignalP v4.1 (Nielsen,
210 2017) in combination with TMHMM v2.0 (Krogh *et al.*, 2001). Carbohydrate active enzymes
211 were predicted using dbCAN (Yin *et al.*, 2012).
212 Log_2-fold changes of differentially expressed genes (DEGs) were calculated using edgeR
213 (Robinson *et al.*, 2010) with default settings. All DEGs with false discovery rate (FDR) $<$
214 0.05 were treated as DEGs. Heatmaps for selected Mercator categories were created using R
215 v3.3.2 (R Core Team, 2016) with the package 'pheatmap' v1.0.8 (Kolde, 2015) applying
216 UPGMA clustering. Labelling of the predicted *B. oleracea* genes was done according to their
217 homologous *A. thaliana* genes from TAIR (The Arabidopsis Information Resource) and
218 adapted if necessary. Abundance of DEGs was visualized using the R package 'ggplot2'
219 v2.2.1 (Wickham, 2009).
220
221

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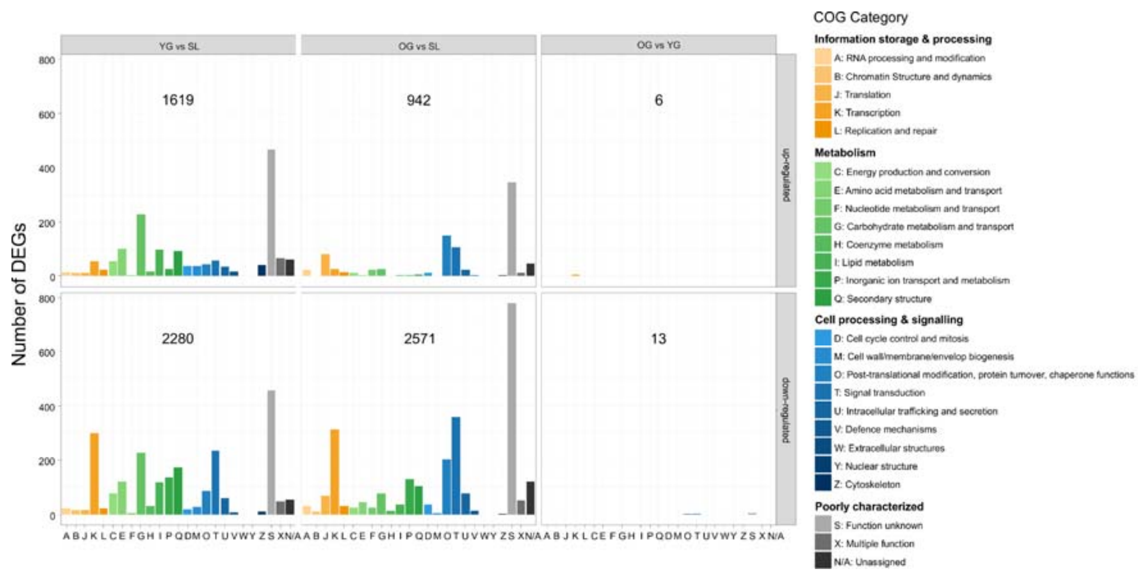
222 ## Results

223 ### Transcriptome analyses

224 162 million good quality reads with an average length of 125 bp were obtained from all
225 libraries (Additional file 2: Table S1). Including isoforms a total of 10940 genes were
226 predicted for *P. brassicae* and 42712 for *B. oleracea*. About 50% of the *P. brassicae* and 85%
227 of the kohlrabi transcripts could be functionally annotated using eggNOG-mapper. Only
228 0.0005% of the reads of the control plant (C) and the symptomless root samples (SL) matched
229 *P. brassicae* transcripts, which indicates that these roots were not infected by *P. brassicae*. In
230 the YG and OG libraries 23% and 33% of the reads matched *P. brassicae* (Additional file 1:
231 Figure S1).

232 The transcriptomes of infected plants (SL, YG, OG) contained a total of 5204 DEGs.
233 Compared with SL, in YG 1619 DEGs were up-regulated and 2280 were down-regulated
234 (Fig. 2, Additional file 2: Table S2), while in OG 942 DEGs were up- and 2571 were down-
235 regulated. 790 plant DEGs were assigned to the COG (Cluster of Orthologous Groups)
236 category "Information and Storage Processing", 1401 to "Metabolism", 1245 to "Cellular
237 Process and Signalling" and 1768 to "Poorly Characterized" by eggNOG-mapper (Fig. 2,
238 Additional file 2: Table S3). Only 19 plant genes were differentially expressed between OG
239 and YG (Additional file 2: Tables S4). Between the control plant and the three root tissue
240 types of the infected plants (C vs SL/YG/OG) 19230 DEGs were found (Additional file 2:
241 Tables S5, S6).
242
243



244
245 **Figure 2: Numbers of differentially expressed genes (DEGs) in clubroot infected kohlrabi roots**
246 **per COG category.** DEGs were split into up- and down-regulated genes. Total number of DEGs in
247 each panel is given.
248
249

