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*Published in:*  
Plant, Cell & Environment

*DOI:*  
[10.1111/pce.12979](https://doi.org/10.1111/pce.12979)

*Publication date:*  
2017

*Document Version*  
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

### *Citation for published version (APA):*

Escudero , C., Morris, J. A., Hedley, P. E., & Bos, J. I. B. (2017). Barley transcriptome analyses upon interaction with different aphid species identifies thionins contributing to resistance. *Plant, Cell & Environment*, 40(11), 2628-2643. <https://doi.org/10.1111/pce.12979>

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**Barley transcriptome analyses upon interaction with different aphid species identifies thionins contributing to resistance**

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.12979

## Summary

Non- and poor-host resistance to aphids is poorly understood and is key for development of novel crop resistances to insect pests. Here, we used barley as a monocot model crop plant to study the interaction with aphid species that differ in their ability to infest, and analysed barley transcriptional responses during interactions. Our work provides insights into how barley responds to different types of aphid interactions. Importantly, we identified barley genes contributing to plant defences against aphids.

## Abstract

Aphids are phloem-feeding insects that cause yield loss on a wide range of crops, including cereals such as barley. While most aphid species are limited to one or few host species, some are able to reproduce on many plants belonging to different families. Interestingly, aphid probing-behaviour can be observed on both host and non-host species indicating that interactions take place at the molecular level that may impact host range. Here, we aimed to gain insight into the interaction of barley with aphid species differing in their ability to infest this crop by analysing transcriptional responses. First, we determined colonization efficiency, settlement, and probing behaviour for the aphid species *Rhopalosiphum padi*, *Myzus persicae* and *Myzus cerasi*, which defined host, poor-host and non-host interactions, respectively. Analyses of barley transcriptional responses revealed gene sets differentially regulated upon the different barley-aphid interactions and showed that the poor-host interaction with *M. persicae* resulted in the strongest regulation of genes. Interestingly, we identified several thionin genes strongly up-regulated upon interaction with

*M. persicae*, and to a lesser extent upon *R. padi* interaction. Ectopic expression of two of these genes in *Nicotiana benthamiana* reduced host susceptibility to *M. persicae*, indicating thionins contribute to defences against aphids.

**Key words:** barley, transcriptomics, aphids, plant defence, host range, thionins

## Introduction

Aphids are phloem-feeding insects that cause substantial yield loss on a wide range of crop plants, including monocots and dicots. Most aphid species have a narrow host range, limited to one or few plant families. However, some aphids are able to infest a wide range of plant species, including many important agricultural crops. One example is *Myzus persicae* (green peach aphid), which can infest plants in over 40 families, including solanaceous crops such as potato and tomato, cucurbits, legumes, as well as ornamentals (Blackman and Eastop, 2000). In contrast, closely related *Myzus cerasi* (black cherry aphid) is only able to infest a small number of herbaceous plants. Other species, like *Rhopalosiphum padi*, are highly specialized to infest grasses. Host selection by aphids involves a complex set of plant cues and signals, and most likely, molecular interactions that take place between plant and

aphid upon probing and feeding (as reviewed by Powell et al., 2006; Jaouannet et al., 2013).

Upon landing on the leaf surface aphids may detect plant cues and structures that impact their behaviour. During the interaction with a compatible host plant, aphids will use their specialized mouthparts, or stylets, to feed from the phloem. Once the stylets penetrate the leaf epidermal cells, they follow a mainly extracellular pathway to reach the phloem (Tjallingii et al., 1995). Also, the stylets briefly puncture individual cells along the stylet pathway to potentially detect plant cues. Saliva is secreted during both probing and feeding, which contains sets of proteins and small molecules, called effectors, that promote aphid virulence in compatible interactions (Will et al., 2007; Bos and Hogenhout 2011; Elzinga and Jander 2013). Interestingly, aphid probing not only takes place on host plant species, but also on non-hosts suggesting that recognition and activation of resistance may be dependent on the perception of molecules in aphid saliva (Powell et al., 2006). Indeed, aphid saliva exhibits elicitor activity and can trigger responses similar to PAMP (Pathogen Associated Molecular Pattern)-Triggered Immunity (PTI) in plant-pathogen interactions (de Vos and Jander, 2009; Chaudhary et al., 2014). Although these studies all focused on *Arabidopsis* as a model host, elicitor activity has also been reported in whole extracts of *Diuraphis noxii* (Russian wheat aphid) on a resistant wheat (*Triticum aestivum* L.) genotype (Lapitan et al., 2007). These observations imply that plants recognize aphid saliva components to trigger defences. Whether such

recognition events are indeed determinants of aphid host range remains to be investigated.

Aphid infestations can cause significant yield losses in cereals worldwide. One of the major aphid pests of cereals is *Rhopalosiphum padi*, which infests wheat, barley, and oats. This aphid not only causes direct feeding damage resulting in yield losses up to 50% (Kieckhefer and Kantack, 1986; Papp and Mesterhazy, 1993), but also transmits *Barley Yellow Dwarf Virus* (BaYDV), which infects cereals and grasses, and causes reduced growth and leaf yellowing (Oswald and Houston; 1953, Riedell et al., 1999). Barley (*Hordeum vulgare* L.) is a major economical cereal crop, but also a model monocot plant for molecular biology and genetics research. Although no resistant commercial barley cultivars are available against *R. padi*, partial resistant genotypes were previously identified (Delp et al., 2009). Gene expression analyses of susceptible versus partially resistant barley genotypes upon *R. padi* interaction identified a few genes specifically induced in resistant plants (Delp et al., 2009). These included genes encoding a Ser/Thr kinase, a calcium-binding EF-hand protein (BC14), a proteinase inhibitor, and lipoxygenase LOX2. A number of genes were more highly expressed in resistant versus susceptible genotypes in the absence of aphid infestation, including genes encoding thionins (Delp et al., 2009; Mehrabi et al., 2014).

