Partial-resistance against aphids in wild barley involves phloem and mesophyll-based defences

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Date of Submission: 20/12/2018

Word Count: 6497

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Partial-resistance against aphids in wild barley involves phloem- and mesophyll-based defences

Highlight:

Partial-resistance against aphids in wild barley is based in the mesophyll and vascular tissue and is potentially associated with higher basal defence gene expression and altered phloem amino acid composition.

Abstract:

Aphids, including the bird cherry-oat aphid (*Rhopalosiphum padi*), are significant agricultural pests. Aphid populations are typically controlled using insecticides, but there is increasing demand for more sustainable pest management practices. The wild relative of barley, *Hordeum spontaneum* 5 (Hsp5) has been described as partially-resistant to *R. padi.* Partial-resistance is proposed to involve higher thionin and lipoxygenase gene expression. However, the underlying mechanistic processes are unknown. In this study we compared Hsp5 with a susceptible cultivar of barley (Concerto) to test the extent to which partial-resistance affects aphid fitness. We used the electrical penetration graph technique to monitor *R. padi* feeding patterns to elucidate the tissue location of partial-resistance factors alongside molecular and biochemical analyses to identify potential mechanisms. We show that partial-resistance in Hsp5 extends to three aphid species and is mediated by phloem/mesophyll-based factors, leading to a three-fold increase in the time aphids take to establish sustained phloem ingestion. Partial-resistance likely involves elevated expression of defence and phytohormone genes alongside altered phloem amino acid composition. Further work is required to establish the function of these traits, however this study highlights plant tissues which are important in conferring broad-spectrum partial-resistance against aphids in barley.

Keywords

Amino Acid Composition, Defence gene expression, Electrical Penetration Graph (EPG), *Hordeum spontaneum,* Partial-resistance, *Rhopalosiphum padi, Sitobion avenae*, *Utamphorophora humboldti*

Abbreviations

ABA: Abscisic Acid
Ala: Alanine
Arg: Arginine
Asn: Asparagine
Asp: Aspartic Acid
Concerto: *Hordeum vulgare* cv. Concerto
EPG: Electrical penetration graph
ET: Ethylene
FTIR: Fourier Transform Infrared
Gln: Glutamine
Glu: Glutamic Acid
Gly: Glycine
His: Histidine
Hsp5: *Hordeum spontaneum* 5
Ile: Isoleucine
JA: Jasmonic Acid
Leu: Leucine
Lys: Lysine:
Met: Methionine
Phe: Phenylalanine
r_m: the intrinsic rate of population increase
SA: Salicylic Acid
Ser: Serine
Thr: Threonine
Trp: Tryptophan
Tyr: Tyrosine
Val: Valine

Introduction

Barley, *Hordeum vulgare,* is the fourth most agriculturally important cereal by production quantity (Newton et al., 2011), with 65% of barley yield being used as feed for livestock, 30% in malting and distilling processes, and 5% contributing towards other uses (Newman and Newman, 2006). By 2050 it is estimated that crop yields will need to increase by 60% in order to meet the demands of global population growth (Alexandratos and Bruinsma, 2012). One way to help achieve this is to reduce yield losses to abiotic and biotic stress factors. Aphids such as the bird cherry-oat aphid, *Rhopalosiphum padi*, and the English grain aphid, *Sitobion avenae*, are major pests of cereals. Aphids feed directly from plant phloem using specialised mouthparts known as stylets (Auclair, 1963). Aphids cause direct plant damage by feeding and ingesting phloem sap and act as vectors of economically important plant viruses. Many cereal aphids are vectors for viruses such as *Barley Yellow Dwarf Virus* (*BYDV*), which are transmitted into plant tissues during stylet probing (Powell, 2005). Disease resulting from virus infection can cause yield losses of up to 30% in barley and up to 80% in wheat (Smith and Sward, 1982; Perry et al., 2000; Murray and Brennan, 2010).

Aphids are primarily controlled through the application of insecticides, with the amount of insecticide used per annum increasing (Jess et al., 2018). However the use of these has led to the emergence of insecticide-resistant aphid populations (Chen et al., 2007; Foster et al., 2014), caused negative impacts on non-target organism (Unal and Jepson, 1991; James et al., 2016), and potentially resulted in an increase in *BYDV* prevalence (Dewar and Foster, 2017), leading to legislative restrictions on insecticide use and promotion of integrated pest management (Directive, 2009/128/EC). As a consequence, there is a growing requirement for alternative pest management solutions which are more sustainable, and improving crop resistance to aphids is one avenue which could be explored to achieve this.

To date, only partial-resistance against aphids has been reported for cereal crops. Plant resistance traits which are effective against insect pests can be allocated to one of three categories: 1) chemical deterrence of insect settling and feeding; 2) physical barriers to insect feeding; and 3) reduction in palatability (Mitchell et al., 2016). Partial-resistance against aphids has been associated with each of these categories (respectively, Gibson and Pickett (1983), Tsumuki et al. (1989) and Greenslade et al. (2016). Inherent molecular defences also

143 play a key role in conferring resistance against aphids (Delp et al., 2009; Guan et al., 2015;
144 Escudero-Martinez et al., 2017). Molecular defences shown to be involved in aphid resistance
145 in *Hordeum* spp. include increased expression of thionin (antimicrobial peptides) genes (Delp
146 et al., 2009; Mehrabi et al., 2014; Escudero-Martinez et al., 2017), increased chitinase and β-
147 1,3-glucanase activity (Forslund et al., 2000), and the presence of plant secondary metabolites
148 (Gianoli and Niemeyer, 1998). Plant phythormone signalling pathways, including Abscisic Acid
149 (ABA), Salicylic Acid (SA), Jasmonic Acid (JA) and Ethylene (ET) signalling processes, mediate
150 co-ordinated molecular responses to herbivory via the regulation of defence signalling genes
151 and the biosynthesis of defensive allelochemicals (Smith and Boyko, 2007; Bari and Jones,
152 2009; Morkunas et al., 2011; Foyer et al., 2016); higher constitutive expression of
153 phytohormone signalling genes can lead to improved resistance against aphids in cereals
154 (Losvik et al., 2017).

155 Constitutive and induced plant defences are frequently lost during plant breeding and
156 domestication (Moreira et al., 2018). However, wild relatives of modern crops possess traits
157 which confer improved resistance against herbivorous pests (Dempewolf et al., 2014).
158 Screening crop wild relatives for (partial-)resistance against aphids provides an opportunity
159 to identify potentially beneficial traits (Zhang et al., 2017) which can be introduced into
160 agricultural cultivars to improve resistance (Xu et al., 2015; Li et al., 2018). Screening of wild
161 relatives in wheat (Xu et al., 2015; Aradottir et al., 2017), barley (Nieto-Lopez and Blake, 1994;
162 Åhman et al., 2000) and maize (Maag et al., 2015) has resulted in successful identification of
163 partial-resistance traits in wild relatives of these species (Tsumuki et al., 1989; Barria et al.,
164 1992; Gianoli and Niemeyer, 1998; Ahmad et al., 2011; Greenslade et al., 2016;
165 Chandrasekhar et al., 2018; Li et al., 2018).

166 Partial-resistance against *R. padi* was identified in the barley wild relative *H. spontaneum* 5
167 (Hsp5) (Åhman et al., 2000; Delp et al., 2009). Although Hsp5 features higher tissue
168 concentrations of indole alkaloid gramine, a plant defensive compound, the presence of
169 gramine did not correlate with increased partial-resistance (Åhman et al., 2000). Instead,
170 partial-resistance in Hsp5 is thought to involve increased expression of plant defence genes
171 (Delp et al., 2009; Mehrabi et al., 2014), including higher constitutive expression of thionin
172 genes (Delp et al., 2009) and higher expression of a proteinase inhibitor (Mehrabi et al., 2014).
173 Initial attempts have been made to characterise the molecular processes contributing to

174 partial-resistance in Hsp5 (Delp et al., 2009), although the underlying mechanism(s) of
175 resistance and the tissue location of these resistance factors remains to be elucidated.

176 The primary aim of this study was to characterise the plant traits and mechanisms
177 contributing to partial-resistance in Hsp5 by comparison with a susceptible commercial barley
178 cultivar, Concerto, and explore whether this partial-resistance is effective against several
179 aphid species, including two agricultural pests (*R. padi* and *Sitobion avenae*) and an invasive
180 species (*Utamphorophora humboldti)*. The factors contributing towards partial-resistance in
181 Hsp5, and their tissue location, were elucidated using the electrical penetration graph (EPG)
182 technique to monitor *R. padi* feeding processes. Subsequently, we assessed differences
183 between the two barley species in leaf surface architecture, defence gene expression, and
184 phloem amino acid composition to gain insight into the potential mechanisms underlying
185 partial resistance in Hsp5.

186

187 Materials and methods

188 *Plant growth and insect rearing conditions*

189 *Hordeum vulgare* Linnaeus cv. Concerto (Concerto) and *H. spontaneum* 5 Linnaeus (Hsp5)
190 seeds were surface-sterilised by washing in 2% (v/v) hypochlorite and rinsing with d.H_2O.
191 Seeds were kept moist in the dark: Hsp5 seeds were incubated at 4^oC for 14 days and Concerto
192 seeds were kept at room temperature for 48h. Germinated seedlings were planted into a
193 bulrush compost mix (Bulrush, Northern Ireland) and grown under glasshouse conditions with
194 a 16:8h day:night length and a 20:15^oC day:night temperature. Plants were grown until the
195 first true leaf stage (1.1 – 1.2 on the Zadoks et al. (1974) growth staging key).