250 ### Plant cell wall metabolism

251 In *B. oleracea* 161 of the 5204 DEGs within infected plants (SL vs YG vs OG) were involved
252 in cell wall synthesis, modification, degradation, or phenylpropanoid metabolism. Cellulose,
253 hemicellulose, pectin, and lignin synthesis genes were up-regulated in SL compared with
254 galls tissues (Fig. 3), whereas genes involved in cell wall modification and degradation were
255 down-regulated (Fig. 3). The changes in expression of cell wall genes were more prominent
256 between SL and OG than between SL and YG. In SL a UDP-D-glucuronate 4-epimerase 6
257 (GAE6) homolog responsible for the synthesis of UDP-D-glucuronic acid, the main building
258 block for pectins (Harholt *et al.*, 2010) was up-regulated compared with gall tissue. Predicted
259 expansin (EXP) and expansin-like (EXL) genes were mainly down-regulated in SL compared

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with YG and OG (Fig. 3). Genes coding for XTHs were among the strongest down-regulated DEGs in SL, with XTH24 being the strongest down-regulated transcript of all DEGs. The phenylpropanoid pathway was up-regulated in SL compared with YG and OG (Fig. 3). This includes the phenylalanine ammonia-lyase 1 (PAL1) homolog, a key enzyme in lignin biosynthesis. Flavonoid metabolism was also induced in SL (Additional file 1: Figure S2). Compared with the uninfected control plant cell wall synthesis genes were up-regulated in SL (Additional file 1: Figure S3). XTH genes were up-regulated in YG and OG compared with the uninfected control plant.