Although oxidative stress responses play an important role in several plant-aphid interactions, *R. padi* does not elicit peroxidase activity or consistently activate peroxidase genes in barley (Ni et al., 2001; Delp et al., 2009). Moreover, only limited callose deposition is triggered upon

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interaction of barley with *R. padi* as compared to other cereal aphids, indicating that plant defence responses may differ depending on both host and aphid species (Saheed et al., 2007; Saheed et al., 2008).

The broad host range aphid *M. persicae* is not considered a pest of barley. However, this aphid has been reported on wheat and barley under field conditions (Halbert and Pike, 1985). Despite this, *M. persicae* performed poorly on barley under controlled growth chamber conditions and showed low levels of colonization and limited ingestion of phloem sap on barley when compared to wheat (Davis and Radcliffe, 2008). Based on these observations, barley can be considered a poor-host species of *M. persicae*. Here, we were interested to investigate how barley responds to different aphid species, including *R. padi* and *M. persicae*, to gain insight into why this plant is a suitable host for specific aphid species. Specifically, we aimed to gain insight into why *M. persicae* performs poorly on barley despite an exceptionally broad host range, which includes species within the *Poaceae*.

We previously dissected plant transcriptional responses to aphids during host, poor-host and non-host interactions in *Arabidopsis* (Jaouannet et al., 2015). This revealed several genes specifically involved in either host susceptibility to *M. persicae* and/or non-host resistance to *Rhopalosiphum padi* (bird cherry-oat aphid). Here, we used a combination of aphid interaction assays as well as barley transcriptomics to assess how a monocot crop species responds to different type of aphid interactions and to identify barley genes that may contribute to defences against aphids.

## Materials and Methods

### Aphid cultures

Aphids used for all the experiments were maintained under controlled conditions in growth chambers (18°C, 16 h of light) and contained in cages. *R. padi* was raised on *Hordeum vulgare* L. cv Optic, *M. persicae* (genotype O) was reared on *Brassica napus* (oilseed rape) and *M. cerasi* was raised on *Barbarea verna* (land cress). *R. padi* and *M. persicae* were kindly provided by Dr. B. Fenton and *M. cerasi* was collected from cherry trees in Dundee (UK).

### Barley colonization assays

Seven-day old barley plants of different cultivars (Golden Promise, Optic and Morex) were infested with each of two 1<sup>st</sup> instar nymphs of the species *R. padi*, *M. persicae* and *M. cerasi*. We performed colonization experiments in three biological replicates with seven individually bagged plants for each aphid species per replicate. The total number of aphids was monitored at 8, 14 and 20 days after placing nymphs on the plants. To compare *M. persicae* colonization of barley versus oilseed rape in parallel, three four-week-old oil seed rape plants and three seven-day-old barley cv Golden Promise plants were each challenged with two 1<sup>st</sup> instar nymphs. The total number of aphids was recorded at 7 and 14 days after challenge. Statistical analysis for the above experiments was performed using the Shapiro-Wilk test for normality and a one-way non-parametric test (Mann-Whitney) in Genstat. We measured the length and width of *M. persicae* aphids reared on oil seed rape and the three different barley



cultivars 7 days after aphid challenge. Each plant was challenged with 10 1<sup>st</sup> instar nymphs. Length and width (mm) of aphid was measured in images taken from a set distance with the software Image J (Schneider et al., 2012). Statistical analyses were done using ANOVA, with single factor for the parameters length and width. All experiments were performed in growth chambers (18°C, 16 h of light).

### **Aphid settlement assays**

Seven-day-old barley plants (cv. Optic) were used to assess *R. padi*, *M. persicae* and *M. cerasi* settlement on barley leaves. Four clip cages per aphid species were prepared containing 10 aphids of each species per clip cage. At time 0 the cages, containing the aphids, were placed on the barley leaves. At intervals of 15 minutes the clip cages were opened and the number of aphids settled on the barley leaf counted. The experiment was performed as three biological replicates. The results were analysed by ANOVA with Fisher's protected least significant difference post-hoc test per timepoint

### **Aphid probing assays**

Barley leaf samples (1 cm<sup>2</sup>) were placed on 24-well plates containing 1% water agar. Subsequently, four age-synchronized adult aphids from the species *R. padi*, *M. persicae* or *M. cerasi* were each placed on the leaf samples (cv Golden promise, Optic and Morex). Six leaf samples were used per aphid species and three independent replicates were set up. Plates with aphids were kept in short day chambers at 22°C. After two

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days, the leaf samples were stained in an acid fuchsin solution (Urbanska, 2010) and analysed under a light microscope for the presence of stylet pathways. Stylet paths were counted for each 1 cm<sup>2</sup> leaf sections challenged with aphids and classified as long pathways or short probes. Differences between path and probes were assessed by ANOVA with Fisher's protected least significant difference post-hoc test ( $p > 0.05$ ). We used a similar set-up to visualize callose with aniline blue staining. We removed the chlorophyll from the barley leaves (cv Optic) using (1:3) acetic acid:ethanol over 8 h with two changes in a constant shaker. Next, samples were incubated for 24 h in a solution of 0.05% aniline blue (protocol adapted from Daudi et al., 2012). Samples were analysed for the presence of cells with callose deposition under a confocal microscope Zeiss LSM 510 (Jena, Germany) using a Zeiss x20 lens and a green excitation filter (wavelength 516 nm).