196 Asexual laboratory cultures of the bird cherry-oat aphid, *Rhopalosiphum padi* (Linnaeus)
197 (genotype B; Leybourne et al. (2018)), the English grain aphid, *Sitobion avenae* Fabricius and
198 the American grass leaf aphid, *Utamphorophora humboldti* Essig were established from
199 individual apterous adults collected from Dundee, UK. Molecular barcoding of the
200 cytochrome oxidase subunit I gene (Folmer et al., 1994) was used to confirm identity of aphid
201 species. Aphid cultures were reared on one week old barley seedlings (cv. Optic) contained in
202 ventilated cups at 20^oC and 16:8h (L:D).

203 ***Insect fitness measurements***

204 The performance of all aphid species was assessed in glasshouse conditions (described above)
205 on Hsp5 and Concerto (n = 12) in a randomised block design (each block contained one
206 replicate of each treatment combination). Plants were infested with a single apterous aphid
207 which was allowed to reproduce overnight, then a total of three nymphs were retained on
208 each plant. Mean nymph mass and number of surviving nymphs were recorded at 72h and
209 168h, after which a random single nymph was returned to the plant; for this nymph, data
210 were collected on the length of the pre-reproductive period (d) and the intrinsic rate of
211 population increase (r_m). Aphids were caged onto the first fully expanded true leaf using
212 Perspex clip-cages of 25mm internal diameter (MacGillivray and Anderson, 1957). Nymph
213 mass gain was calculated as the change in mass between 168 and 72h and aphid r_m was
214 calculated using the equation of Wyatt and White (1977): $rm = 0.74\ \frac{\ln(Fd)}{d}$, where d is the
215 time period between birth and production of first progeny, and Fd is the total progeny
216 produced over a time period equal to d.

217 ***Electrical penetration graph (EPG) monitoring of aphid feeding***

218 The DC-EPG technique (Tjallingii, 1978; Tjallingii, 1988; Tjallingii, 1991; Tjallingii, 2001) was
219 employed to monitor the probing and feeding behaviour of apterous *R. padi* over a 6h period
220 using a Giga-4 DC-EPG device (EPG Systems, The Netherlands) on plants at the true-leaf stage.
221 A plant probe (copper rod approximately 50 mm long x 5 mm diameter), was soldered to
222 electrical wire extending from the plant voltage output of the Giga-4 device and inserted into
223 the plant soil. An aphid probe was made by soldering a piece of copper wire (30 mm long x 2
224 mm diameter) to a brass pin (tip diameter 2 mm). Approximately 30 mm of gold wire (20 µm
225 diameter; EPG Systems, The Netherlands) was adhered to the copper end of the aphid probe
226 using water-based silver glue (EPG Systems, The Netherlands) and aphids were connected by
227 adhering the free end of the gold wire onto the aphid dorsum using the same water-based
228 adhesive. Wired aphids were connected to the Giga-4 device by placing the end of the brass
229 pin into the EPG probes with a 1 GΩ input resistance and a 50x gain (Tjallingii, 1988). The
230 order in which *R. padi* – plant combinations were tested and allocated to an EPG probe was
231 randomised. Data were acquired using Stylet+D software (EPG Systems, The Netherlands). A

232 total of 18 and 16 successful recordings were made for aphids feeding on Concerto and Hsp5,
233 respectively. All EPG recordings were obtained within a grounded Faraday cage.

234 EPG waveforms were annotated using Stylet+A software (EPG Systems, The Netherlands).
235 Waveforms were annotated by assigning waveforms to np (non-probing), C (stylet
236 penetration/pathway), pd (intercellular punctures), E1 (saliva secretion into phloem), E2
237 (saliva secretion and passive phloem ingestion), F (penetration difficulty) or G (xylem
238 ingestion) phases (Tjallingii, 1988; Alvarez et al., 2006). No E1e (extracellular saliva secretion)
239 phases were detected. Annotated waveforms were converted into time-series data using the
240 excel macro developed by Dr Schliephake (Julius Kühn-Institut, Germany).

241 ***Determination of non-glandular trichome density***

242 Non-glandular trichome densities of Hsp5 and Concerto (n = 9) were determined using
243 polarised light microscopy following a procedure adapted from Pomeranz et al. (2013).
244 Briefly, the first true leaf was excised from plant stems and treated to two incubation steps
245 to clear the leaf epidermis. Firstly, leaf area was measured and leaves were soaked in 96%
246 Ethanol (Sigma-Aldrich, UK) for 48h before being treated with 1.25 M NaOH:EtOh (1:1, v:v) at
247 70°C for 2h. Leaves were stored in 50% glycerol prior to trichome visualisation; if NaOH:EtOH
248 treatment did not clear the leaf epidermis leaves were further treated with 80% lactic acid
249 for 6h. To analyse trichome density samples were placed adaxial side up on microscope slides
250 (75 x 25 mm; Corning, UK). A polarising light microscope was created using two 50 mm^2
251 polarising filters (Sigma-Aldrich, UK), one placed below the stage of a stereo-microscope but
252 above the light source and the second attached below the objective lens. Non-glandular
253 trichomes appeared illuminated under the polarised microscope setup and the number of
254 trichomes per unit area were counted. To allow correlation of trichome density with aphid
255 performance, an additional *R. padi* performance experiment (as described above) was carried
256 out on these plants prior to trichome density analysis.

257 ***Epicuticular wax analysis***

258 A Fourier Transform Infrared (FTIR) spectrometer (Bruker Vertex 70 FTIR spectrometer,
259 Bruker, Ettlingen, Germany), incorporating a Diamond Attenuated Total Reflection (DATR)
260 sampling accessory, was used to identify the functional groups present in chemical extracts
261 from the surfaces of Hsp5 and Concerto leaves. Briefly, the waxes from upper and lower leaf

262 surfaces of the first true leaf (n = 4) were extracted with dichloromethane (DCM) by running
263 approximately 1-2 ml of DCM along the leaf surface directly onto the diamond window of the
264 ATR sampling accessory; after evaporation of the DCM, FTIR spectra were recorded of the
265 films deposited on the DATR. Signal-to-noise ratio was enhanced by taking 200 scans for each
266 sample and averaging to obtain a single spectrum. The spectral range scanned was 4000 cm^{-1}
267 to 400 cm^{-1} and a background reading was taken before each sample was analysed.

268 ***Analysis of phloem amino acid composition***

269 Hsp5 and Concerto plants were grown under glasshouse conditions (as described above) in a
270 temporally-split randomised block design: three temporal blocks, one initial temporal block
271 comprising two replicate sub-blocks, and two further temporal blocks each comprising four
272 replicate sub-blocks. Each sub-block contained a single replicate of every treatment
273 combination. Plants at the first true-leaf stage were infested with either ten apterous *R. padi*
274 adults caged onto the plants with microperforated bags (Polybags, UK) or were aphid-free
275 bagged controls. Samples were collected at 0 and 24h post-aphid infestation. Phloem sap was
276 collected using the method of King and Zeevaart (1974); leaves (*n = 10*) were excised from the
277 stem, placed in a dish containing 1 mM EDTA (Sigma-Aldrich, UK) solution and immediately
278 re-cut. The cut surface of the leaf was placed into 200µl of filter-sterilised 1mM EDTA solution
279 (pH 7.5 with NaOH) in a 2 mL Eppendorf™ microcentrifuge tube and incubated for 1.5h at
280 room temperature in a darkened exudation chamber (a polystyrene box containing a dish of
281 6 M K_2HPO_4 (Sigma-Aldrich, UK), and moistened paper towels to maximise humidity and
282 decrease leaf transpiration). After incubation, leaves were removed and the samples of EDTA
283 solution containing exuded phloem sap were stored at -80°C until analysis.

284 Reverse-phase HPLC was used to separate phloem amino acids Asp, Glu, Asn, His, Ser, Gln,
285 Arg, Gly, Thr, Tyr, Ala, Trp, Met, Val, Phe, Ile, Leu and Lys using an Agilent HP1100 series auto-
286 sampling LC system equipped with a ZORBAX™ Eclipse AAA column and fluorescence
287 detector. Amino acids were derivatised using *o*-phthaldialdehyde (Sigma-Aldrich, UK)
288 following the method developed by Jones et al. (1981); all protein amino acids except proline
289 and cysteine could be detected by this method, with a detection limit of approximately 0.5
290 pmol. Samples were processed in their experimental blocks along with blanks (1 mM EDTA
291 solution that was not exposed to an excised leaf) as controls. Amino acids were identified and

292 quantified by comparison with known concentrations of amino acid standards (AA-S-18
293 (Sigma, UK), supplemented with Asp, Glu and Trp).

294 ***qPCR analysis of plant defence genes***

295 Five sub-blocks over the three temporal blocks (one from the first block and two from each
296 subsequent temporal block) were chosen at random from the phloem exudation experiment
297 described above and leaf tissue was sampled for gene expression analysis. Approximately
298 25mm length of leaf tissue was cut from the true leaf and flash-frozen in liquid nitrogen
299 immediately prior to the excision of the leaf for phloem exudation. Plant material was stored
300 at -80°C until RNA extraction.