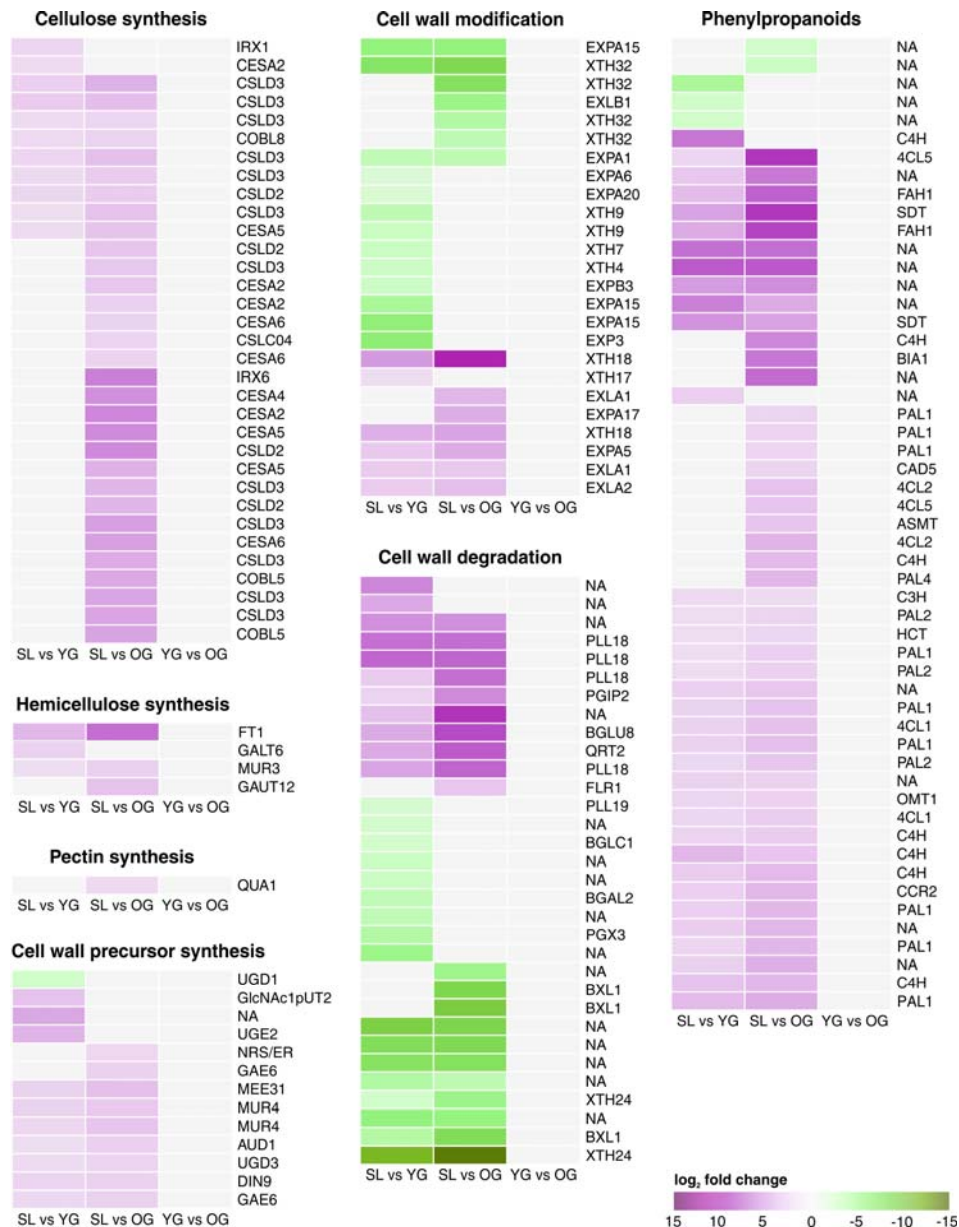

Figure 3: Kohlrabi cell wall metabolism. Clustered heatmaps of $\log_2$ fold change values of DEGs. Genes involved in anabolic processes of cell wall components were generally up-regulated in SL compared with YG and OG, whereas catabolic and modifying genes were mainly down-regulated. No DEGs were present comparing YG with OG. Up-regulated genes are shaded in purple and down-regulated genes in green. *Arabidopsis* homologs are given. NA: not assigned.

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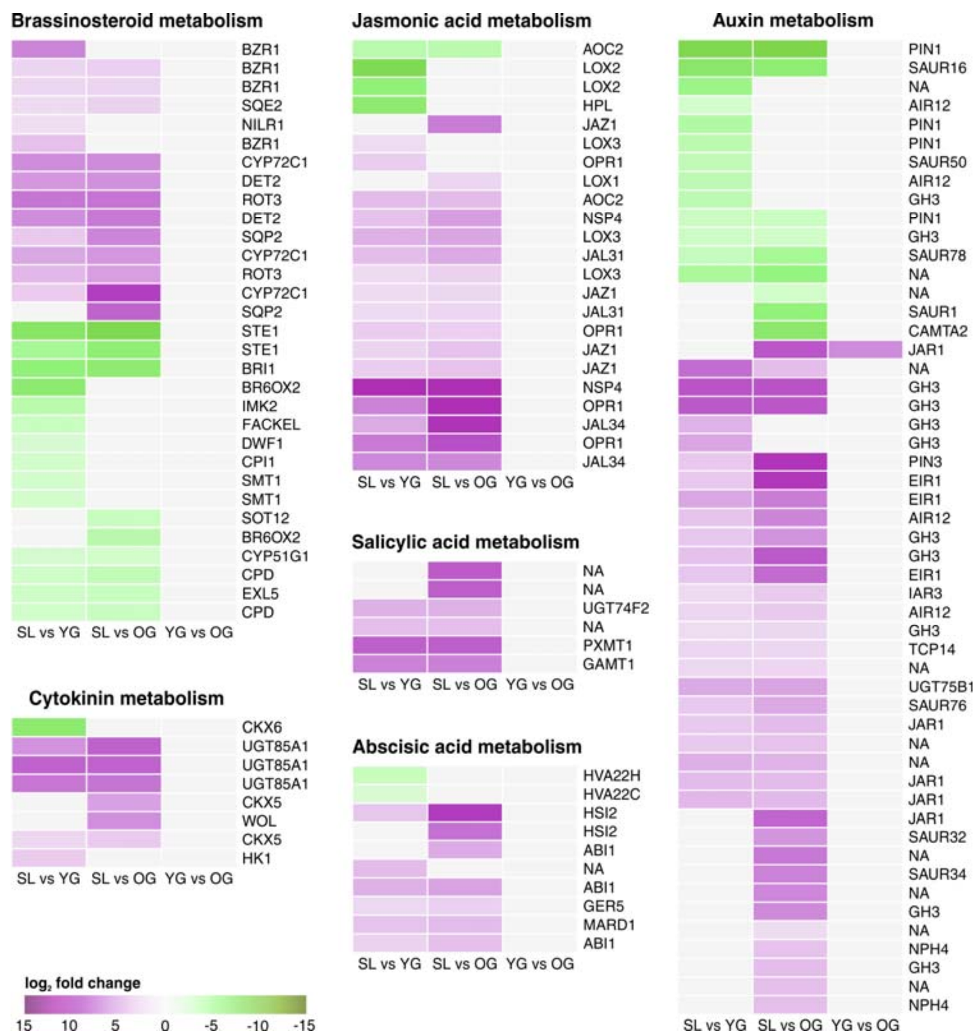
278

279 **Plant hormones**

280 The CK and auxin metabolism was altered in YG and OG compared with SL (Fig. 4). So was
281 a homolog of CKX6 (cytokinin oxidase/dehydrogenase 6) down-regulated in SL compared
282 with YG. Homologs of CKX5, CK receptors, and a CK-regulated UDP-glucosyltransferase
283 were up-regulated in SL. Most auxin related DEGs, auxin response factors (ARFs and IAAs)
284 and IAA amino acid conjugate synthetases (GH3) were up-regulated in SL compared with
285 gall tissue (Additional file 1: Figure S4). However, an IAA7 gene, a repressor of auxin
286 inducible genes was down-regulated, as well as a homolog of GH3.2. Expression of PIN-
287 FORMED 1 (PIN1) genes was reduced in SL (Fig. 4). Whereas SAUR (small auxin up-
288 regulated RNA) and AIR12 (auxin-induced in root cultures protein 12-like) genes, were
289 found in up- and down-regulated DEGs (Fig. 4). Myrosinases and nitrilases were up-regulated
290 in SL compared with galls (Additional file 1: Figure S5). Compared with the control CK and
291 auxin metabolism were up-regulated in SL (Additional file 1: Figure S3). In old galls two
292 transcripts related to auxin synthesis and regulation were down-regulated compared with
293 young galls (Additional file 2: Table S4).