### **Barley transcriptome analyses**

Barley plants (cv Optic) were pre-germinated in Petri dishes covered with wet filter paper for three days in the dark. Germinating seeds were then moved to soil and grown under controlled conditions (short day, at 22 °C, 70% humidity and 125  $\mu\text{mol photons/m}^2\cdot\text{s}$ ). One week later, the plants were challenged with fifteen mixed-age aphids enclosed in clip cages. As a control, we also placed clip cages without aphids on barley plants. Leaf tissues enclosed within the clip cages were collected after 3 h and 24 h from both aphid-challenged and control treatments. Samples from the same treatment (6 samples) were pooled together in a Falcon tube

submerged in liquid nitrogen. We performed this experiment as three biological replicates. Individual replicates were set up at the same time of day to take into account any effects of the plant circadian cycle. The experiment was started at 9am, with the 3h samples being collected from 12noon and the 24h samples being collected from 9am the next day. RNA was extracted using the Qiagen RNeasy Plant Mini Kit® following the manufacturer's protocol. RNA quality was assessed using the Agilent 2100 Bioanalyzer prior to microarray processing. RNA from each of three replicates was hybridized to a custom-design Agilent barley 60k microarray (Comadira et al., 2015). The microarray experimental design and dataset can be accessed at ArrayExpress (<https://www.ebi.ac.uk/arrayexpress>; accession # E-MTAB-5133).

Recommended total RNA labelling (100 ng each) and hybridization was used throughout (Agilent One-Color Microarray-Based Gene Expression Analysis: Low Input Quick Amp Labeling version 6.5). Data were extracted from each microarray using Feature Extraction software (version 10.7.3.1; Agilent Technologies) with default settings, and subsequently data were imported into GeneSpring (version 7.3; Agilent Technologies) software for pre-processing and analyses. Default one-colour normalization was performed and probes filtered on flags to remove inconsistent data. To identify genes differentially expressed between aphid-challenged versus unchallenged barley samples at the 3 h and 24 h time points, a paired Student's t-test ( $p\text{-value} \leq 0.05$  with Benjamini-Hochberg correction) was performed. Data was visualized using line graphs, box whisker plots, tree-heatmaps for hierarchical clustering, and Venn diagrams for gene-list

comparisons. MapMan functional BIN classification was performed using with Wilcoxon Rank Sum Test (cut-off  $p \leq 0.05$  and fold change  $\geq 2$ ) (Thimm et al., 2004).

We assessed variation between the 3h and 24h control samples to determine the impact of potential diurnal effects or different exposure time to clip cages on hierarchical clustering. By comparing the 2 controls, we identified 331 genes differentially expressed. Of these, 120 were also identified in our set of 974 differentially expressed genes across barley-aphid interactions. Removing these 120 genes from the hierarchical clustering approach did not affect the main groups identified across barley aphid interactions as shown in figure 3 (Supplementary Fig. 1, Supplementary table 11).

### **GO enrichment analyses**

We used Biomaps software available on the Virtual Plant web platform, version 1.3 (<http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/>) (Katari et al., 2010) to analyze gene ontologies (GO) using *Arabidopsis thaliana* Columbia tair10 genome (28,775 genes) as a reference genome. Gene enrichment analysis of the set of genes was performed in Biomaps. The different gene sets were interrogated with the available options: GO Biological Process, GO Molecular Function and GO Cellular Compartments (TAIR/TIGR), AraCyc pathways (v11.5) from PlantCyc, functional classification by the Munich Information Center for Protein Sequences (MIPS) and KEGG Pathways. The gene ontology was calculated with Fisher Exact Test  $p \leq 0.05$  (False Discovery Rate

correction).

### **Quantitative RT-PCR to assess gene expression changes**

Validation of the microarray data and analyses of gene expression changes across select barley cultivars, was done by qRT-PCR using the Universal Probe Library (UPL) (Roche Diagnostics). To validate expression of selected genes, we pooled the RNA of the three barley cv Optic biological replicates used for microarray hybridization. For expression analyses of barley thionins at 72h post infestation, we used three biological replicates of barley cultivar Optic challenged with aphids for 72 h using clip cages as described for the microarray experiment. To assess expression of selected genes across cultivars, we used three biological replicates of barley cultivars Morex and Golden Promise challenged with aphids for 24 h using clip cages as described for the microarray experiment with cultivar Optic. DNase treated RNA (Ambion TURBO DNA-free DNase Treatment) was converted into cDNA with SuperScript III Reverse Transcriptase (Invitrogen) using random primers. Databases used for primer design were a local database containing predicted barley cv Morex genes, NCBI, Ensembl Plants *Hordeum vulgare*, and the Plant Genome and Systems Biology (PGSB) barley genome database. Primers and probes were designed using the UPL Roche website and are listed in Supplementary Table 10. Primers and probes were first tested for efficiency (85-105%). Reactions were prepared using 25 µl of total volume, 12.5 µl of FastStart TaqMan Probe Master Mix (containing ROX reference dye), 0.25 µl of gene specific

primers (0.2 mM) and probes (0.1 mM). A StepOne thermocycler (Applied Biosystems by Life Technology) was run as follows: 10 min of denaturation at 95 °C, followed by 40 cycles of 15 s at 94 °C and 60 s at 60 °C. Relative gene expression was calculated with the method  $\Delta\Delta C_t$  (Delta Cycle threshold) with primer efficiency taken into consideration. Every sample was run as three technical replicates. Cycle threshold values were normalized with three reference genes, *actin-2* (MLOC\_78511.2), *ubiquitin* (AK248472.1) and *pentatricopeptide* (AK373147/MLOC\_80089.1). Expression of these reference genes was unaffected in our microarray experiment (data not shown).