301 For RNA extraction leaf samples were ground to a fine powder under liquid nitrogen using a
302 mortar and pestle and total RNA was extracted with the Norgen Plant/Fungi RNA Extraction
303 Kit (Sigma-Aldrich, UK) following the manufacturer's protocol with additional DNAse I
304 treatment (Qiagen, UK); RNA quality and quantity was assessed with a NanoDrop® ND-1000
305 Spectrophotometer (ThermoFischer, UK). cDNA synthesis was carried out with approximately
306 1000 ng RNA using the SuperScript® III cDNA synthesis kit (Sigma-Aldrich, UK), following the
307 manufacturer's protocol. qPCR primers were designed using the Roche Universal Probe
308 Library Assay Design Centre. Reference gene primers were described by Hua et al. (2015). All
309 primer sequences are shown in Supplementary Table 1 and were supplied by Sigma-Aldrich,
310 UK. Primers were validated for PCR efficiency prior to use, and reference gene primers were
311 tested for stability across all treatments following the geNorm procedure (Vandesompele et
312 al., 2002). Gene expression analysis used SYBR® Green chemistry with GoTaq® qPCR Master
313 Mix (Promega, UK) on a StepOne™ Real-Time PCR Machine (Applied Biosystems, UK).
314 Reactions were carried out in 12.5 µl reactions with a final concentration of 1x GoTaq® qPCR
315 Master Mix, 1µM of each primer, 1.4 mM $MgCl_2$, 2.4 µM CXR reference dye and a cDNA
316 quantity of approx. 12.5 ng (assuming 1:1 RNA:cDNA conversion). The qPCR conditions were
317 as follows: 95°C for 15 mins followed by 40 cycles of denaturing for 15s at 95°C, annealing for
318 30s at 60°C, and 30s at 72°C for DNA extension. Fluorescence was recorded at the end of each
319 annealing cycle and a melting curve was incorporated into the end of the qPCR programme.
320 Data were normalised to the mean expression of two reference genes, *HvCYP* (AK253120.1) and
321 *HvUBC* (AK248472.1), and $2^{-\Delta\Delta Ct}$ methodology (Livak and Schmittgen, 2001) was used to
322 determine differential expression, with Concerto at 0h aphid infestation used as the

323 experimental control treatment. Expression levels at 24h were further normalised to
324 uninfested control plants. Samples were processed in experimental blocks with three
325 technical replicates at the PCR level.

326 ***Statistical analysis***

327 All statistical analyses were carried out using R Studio v.1.0.143 running R v.3.4.3 (R Core
328 Team, 2014) using packages: car v.2.1-4 (Fox and Weisberg, 2011), coxme v.2.2-7 (Therneau,
329 2018), dunn.test v.1.3.5 (Dinno, 2017), ggplot2 v.2.2.1 (Wickham, 2009), ggpubr v.
330 0.1.2 (Kassambara, 2017), ggfortify v.0.4.5 (Tang et al., 2016), lawstat v.3.2 (Hui et al., 2008),
331 lme4 v.1.1-13 (Bates et al., 2015), lmerTest v.2.0-33 (Kuznetsova et al., 2017), lsmeans v.2.27-
332 62 (Lenth, 2016), survival v.2.41-3 (Therneau and Grambsch, 2000), survminer v.0.4.2
333 (Kassambara and Kosinski, 2017), vegan v.2.5-3 (Oksanen et al., 2013).

334 Aphid juvenile mass gain and adult r_m, were modelled using linear mixed effects models with
335 experimental block incorporated as a random factor. ANOVA with type III Satterthwaite
336 approximation for degrees of freedom was used to analyse the final models with calculation
337 of Least Squares Means used for *post-hoc* testing. A Cox proportional hazards regression
338 model was used for nymph survival analysis (Therneau and Grambsch, 2000; Therneau, 2018),
339 incorporating experimental block as a random factor; a χ^2 test was used on the final model. A
340 log-rank test with Benjamini-Hochberg correction was used to carry out pairwise comparisons
341 between aphid-plant combinations. Aphid feeding behaviour was assessed globally by fitting
342 a permutated MANOVA to the dataset. Response variables with normal data distribution
343 were analysed by either ANOVA or general linear models, while non-normally distributed data
344 were analysed with Kruskal-Wallis tests.

345 Levene's test (Hui et al., 2008) was used to analyse differences in trichome density; a Kendall's
346 rank correlation tau was then used to test for correlations between trichome density and *R.*
347 *padi* performance. Differences in leaf surface chemistry were analysed using a Welch two
348 sample *t*-test by comparing the total number of identified functional groups in the two plant
349 species.

350 Phloem amino acid concentrations (pmol/µl) were expressed as relative amino acid
351 composition by converting into mole % of total amino acid content and subjected to principal
352 component analysis with a correlation matrix. Three principal components explained 69% of

353 observed variation and were analysed by fitting a linear mixed effects model incorporating
354 aphid treatment, plant, time-point, plant x time-point interaction, and leaf subsampling for
355 RNA (to test effect on amino acid composition) as explanatory variables with temporal block,
356 randomised block and HPLC batch as random factors. Final models were analysed with a χ^2
357 test. Differential gene expression ($2^{-\Delta\Delta Ct}$) data were analysed using Kruskall-Wallis rank-sum
358 tests, with subsequent Dunn's test *post-hoc* analysis of the plant x infestation interaction.

359 Results

360 *A wild relative of barley exhibits partial-resistance against multiple aphid species*

361 Aphid performance experiments were undertaken to assess whether partial-resistance in
362 Hsp5 against *R. padi* (Delp et al., 2009; Leybourne et al., 2018) extends to other aphid species.
363 We assessed the fitness of *R. padi, S. avenae,* and *U. humboldti* on Hsp5 in comparison with
364 a commercial barley cultivar (Concerto) and found evidence for partial-resistance against all
365 three aphid species. Specifically, nymph survival was significantly lower on Hsp5 ($\chi^2_1 = 10.65$;
366 $p = 0.001$; Fig. 1A; Table 1); pairwise comparison showed that nymph survival was reduced on
367 Hsp5 for *U. humboldti* ($p = <0.001$), but not for *R. padi* ($p = 0.057$) or *S. avenae* ($p = 0.973$).
368 Furthermore, nymph mass gain ($F_{56,1} = 9.14$, $p = 0.003$; Fig. 1B; Table 1) and the rate of
369 population increase (r_m) ($F_{54,1} = 27.43$, $p = <0.001$; Fig. 1C; Table 1) were reduced for all aphid
370 species when feeding from Hsp5. Differences were also detected between aphid species, with
371 *R. padi* exhibiting the highest r_m ($F_{54,2} = 99.10$, $p = <0.001$; Fig. 1B; Table 1) and *S. avenae* the
372 largest mass gain ($F_{56,2} = 49.82$, $p = <0.001$; Fig. 1C; Table 1). On average *U. humboldti* was
373 the least fit species (Fig. 1A-C).

374 *Partial-resistance against R. padi involves mesophyll and phloem resistance factors*

375 To elucidate the potential underlying partial-resistance mechanism(s) against aphids in Hsp5
376 *R. padi* feeding behaviour was monitored using the EPG technique. Overall, the feeding
377 behaviour of *R. padi* differed significantly when feeding from Hsp5 compared with Concerto
378 ($F_{1,33} = 2.61$; $p = 0.022$; Fig. 2; Table 2). Aphids showed significant differences in feeding
379 behaviour at the leaf epidermis and within mesophyll tissue (Fig. 3; Table 2). We observed an
380 approximate three-fold decrease in the time to the first epidermal probe when feeding on
381 Hsp5 ($F_{1,33} = 7.99$; $p = 0.008$), alongside a shorter duration of the first probe ($F_{1,33} = 6.94$; $p =$
382 0.013) and an increased time to the first sieve element puncture ($F_{1,33} = 6.33$; $p = 0.017$). In

383 addition, aphids feeding on Hsp5 showed a two-fold delay in initiating passive phloem
384 ingestion ($\chi^2_{1,33}$ = 4.29; p = 0.038) and the number of intracellular punctures observed during
385 the first aphid probe of plant tissue was lower, with an average of ten intracellular probes,
386 compared with 24 on Concerto ($F_{1,33}$ = 3.95; p = 0.047; Table 2).

387 Feeding patterns within the vascular tissue also differed (Fig. 4; Table 2). Specifically, aphids
388 feeding on Hsp5 showed delayed initiation of sustained phloem ingestion (ingestion for >10
389 mins) after the first sieve element puncture ($\chi^2_{1,33}$ = 4.29; p = 0.038). The total length of time
390 aphids spent in the sustained feeding phase was also 2.5x shorter ($\chi^2_{1,33}$ = 7.49; p = 0.006),
391 and the ratio of time aphids spent ingesting phloem relative to the length of time probing
392 plant tissue (ingestion:pathway ratio) was four-fold lower ($\chi^2_{1,33}$ = 7.43; p = 0.006).
393 Furthermore, aphids feeding on Hsp5 spent three times longer ingesting xylem ($\chi^2_{1,33}$ = 5.28;
394 p = 0.022). Table 2 reports additional feeding parameters which highlight further mesophyll
395 and vascular-mediated resistance against *R. padi* in Hsp5.

396 ***Leaf surface architecture differs between susceptible and partially-resistant plants***.

397 The leaf surface represents a key interface between plants and insects, with many factors
398 reported to modulate plant-insect interactions, including leaf trichomes and epicuticular
399 waxes (Agrawal et al., 2009; Glas et al., 2012; Karley et al., 2016). Leaf trichome counts
400 revealed Hsp5 had a significantly higher abundance of non-glandular leaf trichomes ($F_{1,18}$ =
401 6.24; p = 0.022; Fig. 5). Non-glandular trichome abundance showed a negative correlation
402 with *R. padi* fecundity (Z = -3.60; p = <0.001; T = -0.56; Supplementary Fig. 1A) but there was
403 no relation with nymph mass gain (Z = -0.29; p = 0.770; T = -0.04; Supplementary Fig. 1B). In
404 addition, we found differences in the epicuticular wax composition between the two plants.
405 The complexity of the epicuticular wax differed in that aliphatic hydrocarbons were detected
406 in surface extracts of Hsp5 leaves, two Hsp5 replicates also contained weak ester bands
407 (Supplementary Table 3). In Concerto the chemical mixture contained a more consistent
408 mixture of aliphatic hydrocarbons and more pronounced ester bands, alongside a more
409 complex, but variable, mixture of chemical groups, including carboxylates and amides (*t* = -
410 4.89; df = 6; p = 0.002). Overall, the results were consistent within Hsp5 but more variable in
411 Concerto (Supplementary Table 3). However, the detection of some Nitrogen-containing
412 functional groups in Concerto leaf extracts could be a result of extracting some components

413 within the upper tissue layers, this potentially highlights differences in the thickness of the
414 wax between Hsp5 and Concerto.