294



295

296 **Figure 4: Kohlrabi phytohormone metabolism.** Heatmaps of log_2 fold change values of DEGs.
297 Genes involved in cytokinin, jasmonic acid, salicylic acid, and abscicsic acid metabolism were up-
298 regulated in SL. Genes coding for brassinosteroids clustered into two groups: later stages in BR
299 biosynthesis (down-regulated) and early sterol biosynthesis (up-regulated). Genes involved in auxin
300 metabolism were found within the up- and down-regulated DEGs in SL compared with root galls. One

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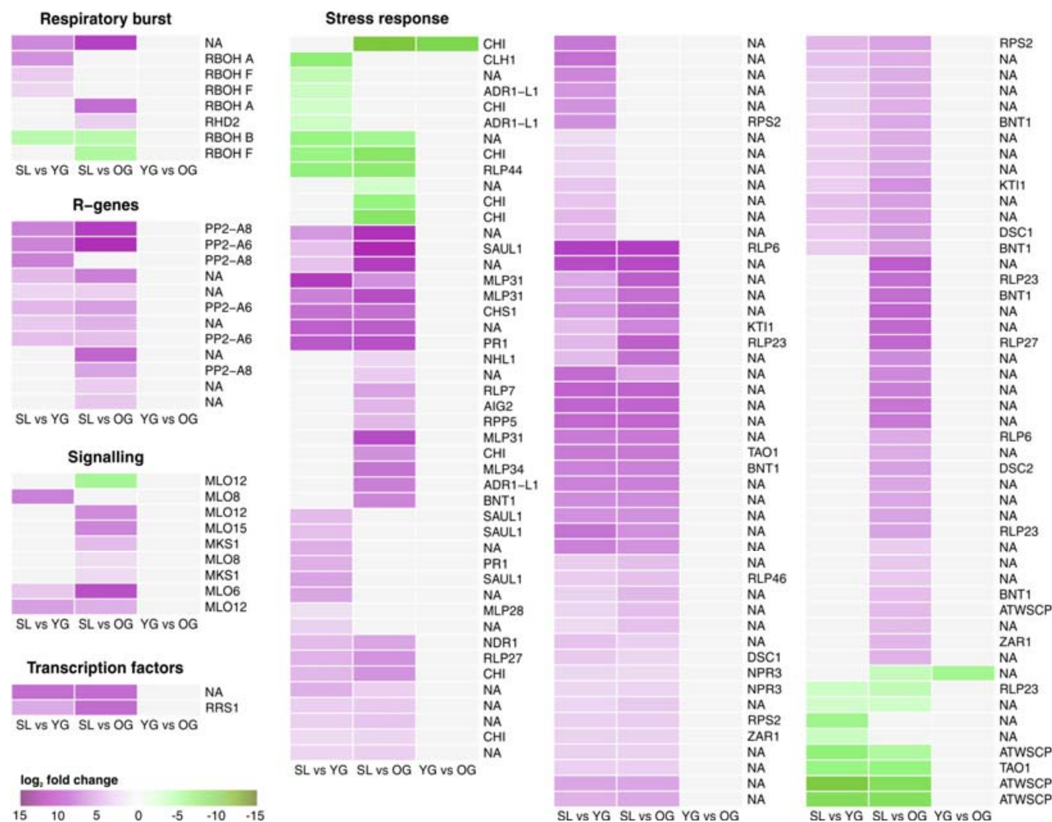
301 DEGs (JAR1) was found between YG and OG. Up-regulated genes are shaded in purple and down-
302 regulated genes in green. *Arabidopsis* homologs are given. NA: not assigned.
303 Early sterol biosynthesis genes, such as the steroid reductase DET2, were up-regulated in
304 SL compared with galls (Fig. 4). Compared with the control, DET2 was up-regulated in SL
305 (Additional file 1: Figure S3). However, BR biosynthesis genes were generally down-
306 regulated in SL (Fig. 4), including key genes like DWF1 (dwarf 1) or BRI1 (BR receptor
307 brassinosteroid insensitive 1).
308 Abscisic acid (ABA) signal transduction related genes like ABI1 (ABA insensitive 1) and
309 HSI2 (high-level expression of sugar-inducible gene 2) were up-regulated in SL compared
310 with YG and OG (Fig. 4) whereas ABA related transcription factors WRKY18 and
311 HVA22A/C homologs were down-regulated in SL (Fig. 4, Additional file 1: Figure S6).
312