#### **Ectopic expression of thionins followed by aphid performance assays**

Two different barley thionin genes (AK252675.1/MLOC\_46400.1 and AK359149/ MLOC\_34881.1) were selected for cloning and aphid performance assays. Coding sequences were cloned into destination vectors pB7WG2 to allow ubiquitous overexpression under the 35S promoter. *Agrobacterium tumefaciens* strain GV3101 carrying the thionin constructs or the empty vector were infiltrated into *Nicotiana benthamiana* leaves at a relative  $OD_{600} = 0.1$ . Twelve infiltration sites were used for each construct (two per plant, a total of 6 plants per construct). One day after infiltration, 2 adult *M. persicae* aphids were placed at the underside of the infiltrated leaf areas and the area was enclosed with a clip cage. One day later, the adult aphids and all except 3 nymphs were removed from the leaf area. Aphids were moved to freshly infiltrated plants 7 days

after initial agroinfiltration. Total nymph numbers were counted 14 days after the start of the experiment. Three independent biological replicates were performed. Differences between thionin-expressing leaves and the vector control were assessed by one-way ANOVA and post-hoc Fisher's test ( $p \leq 0.01$ ).

To assess activation of *Nicotiana benthamiana* defence genes *PAD4*, *PR-1*, *TP1* and *PR-4*, by barley thionins we agroinfiltrated leaves of three plants per biological replicate and collected leaf samples for RNA extraction with the Qiagen RNeasy Plant Mini Kit®. Quantitative RT-PCR was performed as previously described by Rodriguez et al. 2014.

## Results

### **Differences in barley colonization by the aphid species *Myzus persicae*, *Myzus cerasi* and *Rhopalosiphum padi***

Although *M. persicae* and *M. cerasi* have not been reported to cause significant infestations on barley, *M. persicae* has been found on barley plants in a field setting, suggesting this aphid may be able to at least survive on this crop (Halbert and Pike, 1985). We were interested to determine whether, and to what extent, both these aphids were able to colonize barley under controlled glasshouse conditions. Therefore, we set-up aphid infestation assays of three barley cultivars, Golden Promise, Optic and Morex, with *M. persicae* and *M. cerasi*, as well as *Rhopalosiphum padi*, a major aphid pest of barley. Seven-day-old plants were challenged each with 2 nymphs per plant and the numbers of aphids

were counted at 8, 14 and 20 days after challenge (DAC) (Fig. 1a). Whilst the *R. padi* population reached on average between 800-1200 aphids across the cultivars, the *M. persicae* population only reached on average between 30-45 aphids at 20 DAC (Fig. 1a). No living aphids were found for *M. cerasi* at 8 DAC, indicating this species was unable to survive on barley.

Since we found that *M. persicae* was able to reproduce on barley we were interested to compare how the level of reproduction on barley compared to that on the well-documented host plant oilseed rape (*Brassica napus*).

We performed colonization experiments of barley and oil seed rape in parallel for *M. persicae*, and infested 7-day-old barley and 4-week-old oil seed rape plants each with 2 synchronized adult aphids per plant. The total populations were counted after 14 days. Whilst we counted over 80 aphids on oil seed rape, we only found around 10 aphids on barley (cultivar Golden Promise), indicating an 8-fold difference in population size (Fig. 1b). During these experiments we noted that the *M. persicae* adults reared on barley were smaller in size than those on oil seed rape.

To confirm this we measured aphid body length and width of *M. persicae* reared on barley versus oil seed rape. We allowed 6 nymphs to feed on barley or oilseed rape plants for 7 days and measured body length and width. Aphids feeding on barley were significantly smaller than those feeding on oil seed rape (One-Way ANOVA,  $p \leq 0.01$ ) (Fig. 1c). No significant differences in aphid size were found between different barley cultivars (Fig. 1c). Although *M. persicae* was able to survive and reproduce on barley, this species performs poorly on this crop plant



compared to *R. padi*. Based on our colonization data we defined *M. cerasi*-barley as a non-host interaction and *M. persicae*-barley as a poor-host interaction in our follow-up work detailed below.

In our colonization experiments we noticed a smaller number of aphids remained on the leaf surface for *M. cerasi* compared to the other species after placing them on barley leaves. This observation led us to further investigate whether the aphid species settled differently on barley in a no-choice experiment. We placed 10 adult aphids for each species in a clip cage, which was attached to lower side of the leaf surface, allowing aphids to either stay in the clip cage or move onto the plant. Aphid numbers on the leaf surface were counted at 15-minute intervals for three hours. Whilst both *R. padi* and *M. persicae* moved from the clip cages to the leaf surface, with between 7-9 aphids counted after 3 hours, only few aphids (between 1-3) were found on the leaf surface in the case of *M. cerasi* (Fig. 1d). This, together with the inability of *M. cerasi* to survive and reproduce on barley, shows barley is a non-host of this aphid species.