415 ***Basal expression levels of several plant defence genes are elevated in Hsp5***

416 To investigate the level of defence gene expression in Concerto versus Hsp5 we selected
417 marker genes for defensive thionins (*HvTHIO1, HvTHIO2, HvβTHIO*) alongside JA (HvLOXA,
418 *HvLOX2* and *HvJAZ*), SA (*HvNPR1*), ET (*HvERF*), and ABA (*HvA1*) signalling pathways. We
419 assessed expression of marker genes in both plant types constitutively and in response to 24h
420 of aphid infestation. We observed 10-(*HvTHIO1)*, 13-(*HvTHIO)* and 7-fold (*HvβTHIO)* higher
421 expression of the three thionin genes in Hsp5 (Fig 6; Table 3). Thionin gene expression levels
422 remained higher in Hsp5 24h after aphid infestation but were not differentially regulated in
423 either plant in response to aphid infestation (Fig 6; Table 3). In addition, *HvLOXA*, *HvLOX2,*
424 *HvA1,* and *HvERF1*, were more highly expressed in Hsp5, with 3.5-, 6-, 10- and 14-fold higher
425 expression levels, respectively (Fig 6; Table 3).

426 No genes were differentially expressed in response to aphid infestation. However, several of
427 the phytohormone signalling genes were differentially expressed in response to the plant x
428 aphid infestation interaction; namely *HvLOXA, HvLOX2, HvNPR1,* and *HvERF1* (Table 3).
429 *HvLOXA* was significantly down-regulated in Hsp5 after 24h aphid infestation to levels similar
430 to those observed in Concerto (Fig. 6; Table 4). For *HvLOX2, HvNPR1,* and *HvERF,* expression
431 levels were higher in Hsp5 after 24h of aphid infestation compared with levels in Concerto
432 (Fig. 6; Table 4). *HvJAZ* was not differentially regulated in response to any treatment factor.

433 ***Phloem amino acid composition in Hsp5 is characterised by a reduction in the proportion of***
434 ***essential amino acids and an increased abundance of asparagine***

435 To investigate phloem factors contributing to partial-resistance against aphids (Fig. 4; Table
436 2) we analysed the amino acid composition and the proportion of essential and non-essential
437 amino acids in phloem exudates (Fig. 7). Principal component analysis of amino acid
438 composition (mol% of Asp, Glu, Asn, His, Ser, Gln, Arg, Gly, Thr, Tyr, Ala, Trp, Met, Val, Phe,
439 Ile, Leu, and Lys) of phloem exudates revealed that three principal components explained 69%
440 of the observed variation in overall amino acid composition, with all three principle
441 components showing a degree of separation between the two plant types (Table 5).
442 Furthermore, >50% of the variation was explained by the first two principal components (Fig.

443 7C; Table 5) with separation of Hsp5 from Concerto largely occurring along PC1 due to
444 differences in the relative amounts of Asn and His vs. most other essential amino acids (Fig.
445 7C). The total percentage of essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp,
446 Val; described in Morris (1991)) was higher in Concerto compared with Hsp5 (18.97% vs
447 11.45%; $\chi^2_1 = 25.43$; $p = <0.001$; Fig. 7D; Table 6).

448 The difference in composition of non-essential amino acids was most pronounced between
449 the two plant types, with a higher proportion of Asn and lower proportions of Glu and Gly in
450 Hsp5 (42.75%, 7.34% and 12.69%, respectively) compared with Concerto (7.55%, 15.18% and
451 22.43%, respectively) (Fig 7A-B). Amino acid composition and percentage of essential amino
452 acids changed over the 24h of the experiment, but not in response to aphid treatment (Table
453 5; Table 6). Harvesting of leaf material for qPCR analysis had no effect on the phloem amino
454 acid composition (Table 5; Table 6).

455 **Discussion**

456 In this study we provide evidence for mesophyll and phloem mediated partial-resistance
457 against aphids in a wild barley relative, which is effective against three aphid species,
458 including two agricultural pests and an invasive species. Our data suggest the partial-
459 resistance in this progenitor of modern barley is complex and may be associated with elevated
460 defence gene expression and reduced phloem quality.

461 ***Mesophyll and phloem-based factors contribute to partial-resistance in Hsp5***

462 Our observation that *R. padi* feeding on Hsp5 spends more time in the pathway phase
463 (probing into plant tissue) and takes longer to reach the phloem and initiate
464 salivation/phloem ingestion is representative of feeding patterns associated with mesophyll-
465 based resistance (Alvarez et al., 2006). Factors associated with mesophyll resistance are
466 thought to antagonise and impede stylet penetration (Montllor and Tjallingii, 1989; Alvarez
467 et al., 2006). In addition, *R. padi* spends less time ingesting phloem sap and salivating into the
468 phloem, in line with resistance factors residing in the phloem (Alvarez et al., 2006). Phloem
469 based resistance factors generally alter the ingestion efficiency of aphids and can involve
470 feeding antagonists, alterations to the phloem metabolic profile, and the plugging of sieve-
471 tube elements (Klingler et al., 1998; Klingler et al., 2005; Pegadaraju et al., 2007; Medina-
472 Ortega and Walker, 2015; Greenslade et al., 2016; Peng and Walker, 2018).

473 Partial-resistance against aphids in other plant-aphid systems have similarly pointed to the
474 importance of mesophyll and phloem-based resistance factors (Montllor and Tjallingii, 1989;
475 Caillaud et al., 1995; Pegadaraju et al., 2007; Guo et al., 2012; Greenslade et al., 2016). For
476 example, partial resistance in *Triticum monococcum* lines (a wild relative of wheat) against *S.*
477 *avenae* and *R. padi* involved resistance at the phloem level, as determined by EPG (Caillaud
478 et al., 1995; Greenslade et al., 2016). Interestingly, metabolic profiling of resistant and
479 susceptible lines in the latter study indicated different metabolite profiles between
480 susceptible and partially-resistant plants, including lower levels of primary metabolites in the
481 partially-resistant lines and elevated Asn (Greenslade et al., 2016). Resistance against *D. noxia*
482 in five cereal species was shown to involve phloem and mesophyll resistance factors, with
483 elevated 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) levels hypothesised to be
484 a contributing factor towards resistance (Mayoral et al., 1996). Resistance against *D. noxia* in
485 wheat also involved individual resistance genes; the most recent of these to be characterised
486 is *Dn10* (Li et al., 2018), although the tissue types involved in conferring this resistance are
487 unknown.

488 In addition, *Arabidopsis thaliana* defence against *Myzus persicae* involved *phytoalexin*
489 *deficient4 (PAD4),* and reduced susceptibility in *PAD4* overexpressing lines was associated
490 with reduced phloem ingestion (Pegadaraju et al., 2007). Furthermore, gene-for-gene type
491 resistance against the pea aphid, *Acyrthosiphon pisum,* in *Medicago truncatula* line Jemalong
492 A17 (A17) is conferred by a single gene, *RAP1,* which maps to chromosome 3 (Stewart et al.,
493 2009). Further characterisation showed that A17 also conferred resistance against the
494 bluegreen aphid, *A. kondoi,* as well as *A. pisum,* and that this resistance was phloem-mediated
495 and linked to a QTL on chromosome 3 (Guo et al., 2012). The inorganic chemistry of leaf tissue
496 can also play a role in plant defence (Lane, 2002; Boyd, 2012), with oxalates highlighted as
497 important contributors towards plant defence against pathogens in cereals (Lane, 2002). The
498 inorganic chemistry of Hsp5 and Concerto leaves was not assessed in this study, but a
499 comparison of leaf chemistry could highlight further chemical groups which could be involved
500 in mediating plant-aphid interactions.

501 ***Differences in leaf surface characteristics contribute little to partial-resistance against***
502 ***aphids in Hsp5***

503 Leaf surface architectural differences were detected between Concerto and Hsp5 and
504 included a higher abundance of non-glandular leaf trichomes in Hsp5 and a more complex
505 chemistry in the epicuticular extracts of Concerto. Non-glandular trichomes have been
506 reported to decrease *R. padi* fitness in wheat (Roberts and Foster, 1983), but have contrasting
507 effects on herbivore fitness in other plants. For example, the density of non-glandular
508 trichomes in raspberry negatively affected spider mite performance but had minimal impact
509 on aphid fitness (Karley et al., 2016).

510 Although we detected a positive correlation between trichome abundance and *R. padi* r_m,
511 there was no correlation between trichome abundance and juvenile *R. padi* mass gain,
512 indicating that either non-glandular trichomes have differential effects on aphids at different
513 life-stages or that the higher abundance of non-glandular trichomes on the leaf surface of
514 Hsp5 are not a primary cause of partial-resistance to aphids. Indeed, EPG analysis indicated
515 that leaf surface traits contributed little to partial-resistance in Hsp5. Aphids feeding from
516 Hsp5 did not show increased time spent in the non-probing phase (a key indicator of
517 epidermal-mediated resistance: Alvarez et al. (2006). Moreover, the time taken for aphids to
518 penetrate the leaf surface was shorter on Hsp5, indicating aphids experienced fewer barriers
519 to probing the leaf epidermis on Hsp5. Less complex chemistry at the leaf epicuticle in Hsp5
520 may have played a role in promoting aphid probing of plant tissue and previous studies have
521 shown that epicuticular chemical compounds can either promote or deter aphid probing of
522 plant tissue (Powell et al., 1999). Barley epicuticular waxes can contribute towards resistance
523 against *R. padi* (Tsumuki et al., 1989), although the influence of specific chemical functional
524 groups on aphid behaviour is not well characterised.