313 **Plant defence**

314 Generally, genes for disease resistance proteins were up-regulated in SL compared with YG
315 and OG (Fig. 5). From pathogen recognition genes via signalling proteins and transcription
316 factors to pathogenesis related (PR) proteins, the whole signal cascade of pathogen defence
317 was affected. Of the predicted defence related DEGs within infected plants (SL vs YG vs OG)
318 60 were assigned as TIR-NBS-LRR (Toll/interleukin-1 receptor nucleotide-binding site
319 leucine-rich repeat) class proteins.
320 The BIK1 (botrytis-induced kinase 1), which interacts with BRI1 and BAK1 (BRI1-
321 associated receptor kinase) to induce defence responses was up-regulated in SL compared
322 with galls (Additional file 1: Figure S7) and up-regulated in SL compared with C (Additional
323 file 1: Figure S3).
324
325


326
327
328 **Figure 5: Biotic stress response of kohlrabi.** Heatmaps of $\log_2$ fold change values of DEGs. Almost
329 all DEGs were up-regulated in SL compared with root galls. Two down-regulated DEGs, a chitinase
330 (CHI) and a gene of unknown function, were found between YG and OG. Up-regulated genes are

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331 shaded in purple and down-regulated genes in green. *Arabidopsis* homologs are given. NA: not
332 assigned.
333
334 In SL JA related genes such as LOX2 (lipoxygenase 2), AOC (allene oxide cyclase), and
335 HPL (hyperoxide lyase) were down-regulated, while other LOX genes and the JA amido
336 synthetase genes JAR1 were up-regulated in SL compared with galls (Fig. 4). One down-
337 regulated isoform of JAR1 was found between OG and YG. We found no glucosinolate
338 biosynthesis genes in the DEGs in our study.
339 The ICS1 gene and the ICS1 activating transcription factor WRKY28 (van Verk *et al.*,
340 2011) were up-regulated in SL compared with the control plant (data not shown). Genes for
341 SA modification, like the SA methylating SABATH methyl transferase genes (PXMT1,
342 GAMT1) and a SA-glucosidase (UGT74F2) were up-regulated in SL compared with galls
343 (Fig. 4). The SA induced PR1 gene was induced in SL (Fig. 5). The PR-gene expression
344 regulator NPR1 (non-expressor of PR1) was not differentially expressed in our samples. Of
345 genes that regulate PR1 expression via NPR1, WRKY70 was down-regulated in SL whereas
346 NPR3 and TGA3 were up-regulated (Fig. 5, Additional file 1: Figure S6). Compared with the
347 uninfected plant, the TGA3 gene expressions appeared to be induced in SL. Genes for the
348 TAO1 disease resistant protein, which induces PR1 expression were up-regulated in SL, as
349 well as the NDR1 (non-race specific disease resistance 1) gene, required for the establishment
350 of hypersensitive response and SAR. Genes for the disease resistant protein RPS2, activated
351 by NDR1, were also up-regulated in SL(Fig. 5). Additionally, other defence related genes
352 coding for protease inhibitor genes, R-genes or some chitinases were down-regulated in SL
353 (Fig. 5). Comparing biotic stress response genes of SL with the control (C), we observed an
354 up-regulation of 164 of the total 190 DEGs (Additional file 1: Figure S3).
355
356 ***P. brassicae* gene expression**
357 The *P. brassicae* genes with the highest FPKM values belonged to growth and cellular
358 process related COG categories such as translation, transcription, and signal transduction, but
359 also in energy conversion and carbohydrate and lipid metabolism (Additional file 1:
360 Figure S8). The *P. brassicae* PbBSMT gene was amongst the highest expressed pathogen
361 genes (Additional file 2: Tables S7, S8). Other highly expressed genes were HSPs (heat shock
362 proteins), a glutathione-S-transferase, an ankyrin repeat domain-containing protein, ribosomal
363 genes, and genes of unknown function (Additional file 2: Tables S7, S8). The PbGH3 gene
364 was not expressed in our samples. The *P. brassicae* protease gene PRO1, proposed to be
365 involved in spore germination (Feng *et al.*, 2010), was expressed in both YG and OG.
366 Between YG and OG samples only five *P. brassicae* DEGs were identified, coding for a
367 HSP, a chromosomal maintenance protein, a DNA-directed RNA-polymerase, a
368 retrotransposon and a Scl Tal1 interrupting locus protein (Additional file 2: Table S9).
369 Cumulating all FPKM values revealed that most sequenced reads from *P. brassicae* RNA
370 extracted from root galls (YG and OG) mapped to the COG categories "Post-translational
371 modification, protein turnover, chaperon functions" and "Translation" (Additional file 1:
372 Figure S9). Very few *P. brassicae* reads were found in the data obtained from the control
373 plant and SL (Additional file 1: Figure S10), those were most likely from attached spores or
374 contamination via soil particles.
375
376