**The aphid species *Myzus persicae*, *Myzus cerasi* and *Rhopalosiphum padi* produce different stylet pathways when probing barley leaves and activate callose deposition**

Aphid probing has been reported to take place during both host and non-host interactions, and is essential for the delivery of saliva inside plant cells and the apoplastic space (McLean and Kinsey 1968; Wikteliuss 1982). We investigated whether the different aphid species included in our study were all probing barley leaves, and how stylet pathways compared among the different interactions. To do this, we made use of an acid fuchsine stain, which is commonly used to visualize aphid stylet pathways, in combination with light microscopy. We challenged barley leaves with *R. padi* (host interaction), *M. persicae* (poor-host interaction), or *M. cerasi* (non-host interaction), and collected leaf samples for staining 2 days later. For *R. padi*, we mainly observed long highly-branched stylet pathways, whereas for *M. cerasi*, which was unable to survive on barley, we observed a small number of short probes, visible as pink dots (Fig. 2a). For *M. persicae*, we observed a large number of short probes, visible as pink dots, but also some stylet pathways (Fig. 2a, Supplementary Fig. 2). We then quantified the number of pathways and short probes (dots without pathway) in a 1 cm<sup>2</sup> size leaf area of three different barley cultivars (Morex, Optic, and Golden Promise). This confirmed that during the host interaction with *R. padi* long and branched stylet pathways were most abundant, with between 40 to 50 pathways per leaf area, and less than 20 short probes (Fig. 2b). During the poor-host interactions with *M. persicae*, we mostly detected short probes, ranging from 40 to 60 probes per leaf

area, and around 20 stylet pathways (Fig. 2b). During the non-host interaction with *M. cerasi* we observed a much lower number of short probes (around 10) per leaf area compared to the poor-host interaction with *M. persicae*, and only few stylet pathways. We observed similar results across the three barley cultivars (Fig. 2b). Our data show that although *M. cerasi* and *M. persicae* do not or poorly infest barley, these aphids probe the barley leaf tissue, indicating that signals can be exchanged at the plant-aphid stylet interface. Also, clear differences in stylet pathway formation were observed, which may reflect the ability of the aphids to successfully feed from the phloem and establish populations.

During compatible host barley-aphid interactions, the production of callose depositions has been reported (Saheed et al., 2009). Therefore, we assessed whether barley responds to *M. persicae* (poor-host interaction), *M. cerasi* (non-host interaction) and *R. padi* (host interaction), in a similar way with regards to callose deposition. We visualized callose using aniline blue staining on barley epidermal cells from leaves 2 days after infestation with 5 adult aphids. We observed a strong callose accumulation at the site of the stylet penetration in the epidermal cells for all three interactions (Fig. 2c, Supplementary Fig. 3). For *R. padi*, we usually observed one stylet pathway or probe per cell and we also noted callose depositions at the cell wall of punctured cells where we detected a stylet pathway (Fig. 2c). For *M. persicae*, we noted multiple sites of callose deposition per cell, which most likely reflect multiple probing sites per cell (Fig. 2c). These results are consistent with our observations that

*M. persicae* shows increased probing compared to *R. padi*. Occasionally, cell wall depositions could be observed during barley-*M. persicae* interactions. For *M. cerasi*, we only occasionally detected callose depositions, which again likely reflects limited probing consistent with the results obtained using fuchsine staining and light microscopy (Fig. 2c).

### **The barley transcriptome responds most strongly to interaction with *M. persicae* compared to the interactions with *R. padi* and *M. cerasi***

We previously compared Arabidopsis host, poor-host and non-host responses to aphids, which identified genes differentially expressed during specific interactions (Jaouannet et al., 2015). Here, we aimed to perform a similar comparison using barley as a model monocot crop species. We challenged 7-day-old barley plants (cv. Optic) each with 15 adults of *M. persicae*, *M. cerasi*, *R. padi* aphids or no aphids, and collected leaf material 3 and 24 hours later. RNA was extracted and prepared for hybridization with a custom Agilent 60K barley microarray. We identified 974 genes that were significantly differentially expressed ( $p$ -value  $\leq 0.05$ ) in at least one of the aphid treatments compared to the no-aphid control (Supplementary Table 1). Hierarchical gene tree cluster analysis of the differentially expressed genes revealed two main clusters within this set of genes based on their expression profiles (Fig. 3). Cluster A comprised 779 genes and cluster B 195 genes. Within these two main clusters we identified sub-clusters that behave differently across the treatments within the main cluster (Figure 3, Supplementary Table 2). Two

subclusters, A-2 and B-2, showed significant over-representation of gene functional categories based on GO annotation. Sub-cluster A-2 comprised 717 genes which were predominantly up-regulated during the interactions with *R. padi* and *M. persicae* at the 24h timepoint, and cluster B-2, which contained 110 genes specifically up-regulated at the 24h timepoint during the *M. persicae* interaction (Fig. 3, Supplementary Table 2). Both these clusters showed over-representation of genes predicted to be involved in a range of metabolic processes with functions in catalytic activity (GO:0003824), (Supplementary Table 2 and 3).

#### **Differential barley transcriptome responses specific to the interaction with *R. padi* or *M. persicae***

Overall analyses of the barley transcriptional responses during different aphid interactions suggested that some responses were more pronounced during specific aphid interactions or potentially aphid species-specific. This led us to investigate potentially unique barley responses to each one of the aphid species. We performed pairwise analysis of the set of 974 genes to identify down- and up-regulated genes per aphid species treatment per timepoint as compared to the no-aphid control (Fig. 4, Supplementary Table 4 and 5). The barley transcriptional response to aphids was more pronounced at the 24 h timepoint compared to the 3 h timepoint, with 905 genes (24 h timepoint) versus 91 genes (3 h timepoint) being significantly differentially expressed in at least one of the aphid treatments compared to the non-infested control ( $p \leq 0.05$ ).