525 ***Basal defence gene expression is elevated in Hsp5***

526 In line with Delp et al. (2009) and Mehrabi et al. (2014), basal expression levels of thionin
527 genes, which contribute to barley defences against aphids (Escudero-Martinez et al., 2017),
528 and *LOX2*, a JA-signalling marker, were elevated in Hsp5. The feeding parameters exhibited
529 by *R. padi* while feeding on Hsp5 indicated that mesophyll-based mechanisms conferred
530 short-term (6h) partial-resistance against aphids. Thionins are antimicrobial peptides present
531 in plant cell walls and within the intracellular space (Reimann-Philipp et al., 1989; Stec, 2006).
532 It is therefore likely that aphids are exposed to thionins when probing mesophyll tissue, and
533 these could contribute to reduced aphid performance on Hsp5.

534 In addition, several plant defence signalling genes, *LOX* (JA), *ERF1* (ET) and *A1* (ABA) showed
535 higher basal expression in Hsp5, suggesting that Hsp5 may be able to respond more efficiently
536 to biotic stimuli. In line with our data, *HvLOX2* genes and other components of the JA (*HvAOS,*
537 *HvAOC*), ET (*HvACCO, HvACCS*), and Auxin (*HvTDS, HvTSM*) signalling pathways showed higher
538 basal expression in the barley cultivar Stoneham, which is partially-resistant against *D. noxia,*
539 relative to susceptible cultivar Otis (Marimuthu and Smith, 2012). Furthermore,
540 overexpression of *HvLOX2* in barley resulted in decreased *R. padi* fecundity five days after
541 infestation (Losvik et al., 2017), pointing to a functional role of this gene and JA signalling in
542 short-term barley defence against aphids. Expression levels of *CmERF1* in early responses (6h)
543 to aphid infestation were around ten-fold higher in a resistant variety of melon compared
544 with a susceptible variety (Anstead et al., 2010), and higher ET levels have been reported to
545 contribute to *R. maidis* resistance in maize (Louis et al., 2015). ABA mediated-processes have
546 been implicated in increasing both plant resistance (Zhu-Salzman et al., 2004; Park et al.,
547 2006) and susceptibility (Kerchev et al., 2013; Hillwig et al., 2016) to aphids. Therefore the
548 consequence of elevated *HvA1* in the context of partial aphid resistance in Hsp5 is not clear.
549 Guo et al. (2016) reported that elevated ABA levels in response to drought conditions can lead
550 to increased aphid xylem ingestion. This finding suggests that elevated expression of
551 components of the ABA signalling pathway could indirectly contribute to partial-resistance by
552 encouraging xylem ingestion, thereby reducing phloem ingestion. Indeed, longer periods of
553 xylem ingestion were detected in *R. padi* feeding from Hsp5, indicating that there may be
554 potential fitness consequences for aphids as a result of higher basal expression levels.

555 In contrast, basal expression levels of *HvNPR1*, a SA-signalling marker, were not significantly
556 different between Hsp5 and Concerto. However, *HvNPR1* levels in Concerto were down-
557 regulated 24h after aphid infestation to a level that was significantly lower than *HvNPR1* levels
558 in Hsp5 after 24h of aphid infestation. Therefore, SA-mediated defences in Concerto, but not
559 Hsp5, may be repressed upon aphid infestation leading to increased susceptibility of this
560 cultivar. Indeed, NPR1 is required for initiation of SA-mediated defence against aphids in *A.*
561 *thaliana* (Moran and Thompson, 2001; Wu et al., 2012).

562 ***Essential amino acid availability is reduced in Hsp5***

563 The observation that the relative concentration of essential amino acids was lower in the
564 phloem of Hsp5 compared with Concerto could be associated with lower aphid performance.

565 Alterations to amino acid composition can reduce plant palatability and nutritional quality
566 and contribute to increased resistance against aphids (Sandström and Pettersson, 1994;
567 Ponder et al., 2000; Karley et al., 2002). For example, decreased survival and fecundity of *M.*
568 *persicae* and *Macrosiphum euphorbiae* feeding from older "tuber-filling" potato plants
569 compared with younger "pre-tuber-filling" plants was linked to changes in amino acid
570 composition (Karley et al., 2002). When presented with chemically-defined diets
571 representative of the phloem sap composition of young and mature potato plants, *M.*
572 *persicae* and *Ma. euphorbiae* showed decreased feeding rate on the "tuber-filling" diets
573 (Karley et al., 2002). In addition, *R. padi* feeding from barley plants grown under nitrogen-
574 limited conditions exhibited reduced r_m compared with *R. padi* feeding on barley grown under
575 nitrogen-rich conditions (Ponder et al., 2000). Examination of phloem amino acid composition
576 indicated that composition was altered under nitrogen-limited conditions. Interestingly,
577 plants under nitrogen-limited conditions contained a higher percentage of Asn, a lower
578 percentage of Gly and a small reduction in the concentration of essential amino acids
579 compared with nitrogen-fertilised plants (Ponder et al., 2000).

580 Changes in the abundance of specific amino acids cannot be readily associated with increased
581 insect resistance/susceptibility in a given plant species. It is most likely that any observed
582 effect on aphid fitness is due primarily to the ratio of essential:non-essential amino acids as a
583 result of compositional changes. Indeed, essential amino acids are generally elevated in
584 susceptible plants compared with varieties showing increased resistance against aphids
585 (Auclair, 1976). Vogel and Moran (2011) found that the mass of multiple *A. pisum* biotypes
586 was reduced when essential amino acids were removed from aphid artificial diets; this was
587 observed even though aphids have the capacity to synthesis essential amino acids via their
588 essential endosymbiont, *Buchnera aphidicola* (Douglas and Prosser, 1992). Furthermore,
589 some aphid species actively remobilise plant nutrients to increase the abundance of essential
590 amino acids in susceptible plant hosts (Telang et al., 1999; Sandström et al., 2000). Enhanced
591 mobilisation of plant nutrients is thought to be the mechanism explaining resistance
592 breakdown in wheat by a virulent biotype of the greenbug, *Schizaphis graminum* (Dorschner
593 et al., 1987). In a study by Sandström et al. (2000), *R. padi* feeding did not lead to increased
594 phloem mobilisation of amino acids, similar to observations made in our experimental system

595 where *R. padi* infestation did not alter the phloem amino acid composition in Hsp5 or
596 Concerto.

597 Conclusion

598 This study shows that partial-resistance against aphids in wild barley can be wide-ranging and
599 effective against multiple aphid species. Broad partial-resistance traits are agriculturally and
600 economically important as crops are likely to be infested by multiple insect species under field
601 conditions (Stam et al., 2014). However, compared with gene-for-gene resistance traits,
602 comparatively little is known about the genetic determinants of partial-resistance. The
603 underlying factors contributing towards partial-resistance are complex and likely to involve
604 multiple, independent resistance pathways (Mayoral et al., 1996; Moharramipour et al., 1997;
605 Seah et al., 1998). We show that partial-resistance against aphids in wild barley involves
606 resistance factors that reside in both the mesophyll and vascular tissues. This partial-
607 resistance is potentially caused by heightened basal expression of defence and
608 phytohormone signalling genes, alongside altered phloem amino acid quality. However, the
609 functional molecular role of these differences, and how they contribute towards partial-
610 resistance, remains to be established.

611 Supplementary Data

612 Supplementary Table 1: primers used in RT-qPCR analysis.

613 Supplementary Table 2: Additional EPG parameters.

614 Supplementary Table 3: FTIR results for leaf surface extracts

615 Supplementary Fig. 1: Correlation of insect fitness with non-glandular trichome density.

616

617

618 Acknowledgements

619 D.J.L was funded by the James Hutton Institute and the Universities of Aberdeen and Dundee
620 through a Scottish Food Security Alliance (Crops) PhD studentship. A.J.K, T.A.V, A.H.J.R,
621 A.M.M, and E.P-F were supported by the strategic research programme funded by the

Scottish Government's Rural and Environment Science and Analytical Services Division. J.I.B.B. was supported by the European Research Council (310190-APHIDHOST). We would like to thank Dr Freddy Tjallingii (EPG Systems, The Netherlands), Professor Alberto Fereres (CSIC, Spain) and Professor Gregory Walker (University of California, Riverside, USA) for providing EPG training and Gaynor Malloch (James Hutton Institute) for kindly providing COI barcoding primers.

Author Contributions

JIBB, AJK, and DJL conceived and designed the experiments. DJL, EP-F, and AMM performed the experiments. JIBB, AJK, DJL, and JAHR analysed the data. DJL wrote the manuscript with input from JIBB, AJK, TAV, and JAHR. All authors read and approved the final manuscript.

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Tables

Table 1: Statistical results of aphid performance experiments (significant values are shown in bold).