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377 **Discussion**

378 **Symptomless roots of clubroot infected plants show transcriptomic traits of clubroot**
379 **resistant/tolerant plants.**
380
381 We found that symptomless roots and clubroots originating from the same plant show
382 differences in their transcriptomic profile similar to the differences of whole roots between
383 resistant and susceptible plants. Gene expression patterns of symptomless roots were similar
384 to the patterns described for resistant hosts, while in clubroot tissue patterns were similar to
385 those observed in susceptible plants. Symptomless roots did also show an induction of several
386 defence related processes (Figs. 3-5) compared with the uninfected control plant. Genes
387 involved in cell wall stability were up-regulated in SL (Fig. 3).
388 Reinforcement of cell walls has previously been reported to hamper the development of
389 *P. brassicae* in resistant *B. oleracea* (Donald et al., 2008) and *B. rapa* callus cultures
390 (Takahashi et al., 2001). Lignin biosynthesis genes were up-regulated in SL tissue compared
391 with root gall tissue and the control plant (Fig. 3, Additonal file 1: Figure S2). Induced
392 lignification processes were observed in shoots of infected plants (Irani et al., 2018) and
393 between resistant and susceptible *B. oleracea* cultivars (Zhang et al., 2016). PAL1, a key
394 enzyme in lignin, SA (discussed below) and flavonoid biosynthesis (Chu *et al.*, 2014, Song *et*
395 *al.*, 2016, Lahlali *et al.*, 2017) was up-regulated in symptomless roots compared with clubroot
396 tissue and the control plant (Fig. 3). Increased lignin biosynthesis and up-regulation of PAL1
397 has been described for an clubroot resistant oilseed rape line carrying the resistance gene Rcr1
398 (Lahlali et al., 2017), while callus cultures overexpressing PAL1 were resistant to an infection
399 with *P. brassicae* (Takahashi et al., 2001).
400 Root reinforcement, therefore, seems to be part of the tolerance mechanism of plants
401 against *P. brassicae*. *P. brassicae* has only a limited arsenal of plant cell wall degrading
402 enzymes in its genome (Schwelm et al., 2015, Rolfe et al., 2016) and infects its hosts via
403 mechanical force with a specialised extrusosome called "Stachel and Rohr" (Kageyama &
404 Asano, 2009). Increased stability of the cell wall, as indicated by gene expression patterns in
405 symptomless roots (Fig. 3) or described for resistant plants (Donald et al., 2008, Takahashi et
406 al., 2001), requires higher mechanical force for successful infection of the host plant,
407 providing a considerable obstacle for *P. brassicae*.
408 In symptomless roots of infected plants, defence related pathways regulated by hormones
409 show patterns usually linked to induced plant defence (Fig. 4). Clubroot tissue on the other
410 hand showed suppression of SA-defence related processes. Genes of SA biosynthesis were
411 up-regulated in SL, but were down-regulated in galls. Salicylic acid can be synthesised via
412 isochorismate (ICS pathway) or from phenylalanine (via PAL pathway) (Pieterse et al., 2012,
413 Lovelock et al., 2016). The up-regulation of the PAL1 gene in symptomless roots could also
414 be linked to the PAL-dependent synthesis of SA, but the majority of SA in clubroot is
415 produced via the ICS pathway (Lovelock et al., 2016). Based on the higher expression of
416 ICS1 and WRKY28 in SL compared with the control, the synthesis of SA was likely induced
417 in SL. In clubroot resistant hosts SA-defence is usually induced (Jubault et al., 2013, Chen et
418 al., 2016a, Lovelock et al., 2016), whereas the SA deficient *sid2* (ICS1) mutant of
419 Arabidopsis was more susceptible to *P. brassicae* infection (Chen et al., 2016b).
420 High SA levels in plant tissues helped to reduce new *P. brassicae* infections (Lovelock et
421 al., 2013), but SA alone is not sufficient to induce resistance against *P. brassicae* (Lovelock
422 et al., 2016). Because SA levels increase in clubroot tissue *P. brassicae* is thought to secret a
423 SABATH-type methyltransferase (PbBSMT; Ludwig-Müller et al., 2015, Bulman et al.,
424 2018), which was one of the highest expressed genes of *P. brassicae* in this study (Additional
425 file 2: Tables S7, S8). PbBSMT has been shown to methylate salicylic acid, contributing to a
426 local reduction of SA in the galls (Ludwig-Müller et al., 2015). MeSA is the major transport
427 form of SA in the plant and has a key role in inducing SAR (Vlot *et al.*, 2009, Park *et al.*,
428 2007). So based on our data we hypothesise that *P brassicae* reduces SA concentrations in the
429 galls via PbBMST mediated methylation (Ludwig-Müller et al., 2015). The so produced