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For the 3 h timepoint, the barley transcriptional response was most pronounced upon interaction with *R. padi*, with 50 genes significantly differentially expressed. Forty of these were host interaction-specific, with 36 being up-regulated and 4 down-regulated (Fig. 4a, Supplementary Table 4 and 5). GO annotation showed over-representation of genes with predicted molecular functions in catalytic, transferase, hydrolase and chitinase activity (GO:0003824, GO:0016740, GO:0016798, GO:0004568) in the set of 36 up-regulated genes (Supplementary Table 6). During the interaction with *M. persicae* 24 genes were significantly differentially expressed, of which 15 were not affected by the other aphid species to a statistically significant level (Fig. 4a, Supplementary Table 4 and 5). Upon interaction with *M. cerasi*, a total of 26 genes were down-regulated, of which only 1 was similarly affected by one of the other aphid interactions. Eleven barley genes were similarly affected by different aphid interactions at the 3 h time point, of which 8 were shared between the *R. padi* and *M. persicae* interactions (Fig. 4a, Supplementary Table 7).

At 24 h after aphid challenge, we observed a much stronger barley transcriptional response, especially in the case of the interactions with *R. padi* and *M. persicae* (Fig. 4b, Supplementary Table 4 and 5). The response to *M. cerasi* was weak, with just 20 genes differentially expressed (Fig. 4b, Supplementary Table 4 and 5). Therefore, for further detailed analyses we specifically focused on the barley interactions with *R. padi* and *M. persicae* as detailed below.

At the 24 h timepoint, 76 genes were significantly up-regulated upon interaction with *R. padi* and *M. persicae*, whereas only 9 genes were commonly down-regulated between these interactions (Fig. 4b, Supplementary Table 7). GO enrichment analyses showed that catalytic activity and oxidoreductase activity were over-presented molecular functions for the up-regulated set of genes (Supplementary Table 8).

Out of the 317 genes significantly affected only upon *R. padi* interaction, 222 were up-regulated (Fig. 4B, Supplementary Table 4). GO enrichment showed an over-representation of genes predicted to be involved in metabolic processes (GO:0008152, GO:0009987, GO:0044237, GO:0044238), response to stimulus (GO:0050896), and response to stress (GO:0006950), and with over-represented molecular functions in catalytic, transferase, and kinase activity (GO:0003824, GO:0016740, GO:0016301) (Supplementary Table 6). In addition, BLAST similarity searches against rice and Arabidopsis databases ( $E < 10^{-5}$ ) revealed several genes predicted to function as WRKY transcription factors (WRKY3, 4, 31 and 50), cytochrome P450s, heat shock proteins, and receptor-like kinases. We then assessed whether this gene set showed a similar direction of regulation during the interactions with *M. persicae* by applying a  $\log_2$  ratio = 1.0 cut off. This showed that actually 98% (218/222) of genes up-regulated upon interaction with *R. padi* showed a similar direction of expression change upon *M. persicae* interaction (Supplementary Table 4). For several of these genes we even noted a higher fold-change upon *M. persicae* interaction than upon *R. padi* interaction. Despite the higher fold-changes these genes were not found

to be significantly up-regulated upon *M. persicae* interaction, which is likely due to the overall stronger transcriptional response to this aphid species. Additionally, the response to *R. padi* included the specific down-regulation of 95 genes, with no significant GO terms found (Fig. 4b, Supplementary Table 5 and 6). Of these 95 genes, only 11 were affected in a similar direction upon interaction with *M. persicae* based on a log<sub>2</sub> ratio = 1.0 cut off. This suggests that the majority of these genes are specifically down-regulated upon interaction with *R. padi*.

All of the 480 barley genes significantly affected specifically upon interaction with *M. persicae* were up-regulated (Fig. 4b, Supplementary Table 4). GO annotation revealed an overrepresentation of genes with predicted functions in catalytic activity (GO:0003824) and copper binding (GO:0005507) (Supplementary Table 6). BLAST similarity searches ( $E < 10^{-5}$ ) revealed many genes predicted to function as thionins, peroxidases, lipoxygenases, receptor-like kinases and protein kinases (Supplementary Table 4). To determine whether the 480 genes were regulated in a similar direction upon interaction with *R. padi* we applied again a log<sub>2</sub> ratio = 1.0 cut off. Only 14% (67/480) of genes showed a similar trend in expression during *R. padi* interaction, although not to a statistically significant level (Supplementary Table 4). Based on this, we conclude that the barley transcriptional response to *M. persicae* is stronger than the response to *R. padi*, with over 400 genes specifically up-regulated during the *M. persicae* interaction.



MapMan analyses further confirmed differences in barley transcriptional responses to *R. padi* and *M. persicae* after 24 h (Supplementary Fig. 4). We observed differences in functional categories between the two barley-aphid interactions, especially in the case of metabolic enzymes, and stress-related genes.

### **Validation of barley transcriptional responses to aphids for selected genes across different barley cultivars**

Based on the microarray data analyses, we selected 11 significant differentially expressed genes across different types of aphid interactions for validation by qRT-PCR analyses. Three of the 11 genes, predicted to encode a cysteine proteinase (MLOC\_74627.1), a Jasmonate ZIM-domain (JAZ) transcription factor (MLOC\_9995.2) and a WRKY4 transcription factor (AK371133), were similarly affected during interaction with *R. padi* and *M. persicae*. Eight of the 11 genes, predicted to encode three different thionins (AK252675, AK359149, and AK357884), a late embryogenesis abundant protein 14 (LEA 14) (MLOC\_5174.1), a protein kinase (AK373791), a lipoxygenase 2 (LOX2) (AK357253.1), a plant cadmium resistance protein 2-like (PCR2-like) (MLOC\_79086.1), and a receptor-like kinase (MLOC\_55207.1), were significantly up-regulated upon interaction with *M. persicae*. In addition to validating the microarray results for barley cultivar Optic, we also challenged cultivars Golden Promise and Morex with the different aphid species in three replicated

experiments to determine if transcriptional responses were conserved across different barley cultivars.