Response Variable	Explanatory Variable	Model Basis	Error Distribution or Statistical Method	Statistical Test	Test Statistic	P value
Nymph Survival	Plant	Cox Proportional Hazards Regression	N/A	Type II Wald χ^2 Analysis of Deviance	$X^2_1 = 10.65$	**0.001**
	Aphid				$X^2_2 = 13.94$	**<0.001**
	Plant x Aphid				$X^2_2 = 5.85$	0.054
Nymph mass gain (mg)	Plant	Linear Mixed Effects Model	Restricted Maximum Likelihood	Type III Analysis of Variance with Satterthwaite approximation for degrees of freedom	$F_{1,56} = 9.14$	**0.004**
	Aphid				$F_{2,56} = 49.83$	**<0.001**
	Plant x Aphid				$F_{2,46} = 3.12$	0.054
Rate of population increase (r_m)	Plant				$F_{1,54} = 27.43$	**<0.001**
	Aphid				$F_{2,54} = 99.10$	**<0.001**
	Plant x Aphid				$F_{2,52} = 1.49$	0.234

Table 2: Statistical results of the significant EPG parameters. The mean value for each plant host and the standard error of the mean are also displayed; in the test statistic column χ^2 values are italicised and F values are underlined.

EPG Parameter	Hypothesised location of resistance factor	Transformation	Mean Value		Standard Error of the Mean		Statistical Test	Test Statistic	Degrees of Freedom (Residuals)	P Value
			cv. Concerto	HsP5	cv. Concerto	HsP5				
All parameters (Global analysis)	All tissue	-	-	-	-	-	Permutated Multiple Analysis of Variance	2.61	1(33)	**0.022**
Time to the first aphid probe of plant tissue	Epidermis	Log[2]	1211 s	421 s	354 s	85 s	Analysis of Variance (ANOVA)	7.99	1(33)	**0.008**
Duration of the first aphid probe	Mesophyll	Log[10]	7717 s	2147 s	1737 s	1161 s	ANOVA	6.94	1(33)	**0.013**
Number of potential drops (intracellular punctures) in first probe	Mesophyll	Sqrt	24	10	5.36	2.82	ANOVA on glm	3.95	1(33)	**0.047**
Time to the first E1 phase (sieve element puncture and salivation into phloem)	Mesophyll	Log[2]	2856 s	7452 s	660 s	1683 s	ANOVA	6.33	1(33)	**0.017**
Time to the first phloem phase containing E1 and/or E2	Mesophyll	-	3761 s	9864 s	675 s	1884 s	Kruskall-Wallis Test (KW)	*5.50*	1(33)	**0.019**
Time to the first phloem ingestion (E2) phase	Mesophyll	-	4395 s	9946 s	846 s	1880 s	ANOVA	5.32	1(33)	**0.028**
Time to first sustained phloem ingestion (>10 minutes)	Mesophyll	-	8210 s	13660 s	1345 s	1954 s	KW	*4.29*	1(33)	**0.038**

Number of probes before first E1	Mesophyll	-	0.66	2.38	0.34	0.76	KW	*9.36*	1(33)	**0.002**
Number of probes before first E2	Mesophyll	-	1	2.5	0.38	0.79	KW	*4.14*	1(33)	**0.042**
Total time ingesting xylem	Xylem	-	2197 s	6859 s	721 s	1408 s	KW	*5.28*	1(33)	**0.022**
Total number of xylem phases	Xylem	-	0.72	1.56	0.13	0.30	KW	*4.77*	1(33)	**0.029**
Total time ingesting phloem (E2)	Phloem	-	7965 s	3299 s	1306 s	1188 s	KW	*8.00*	1(33)	**0.004**
Average time of phloem ingestion (E2)	Phloem	-	5317 s	1717 s	1533 s	702 s	KW	*6.52*	1(33)	**0.011**
Total time of phloem phases containing E1 and/or E2	Phloem	-	8824 s	3620 s	1348 s	1241 s	KW	*9.22*	1(33)	**0.002**
Maximum time of a single phloem phase with E1 and/or E2	Phloem	-	7913 s	3225 s	1442 s	1172 s	KW	*9.01*	1(33)	**0.002**
Maximum length of a single E2 period	Phloem	-	7215 s	2884 s	1371 s	1134 s	KW	*8.40*	1(33)	**0.004**
Average time of phloem phase containing E1 and/or E2	Phloem	-	5911 s	1913 s	1653 s	722 s	KW	*6.88*	1(33)	**0.008**
Time from first E1 phase to sustained E2 phase	Phloem	-	8210 s	13660 s	1345 s	1954 s	KW	*4.29*	1(33)	**0.038**
Total number of sustained E2 phases	Phloem	-	1.39	0.75	0.16	0.17	KW	*6.06*	1(33)	**0.014**
Mean duration of sustained E2	Phloem	-	6500 s	2779 s	1424 s	1141 s	KW	*7.12*	1(33)	**0.008**
Median duration of sustained E2	Phloem	-	6425 s	2779 s	1440 s	1141 s	KW	*6.40*	1(33)	**0.011**

Total time of sustained E2	Phloem	-	7694 s	3060 s	1342 s	1162 s	KW	*7.49*	1(33)	**0.006**
Ingestion:Pathway Ratio (%)	Phloem	-	3.01	0.67	1.40	0.33	KW	*7.43*	1(33)	**0.006**

Table 3: Statistical results of plant defence gene expression assessed by qPCR showing χ^2 and p values (significant values are shown in bold)

Gene	Explanatory variable							
	Plant		Aphid infestation		Plant x infestation		Experimental Block	
HvTHIO1	$X^2 = 11.06$	p = **<0.001**	$X^2 = 0.57$	p = 0.450	$X^2 = 11.67$	p = **0.009**	$X^2 = 1.10$	p = 0.894
HvTHIO2	$X^2 = 13.62$	p = **<0.001**	$X^2 = 0.57$	p = 0.449	$X^2 = 14.31$	p = **0.002**	$X^2 = 0.47$	p = 0.976
HvβTHIO	$X^2 = 14.29$	p = **<0.001**	$X^2 = 1.12$	p = 0.290	$X^2 = 16.10$	p = **0.001**	$X^2 = 0.15$	p = 0.997
HvA1	$X^2 = 5.77$	p = **0.016**	$X^2 = 1.46$	p = 0.226	$X^2 = 4.57$	p = 0.210	$X^2 = 4.58$	p = 0.206
HvNPR1	$X^2 = 1.12$	p = 0.290	$X^2 = 1.85$	p = 0.174	$X^2 = 8.46$	p = **0.037**	$X^2 = 3.64$	p = 0.457
HvERF1	$X^2 = 7.00$	p = **0.008**	$X^2 = 4.81$	p = **0.028**	$X^2 = 12.26$	p = **0.006**	$X^2 = 2.24$	p = 0.691
HvLOXA	$X^2 = 4.81$	p = **0.028**	$X^2 = 4.48$	p = **0.034**	$X^2 = 8.05$	p = **0.045**	$X^2 = 3.11$	p = 0.539
HvLOX2	$X^2 = 5.49$	p = **0.019**	$X^2 = 2.76$	p = 0.096	$X^2 = 8.46$	p = **0.037**	$X^2 = 3.97$	p = 0.410
HvJAZ	$X^2 = 2.85$	p = 0.131	$X^2 = 0.57$	p = 0.450	$X^2 = 3.55$	p = 0.315	$X^2 = 9.42$	p = 0.055

Table 4: Dunn's test *post-hoc* analysis of qPCR results significant for plant x time-point interaction, showing *t* and p values for each pairwise comparison; C = Concerto, H = Hsp5, 0 = 0 h infestation, 24 = 24 h aphid infestation (significant values are shown in bold).

Gene	Pairwise comparison											
	C0:C24		**C0:H0**		**C0:H24**		**C24:H0**		**C24:H24**		**H0:H24**	
HvTHIO1	*t* = 0.69	P = 0.244	*t* = -2.19	P = **0.014**	*t* = -1.82	P = **0.034**	*t* = -2.89	P = **0.001**	*t* = -2.51	P = **0.006**	*t* = 0.37	P = 0.354
HvTHIO2	*t* = 1.28	P = **0.099**	*t* = -1.76	P = **0.038**	*t* = -1.97	P = **0.024**	*t* = -3.05	P = **0.001**	*t* = -*3.26*	P = **<0.001**	*t* = -0.21	P = 0.415
HvβTHIO	*t* = 1.33	P = **0.09**	*t* = -2.08	P = **0.018**	*t* = -1.92	P = **0.022**	*t* = -3.42	P = **<0.001**	*t* =-3.26	P = **<0.001**	*t* = 0.16	P = 0.436
HvNPR1	*t* = 2.62	P = **0.004**	*t* = 0.91	P = 0.182	*t* = 0.21	P = 0.415	*t* = -1.71	P = **0.043**	*t* = -2.41	P = **0.008**	*t* = -0.69	P = 0.244
HvERF1	*t* = 2.03	P = **0.021**	*t* = -1.38	P = 0.082	*t* = -0.32	P = 0.374	*t* = -3.42	P = **<0.001**	*t* = -2.35	P = **<0.001**	*t* = 1.06	P = 0.143
HvLOXA	*t* = 1.12	P = 0.132	*t* = -1.66	P = **0.048**	*t* = 0.21	P = 0.415	*t* = -2.77	P = **0.002**	*t* = -0.91	P = 0.182	*t* = 1.87	P = **0.030**
HvLOX2	*t* = 1.49	P = 0.067	*t* = -1.33	P = 0.091	*t* = -0.48	P = 0.315	*t* = -2.83	P = **0.002**	*t* = -1.97	P = **0.024**	*t* = 0.85	P = 0.196

Table 5: Analysis of deviance table for the scores on each principal component (% variation explained is indicated in brackets) derived from Principal Component Analysis of phloem amino acid composition (significant values are shown in bold)

Explanatory Variable	PC1 (34.38%)		PC2 (17.80%)		PC3 (10.89%)	
	X^2 (df)	p-value	X^2 (df)	p-value	X^2 (df)	p-value
Plant	40.11 (1)	**<0.001**	5.40 (1)	**0.020**	11.98 (1)	**<0.001**
Aphid infestation	0.29 (1)	0.585	0.24 (1)	0.626	2.26 (1)	0.131
Time-point	5.42 (1)	**0.019**	3.05 (1)	0.080	0.08 (1)	0.765
Plant x time-point	0.05 (1)	0.816	2.17 (1)	0.140	1.97 (1)	0.159
RNA Harvest	0.17 (1)	0.671	1.92 (1)	0.166	0.67 (1)	0.413

Table 6: Analysis of deviance table for essential amino acids as a percentage of total amino acids in phloem sap of Hsp5 and Concerto leaves (significant values are shown in bold).