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430 MeSA could trigger SA-related defences in distant plant parts, which become resilient
431 towards new pathogen infection.
432 Processes downstream from SA are mediated via NPR1 (not differentially expressed in our
433 dataset). NPR1 induces PR-gene expression when bound to TGA3 (Saleh *et al.*, 2015). In its
434 interaction with WRKY70, NPR1 serves as a negative regulator of the SA biosynthesis gene
435 ICS1 (Wang *et al.*, 2006). Thus the observed up-regulation of TGA3 in SL (Additional file 1:
436 Figure S6) would lead to an induction of expression of PR-genes in SL. The reduced
437 expression of WRKY70 in SL compared with the galls would lead to a higher SA production
438 in the uninfected root tissue. Additionally, we found an up-regulation of NPR3 genes, coding
439 for repressors of SA-defence genes (Ding *et al.*, 2018), in SL compared with the galls
440 (Fig. 5). When bound to SA, NPR3 would lose its function of repressing SA-defence genes
441 (Ding et al., 2018). The higher expression of NPR3 in the SL might be necessary to
442 compensate for negative effects of the SA synthesis in SL roots.
443 Against some strains of *P. brassicae,* JA contributed to a basal resistance in *A. thaliana*
444 Col-0 and mutants impaired in JA-Ile accumulation, showed a higher susceptibility to
445 *P. brassicae* (Agarwal et al., 2011, Gravot et al., 2012). In *A. thaliana* Col-0 several JA-
446 responsive genes were induced in infected root tissues and JA accumulates in galls (Siemens
447 et al., 2006, Gravot et al., 2012). But in partially resistant *Arabidopsis* Bur-0 only weak JA
448 responses compared with the susceptible Col-0 were found (Lemarié et al., 2015). Those
449 differences might be due to if aliphatic or aromatic glucosinolate production is induced by JA
450 in the particular host (Xu et al., 2018). Generally clubroot susceptible hosts show a high level
451 of JA response, whereas it is reduced in resistant hosts (Jubault et al., 2008, Chen et al.,
452 2016a). In our samples, JAZ genes were up-regulated in SL compared with the gall tissue
453 (Fig. 4) and the control plant. JAZ proteins act as JA co-receptors and transcriptional
454 repressors in JA signalling (Kazan & Manners, 2012) reducing the JA synthesis in SL. This
455 was previously observed in *B. oleracea* plants, where JAZ expression was up-regulated in
456 resistant plants and JA synthesis was highly induced in susceptible plants (Zhang et al.,
457 2016). Among JA-metabolism genes HPL, and LOX2 expression were up-regulated in the
458 galls. LOX2 is essential for the formation of oxylipin volatiles (Mochizuki *et al.*, 2016). The
459 HPL protein competes with substrates essential for JA-synthesis, producing volatile and non-
460 volatile oxylipins (Wasternack & Hause, 2013). The higher expression of HPL and LOX2 in
461 the galls might lead to the production of volatile aldehydes rather than a JA accumulation in
462 the galls.
463 In SL BR-synthesis and BR-signalling genes were down-regulated (Fig. 4). BRs are
464 necessary for the development of clubroot tissue (Schuller *et al.*, 2014), hence a reduction in
465 BRs impairs *P. brassicae* growth and development in SL. The receptor-like cytoplasmic
466 kinase BIK1, a negative regulator of BR-synthesis (Lin *et al.*, 2013), was induced in SL
467 (Additional file 1: Figure S7). *Arabidopsis bik1* knockout mutants have an increased tolerance
468 to *P. brassicae* lacking the typical pathogen phenotype (Chen et al., 2016a, Chen et al.,
469 2016b). For BIK1 the expression of the SL roots differed to resistant plants, as *Arabidopsis*
470 *bik1* knockout mutants have an increased tolerance to *P. brassicae* (Chen et al., 2016a, Chen
471 et al., 2016b). In *Arabidopsis bik1* clubroot resistance was likely not due to the regulatory
472 function of BIK1 for BR, but to increased PR1 expression in this mutant (Chen et al., 2016b).
473

474 **Transcriptomes of symptomless roots and clubroots of the same plant are markedly**
475 **different.**

476 Clubroots and symptomless roots of the same *P. brassicae* infected plants showed markedly
477 different gene expression patterns. The morphological changes of the clubroots go in hand
478 with expression of genes that reduce cell wall stability and growth related processes. Genes
479 for cell wall-loosening processes such as expansins and XTHs (Downes *et al.*, 2001, Sun *et*
480 *al.*, 2005), were up-regulated in gall tissue (Fig. 3). Up-regulation of expansins was reported
481 from *Arabidopsis* clubroot tissue (Siemens et al., 2006, Irani et al., 2018) while XTH activity
482 was reported in *B. rapa* clubroots (Devos et al., 2005). Suppression of xyloglucan, xylan,
483 hemicellulose, pectin and lignin synthesis in gall tissues implies additional reduction of the
484 cell wall stability and rigidity in gall tissues similar to previous findings (Schuller et al., 2014,

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485 Zhang et al., 2016). GAE6 expression was reduced in the galls supporting clubroot
486 development: *Arabidopsis gae1*, *gae6*, and *gae1/gae6* mutants contained lower levels of
487 pectin in their leaf cell walls making them more susceptible to *Pseudomonas syringae* and
488 *Botrytis cinerea* (Bethke *et al.*, 2016). A similar mechanism might benefit *P. brassicae*.
489 Induction of the lignin pathway was an early response (48h) in *Arabidopsis* (Zhao et al.,
490 2017). In our much older kohlrabi galls lignification genes were down-regulated suggesting
491 *P. brassicae* suppresses host lignification in tissues where it has itself established. In *B. napus*
492 reduced lignification was also implied by the down-regulation of CCoAOMT (caffeoyl-CoA
493 O-methyltransferase) (Cao et al., 2008). Here, although not statistically significant,
494 CCoAOMT was also lower expressed in root galls compared with SL, supporting a decreased
495 lignin biosynthesis in clubroots.