For 10 of the 11 selected barley genes, qRT-PCR analyses confirmed the expression profile of the microarray data (cultivar Optic) (Fig. 5). For thionin 3, expression was outside reliable detection limits in the qRT-PCR experiment, and as a result of this we did not confirm up-regulation as observed in the microarray data (Fig. 5). When assessing expression profiles for the selected genes in cultivars Golden Promise and Morex, we noted that although the direction of expression was similar across cultivar-aphid interactions, the strength of response was slightly weaker in cultivar Morex for the majority of genes. In addition, we obtained variable expression profiles across replicates for the three selected thionins, but despite this observed up-regulation in the majority of replicates, especially upon interaction with *M. persicae*. We also assessed the expression of thionins at a later timepoint (72 h) upon interaction with *M. persicae* and *R. padi* to determine if there was a more pronounced difference in expression at a later stage during the infestation process. Although thionins were up-regulated upon infestation with both aphid species for 72h, expression was higher for the *M. persicae* interaction compared to the *R. padi* interaction (Supplementary Fig. 5). As mentioned above, we identified 12 thionins significantly up-regulated upon interaction with *M. persicae* in the microarray data, and further sequence analyses revealed these genes are part of a large gene family in barley with over 39 members that show a high level of sequence similarity (Supplementary Fig. 6). The primers and probes designed for qRT-PCR analyses are

unlikely to discriminate between the different members of this family (Supplementary Fig. 7), which may be differently affected by aphid interaction, and this could explain the level of variation observed in this experiment. Overall, we were able to verify differential expression of selected barley genes upon aphid interaction across different barley cultivars, and confirmed that some barley genes responded more strongly to *M. persicae* than to *R. padi*.

### **Transient overexpression of barley thionins in *Nicotiana benthamiana* reduces *M. persicae* virulence**

Among the 480 genes significantly up-regulated during the barley-*M. persicae* interaction we identified 12 genes predicted to encode thionins, which are small proteins found specifically in plants that have antimicrobial activity (Bohlmann and Apel, 1991; Thevissen et al., 1996). Based on this, we were interested to determine whether thionins impact aphid virulence. We cloned two of the barley thionin genes (AK252675 and AK359149) and ectopically expressed these under the 35S promoter by agroinfiltration in the solanaceous *M. persicae* host plant *N. benthamiana*. Infiltrated leaf areas were then challenged with 3 synchronized nymphs of *M. persicae*, and progeny per adult was counted over a 14-day period. While aphids produced 11 nymphs per adult on leaf areas infiltrated with the *Agrobacterium* strain carrying the empty vector control, on the leaves transiently over-expressing the thionins progeny production was only 6 nymphs per adult, indicating a reduction of nearly

50% compared to the vector control (Fig. 6). To determine whether this observation was linked to possible defence activation due to ectopic expression of the thionins in *N. benthamiana*, we performed expression analyses of marker genes for SA-signalling (PR-1, PAD4), and JA-signaling (PR4 and TP1). None of the markers were consistently differentially expressed upon transient ectopic expression of thionins (Supplementary Fig. 8). Therefore it is unlikely that activation of the corresponding defence signalling pathways is responsible for the observed reduction in reduced aphid performance.

## Discussion

In this study we characterized the interaction of the monocot crop plant barley with three aphid species that differ in their ability to infest this plant species, to identify barley genes potentially involved in plant defences against aphids. This not only generated a comprehensive overview of how barley responds to aphid species during host, poor-host and non-host interactions at the transcriptional level, but also revealed that thionins, which are highly up-regulated upon aphid interaction, especially in the case of poor-host interactions, may contribute to crop resistance.

Whilst we selected aphid species based on their ability to infest or not infest barley in a field environment, our experiments performed under controlled conditions showed that *M. persicae* was able to use barley as a host, but with poor performance, whereas *M. cerasi* was not even settling on this crop. Both *M. persicae* and *M. cerasi* are considered non-pests of

barley and our findings suggest that the mechanisms underlying resistance against these two species are different. In the case of *M. cerasi* it is possible that external plant cues, such as volatiles and epicuticular waxes, deter aphids from settling on barley and initiating probing (Powell et al., 2006). Alternatively, this species could be deterred by peripheral cues upon only one or few brief probes. Aphid probing, which allows aphids to contact the host cell cytoplasm as well as apoplast, is thought to be key in differentiation of host versus non-host species (Powell et al., 2006). The frequent short probes by *M. persicae* and low number of stylet pathways, in combination with poor growth of this aphid on barley, suggests that this aphid species is not able to feed optimally on this plant.

Multiple short probes are also observed during incompatible interactions of other aphid species with both resistant host and non-host plants. For example, frequent brief probes were observed for *Macrosiphum euphorbiae* feeding on resistant tomato plants containing the *Mi-1* resistance gene (Kaloshian et al., 2000), indicating that although aphids were able to locate the phloem, their ability to successfully feed was impaired. Also, during non-host interactions, frequent brief probes have been reported, which are sufficient to support transmission of non-persistent viruses (Schwarzkopf et al., 2013; Harrington et al., 1986).

Potentially, the probing of the leaf tissue may cause damage that is responsible for the common activation of redox-stress and JA-regulated genes as determined by GO analyses (Supplementary Table 2 and 3).