Explanatory Variable	Essential Amino acid (%)	
	X^2 (df)	p-value
Plant	23.35 (1)	**<0.001**
Aphid infestation	0.13 (1)	0.716
Time-point	8.82 (1)	**0.002**
Plant x time-point	0.39 (1)	0.530
RNA Harvest	2.01 (1)	0.155

Figure Legends

Fig 1. Aphid performance on a susceptible barley cultivar (Concerto) and partially-resistant wild relative (HsP5). A) Survival of aphid nymphs over seven days. Number of model observations = 216. C = Concerto; H5 = Hsp5; Rp = *R. padi;* Sa = *S. avenae;* Uh = *U. humboldti*. B) Nymph mass gain and C) intrinsic rate of population increase (rm) of the three aphid species while feeding from the two plant types. Values represent means ± SE. Number of model observations = 72. Letters indicate which groups are similar to each other based on least squares means analysis with Tukey correction. This figure is available in colour at JXB online.

Fig. 2: Representative examples of feeding patterns derived from EPG waveforms of *R. padi* feeding from susceptible Concerto (A) and partially-resistant Hsp5 (B). Diagrams show the frequency and length of each feeding parameter over a 6h (21600s) period. Feeding patterns are split into the five main waveforms observed: non-probing of plant tissue (np), probing into epidermal and mesophyll tissue (the pathway phase (C), saliva secretion into phloem (E1), phloem ingestion (E2), and xylem ingestion (G).

Fig 3: EPG parameters indicative of epidermal, mesophyll and mesophyll/phloem resistance factors. A) Time (s) from start of EPG recording to first stylet puncture of leaf tissue. B) Duration (s) of first stylet probe into plant tissue. C) Time (s) since start of EPG recording until stylet puncture of a sieve tube element. D) Time (s) from start of EPG recording until sustained phloem ingestion. A, B and C values represent means ± SE. Number of model observations = 34. This figure is available in colour at JXB online.

Fig 4: EPG parameters indicative of vascular (phloem and xylem) resistance factors. A) Time (s) from first stylet penetration of a sieve tube element to first sustained phloem ingestion. B) Total time (s) of sustained phloem feeding. C) Ratio of time spent in phloem phase relative to the pathway phase; values represent means ± SE. D) Total time (s) ingesting xylem. Number of model observations = 34. This figure is available in colour at JXB online.

Fig. 5: Trichome densities in the two plant types. A) Median non-glandular trichome densities (No. cm^{-2}) and confidence intervals for Concerto and Hsp5. B) Polarised light micrograph showing the contrasting leaf hair densities between HsP5 (top) and Concerto (bottom). Number of model observations = 30. This figure is available in colour at JXB online.

Fig 6. Expression patterns of thionin and phytohormone signalling genes 0 h and 24 h after aphid infestation. All gene expression values are relative to the mean expression of two reference genes, *HvUBC* and *HvCYP,* and normalised to uninfested control plants. Fold-change, 2-ΔΔCt, is relative to the expression of cv. Concerto at 0 h. Boxplots show median and confidence intervals; total number of model observations for each gene = 20. This figure is available in colour at JXB online.

Fig. 7: Amino acid composition of leaf phloem exudates of Hsp5 and Concerto. A and B) phloem amino acid composition in uninfested leaves of Concerto and Hsp5, respectively, at time zero. C) Biplot of scores on the first two principal components explaining >50% of the detected variation in amino acid composition. D) % proportion of essential and non-essential amino acids in phloem exudates of uninfested Concerto and Hsp5leaves at time zero.

Figures

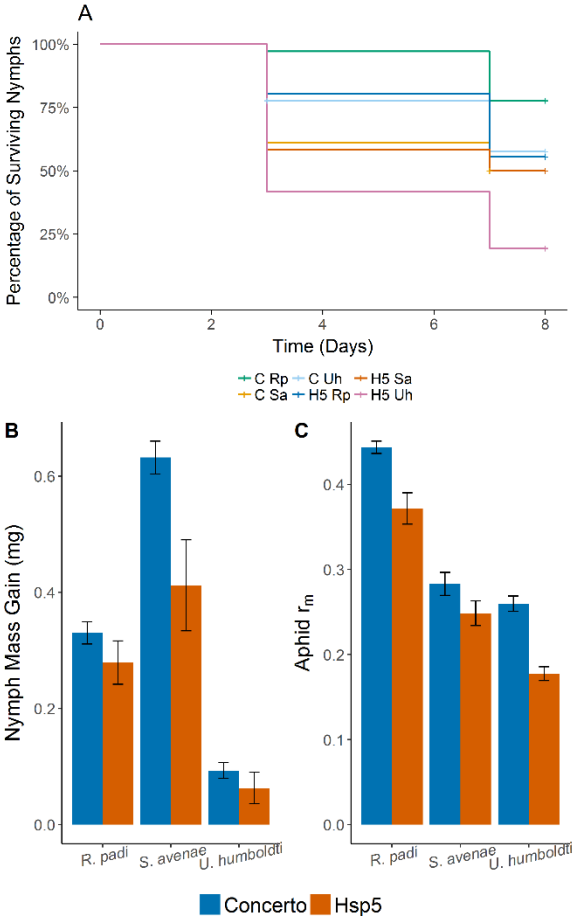

Fig. 1

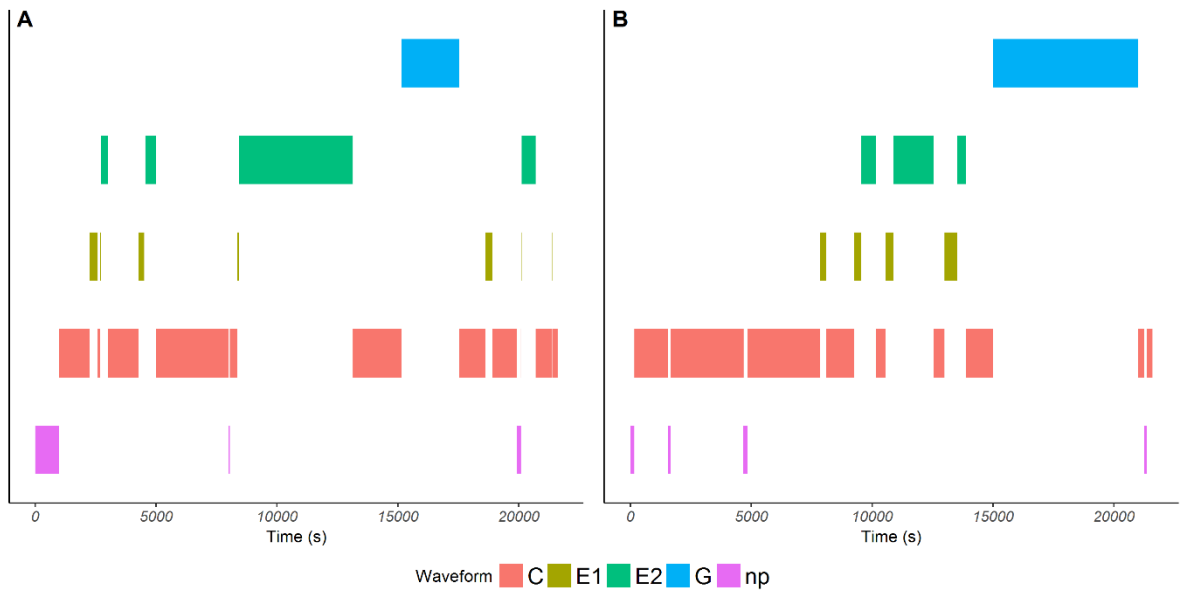

Fig. 2

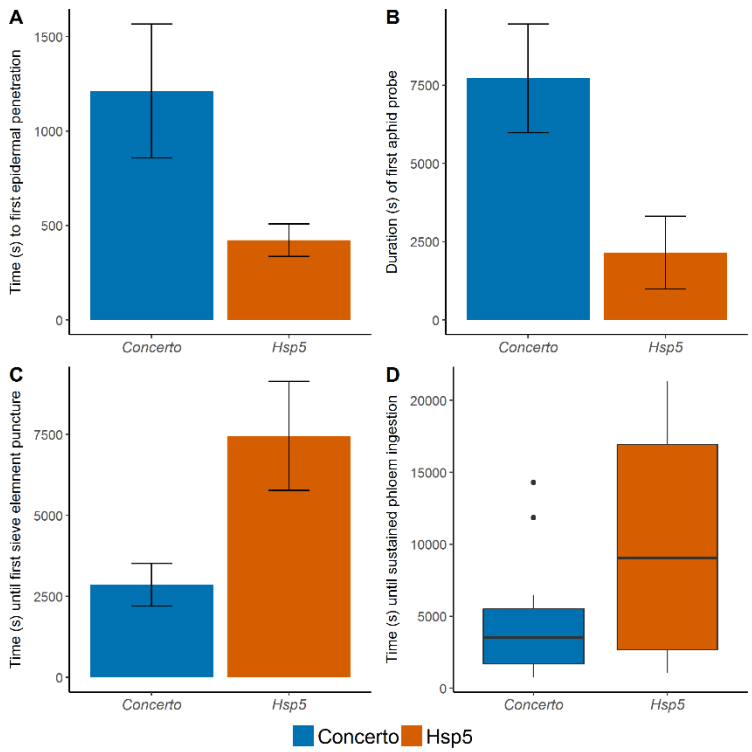
A
Time (s) to first epidermal penetration
1500
1000
500
0
Concerto
Hsp5
B
Duration (s) of first aphid probe
7500
5000
2500
0
Concerto
Hsp5
C
Time (s) until first sieve element puncture
7500
5000
2500
0
Concerto
Hsp5
D
Time (s) until sustained phloem ingestion
20000
15000
10000
5000
0
Concerto
Hsp5
Concerto
Hsp5