496 The marked hypertrophies of the plant roots go hand in hand with changes in the
497 homeostasis of the growth hormones CK and auxins which appear to be host and time
498 dependent (Ludwig-Müller et al., 2009, Jia et al., 2017). With the exception of CKX6,
499 cytokinin related genes are up-regulated in SL (Fig. 4). Fine tuning of the hormone balance
500 appears to be essential in clubroot disease development (Ludwig-Müller et al., 2009).
501 Elevated CK levels are important for the onset of disease development via increasing cell
502 divisions. However, at the onset of gall formation CK metabolism genes, including CK
503 synthesizing and degrading enzymes, are repressed (Siemens et al., 2006, Malinowski et al.,
504 2016). The more active CK metabolism in the SL, might interfere with clubroot development
505 as CKX overexpressing *Arabidopsis* mutant showed reduced gall formation (Siemens et al.,
506 2006). In our kohlrabi samples CKX6 was strongly down-regulated in SL compared with YG
507 but not compared with OG (Fig. 4). The high expression of CKX6 in the YG indicates the
508 presence of plasmodia, as the gene was found to be strongly up-regulated only in cells
509 containing *P. brassicae* plasmodia (Schuller et al., 2014). Evidence that *P. brassicae*
510 interferes with the CK balance via the PbCKX of *P. brassicae* in gall tissues has not been
511 seen in this study as the PbCKX gene was not expressed.

512 Myrosinases and nitrilases that can synthesize auxins from secondary metabolites or
513 aromatic amino acids were up-regulated in SL compared with galls (Additional file 1:
514 Figures S5), but were previously described to be induced in *Arabidopsis* galls (Grsic-Rausch
515 *et al.*, 2000, Siemens et al., 2006). The auxin-induced GH3 gene family, which conjugates
516 IAA to several amino acids, is involved in various responses of plants to abiotic and biotic
517 stresses. The GH3.2 gene was shown to be specifically expressed in clubroots of *Arabidopsis*
518 (Jahn et al., 2013), and was also up-regulated in the kohlrabi galls. Expression of the
519 *P. brassicae* PbGH3 gene was not detected in this study and appears not to play a role in the
520 auxin (or JA) homeostasis at the stage of our gall samples.

521 Besides the described defence responses in SL that are similar to defence responses of
522 resistance plants, we observed up-regulation of other defence related genes in the SL (Fig. 5).
523 Homologues of the Arabidopsis Toll-IL-1 receptor (TIR)-NB-LRR disease resistance proteins
524 TAO1, NDR1 and RPS2 genes were up-regulated in SL. Those genes confer resistance to
525 biotrophic bacterial pathogens in *Arabidopsis* when recognizing effectors (Coppinger *et al.*,
526 2004, Eitas *et al.*, 2008). In roots of beans NDR1 also suppressed nematode parasitism by
527 activating defence responses (McNeece *et al.*, 2017). Thus, we found indications that those
528 proteins might also be involved in a defence response towards *P. brassicae* in the roots.

529 As a result of gall formation and a reduced number of fine roots, clubroot infected plants
530 face abiotic stress like lower water and nutrient supply. The differential expression of ABA
531 related genes (Fig. 4) are therefore likely a response to the abiotic stress in the galls.
532
533

534 **Conclusions**

535 Clubroots and symptomless roots of the same, *P. brassicae* infected plants showed very
536 different gene expression pattern. The here described differences in the plant hormone
537 metabolism might be responsible for the different outcomes in gall tissue and in
538 symptomeless roots as is the increased cell wall stability in symptomless roots. These results

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539 highlight, that interpreting clubroot transciptomes or any other data originating from whole
540 root systems might result in a dilution of biologically relevant signatures. This clearly calls
541 for further studies analysing intra- and inter-tissue specific pattern of clubroot infected plants.
542 As genes involved in resistance responses to *P. brassicae* in were up-regulated in
543 symptomless roots, this might aid the identification of novel traits for resistance breeding.
544
545

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555

556 **Availability of data and materials**

557 The datasets generated and analysed during the current study are available in the European
558 Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena) repository under the project
559 PRJEB26435 (Accessions ERR2567399-ERR2567408) and are available from the
560 corresponding author on request.
561

562 **Authors' contributions**

563 Experimental concept and design: SN. Wet lab work: SC. Bioinformatic and statistic analysis:
564 SC. Analysis of Results: SC, AS, SN. Manuscript writing: SC, AS, SN. Figures and tables:
565 SC. All authors read and approved the final manuscript.
566

567 **Ethics approval and consent to participate**

568 Not applicable.
569

570 **Consent for publication**

571 Not applicable.
572

573 **Competing interests**

574 The authors declare that they have no competing interests.
575

576 **Additional files**

577 Additional file 1: Figure S1 - Figure S10
578 Additional file 2: Table S1 - Table S9
579
580

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