Fig. 3

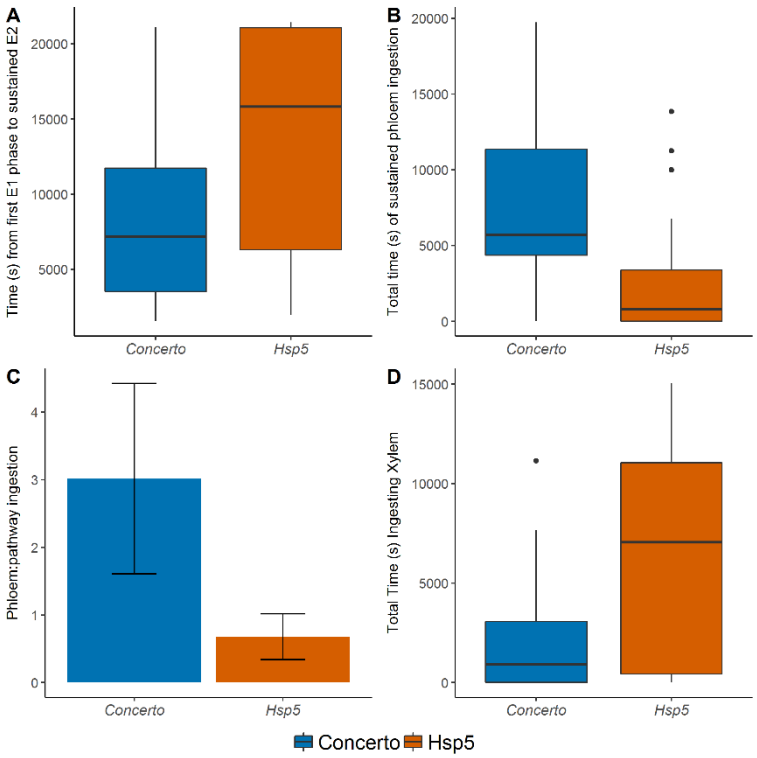
A
Time (s) from first E1 phase to sustained E2
20000
15000
10000
5000
Concerto
Hsp5
B
Total time (s) of sustained phloem ingestion
20000
15000
10000
5000
0
Concerto
Hsp5
C
Phloem:pathway ingestion
4
3
2
1
0
Concerto
Hsp5
D
Total Time (s) Ingesting Xylem
15000
10000
5000
0
Concerto
Hsp5
Concerto
Hsp5

Fig. 4

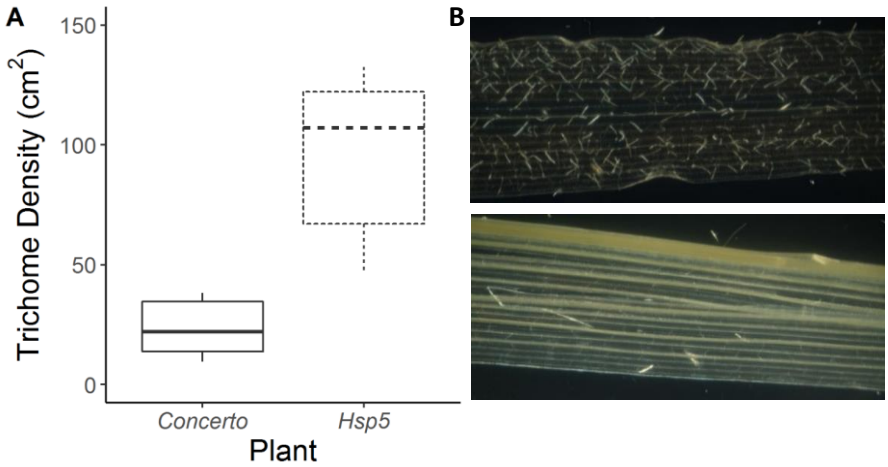

Fig. 5

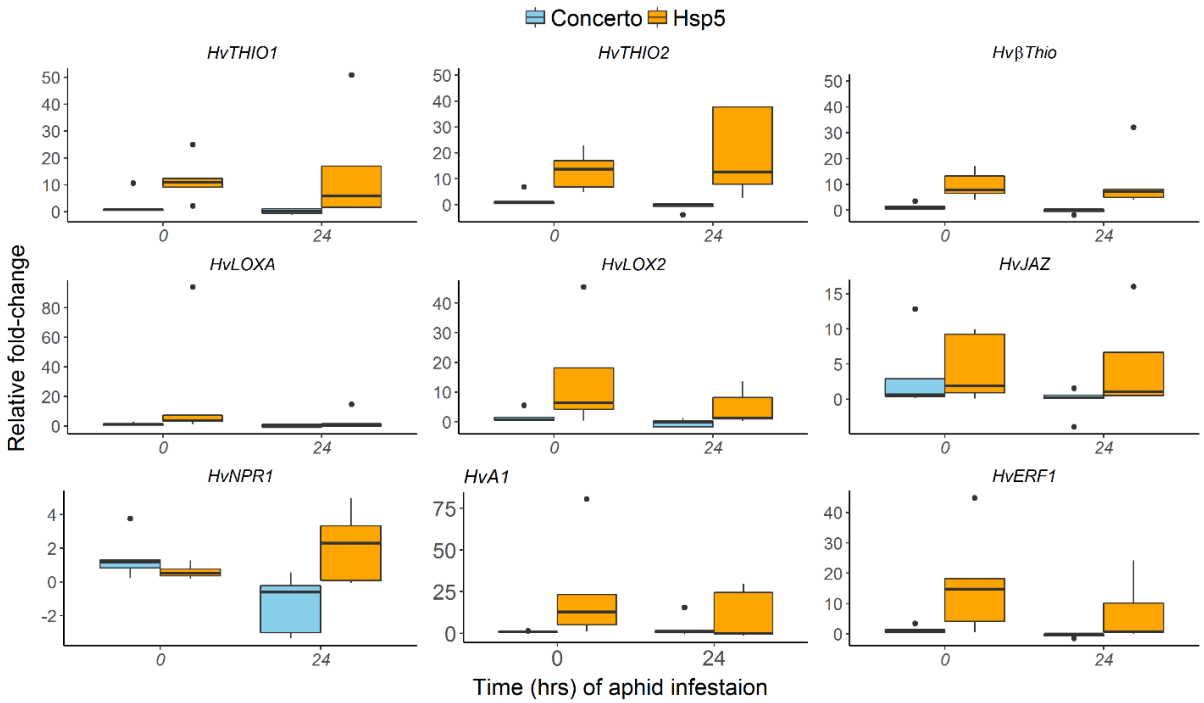
Concerto
Hsp5
HvTHIO1
HvTHIO2
HvβThio
HvLOXA
HvLOX2
HvJAZ
HvNPR1
HvA1
HvERF1
Relative fold-change
Time (hrs) of aphid infestaion
0
24

Fig. 6

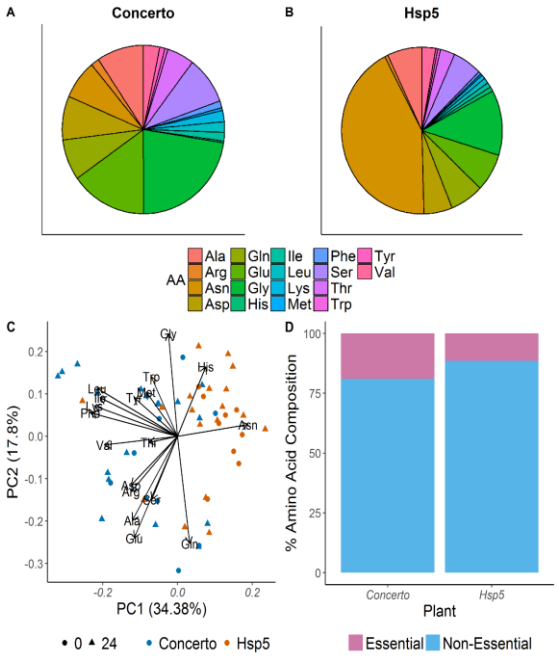
A
Concerto
B
Hsp5
AA
Ala
Arg
Asn
Asp
Gln
Glu
Gly
His
Ile
Leu
Lys
Met
Phe
Ser
Thr
Trp
Tyr
Val
C
PC2 (17.8%)
PC1 (34.38%)
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.2
0.0
0.2
Gly
His
Trp
Met
Tyr
Leu
Ile
Phe
Asn
Val
Thr
Asp
Arg
Ser
Ala
Glu
Gln
0 24
Concerto
Hsp5
D
% Amino Acid Composition
100
75
50
25
0
Concerto
Hsp5
Plant
Essential
Non-Essential

Fig. 7