The most highly up-regulated gene for the *R. padi* and *M. persicae* interactions encoded a cysteine protease, with similarity to Arabidopsis

*Senescence-Associated Gene 12 (SAG12)* (Supplementary Table 7).

Interestingly, infestation of Arabidopsis by *M. persicae* results in activation of SAG genes, including SAG12, and hypersenescence, potentially as part of the plant defence response (Pegadaraju et al., 2005). However, in barley the function of this cysteine protease, and its potential link with senescence, has not yet been investigated.

A large number of genes were highly up-regulated upon barley interaction with *M. persicae* compared to the interactions with the other aphid species, which could reflect specific activation of defences against this aphid species resulting in limited infestation success. One possibility is that the stronger barley transcriptional response to *M. persicae* is due to the frequent short probes observed. A number of genes predicted to function in cell wall-related processes were specifically up-regulated in response to *M. persicae*, which could be part of a damage response upon repeated probing of epidermal and mesophyll cell layers (Supplementary Table 6). However, the combined number for the short probes and stylet pathways between the *M. persicae* and *R. padi* were quite similar among cultivars (Fig. 2b), suggesting that the frequency of total probes is comparable. Barley genes with more pronounced expression upon *M. persicae* interaction, as confirmed by qRT-PCR analyses, were those encoding LOX2, a protein kinase, protein cadmium resistant 2, LEA 14 protein, thionins, and a receptor-like kinase. Most of these genes are not well characterized in monocot crops. In Arabidopsis, we previously identified a *LEA* gene specifically activated during a non-host interaction with aphids, but we found no evidence for activation of Arabidopsis

thionins upon interaction with different aphid species (Jaouannet et al., 2015). Our experimental design took into account local barley responses (i.e. leaf areas within clip cages) to the different aphid species, and the impact on systemic responses remains to be investigated.

We identified several barley genes responsive to aphid infestation that show similarity to RLKs and E3 ubiquitin ligases implicated in PTI in other plant species (Supplementary Table 4). PTI is not well characterized in barley but the activation of these genes by aphids may reflect activation of components of PTI signalling pathways, which will need to be investigated further.

Our transcriptomics approach allowed us to identify barley thionin genes that, upon transient ectopic expression, decreased the susceptibility of a solanaceous plant species to *M. persicae*. Thionins are small peptides of around 5 kDa that contain 6-8 cysteines involved in disulfide bridge formation, which are present in endosperm and leaves of cereals and several other plant species (Bohlmann and Apel, 1991). The leaf-specific thionins in barley are encoded by a large gene family, spanning 50-100 members per haploid genome (Bohlmann et al., 1988), and can be detected in either the plant cell wall as well as inside plant cells (Reimann-Phillipp et al., 1989). Due to high levels of sequence similarity between different members of this family we were unable to assess gene expression of specific thionins in either the microarray or qRT-PCR experiments. Although in the microarray experiments we noted a consistent up-regulation of thionins, especially upon interaction with *M. persicae*, we obtained rather varying results in the validation qRT-PCR

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experiments. Possibly, differences in gene expression among different members of the thionin family detected by the qRT-PCR primers and probes could explain the observed variation. The induction of thionin expression increased beyond the timepoint selected for sampling for the microarray experiment, upon exposure to both *M. persicae* and *R. padi*. This suggests that thionin expression is not suppressed by *R. padi* during interactions with barley host plants. However, we can not rule out this aphid species may be able to suppress thionin function at the post-transcriptional level or that *R. padi* is not affected by barley thionins.

Interestingly, over-expression of thionins in various plant species implicated these peptides in plant defences against plant pathogenic fungi (Bohlmann and Apel 1991), bacteria (Hao et al., 2016, Iwai et al., 2002) and chewing insects (Charity et al., 2005). Although it is thought that their activity relies on forming pores in the cell membranes of pathogens (Pelegri and Franco, 2005), it was recently shown that an Arabidopsis cell wall thionin suppressed cell death triggered by a fungal fruit body lectin from *Fusarium graminearum* upon direct binding (Asano et al., 2013). Also, it was demonstrated that thionins can inhibit bacterial protein synthesis (Garcia-Olmedo, 1983). Our results suggest that thionins may be able to contribute to defences against phloem-feeding aphids, but the underlying mechanism remain to be elucidated. An important next step is to confirm whether these thionins contribute to defences against aphids in barley, and whether such defences are active against different aphid species that differ in their ability to infest this crop plant.



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By performing basic characterization of barley interactions with different aphid species that differ in their ability to infest, we have shown that there are most likely different types of defences that either deter aphids or impair virulence. Transcriptome analyses revealed that barley responded more strongly to *M. persicae* (poor-host interaction) compared to *R. padi* (host interaction) and identified sets of genes that were specifically activated upon interaction with *M. persicae*. From the transcriptome dataset we identified two genes, encoding thionins, which may contribute to defences against the broad host range pest *M. persicae*. By characterizing the interaction of *M. persicae* with poor-host plants such as barley we can identify plant genes that contribute to defences against this aphid pest. With the lack of crop resistance against broad host range pests such as *M. persicae*, the utilization of genes from poor- or non-host plant species that confer aphid resistance may be a promising alternative for crop improvement.

### Acknowledgements

We thank Maelle Jaouannet and Peter Thorpe for help with data analyses, and Patricia Rodriguez, Lilian Fontanet and Camille Lenoir for technical advice and help with setting up plant assays. The work was supported by an ERC Starting grant (APHIDHOST) and a Royal Society of Edinburgh Personal Fellowship awarded to JIBB.

## Competing interests

The authors declare that they have no competing interests.

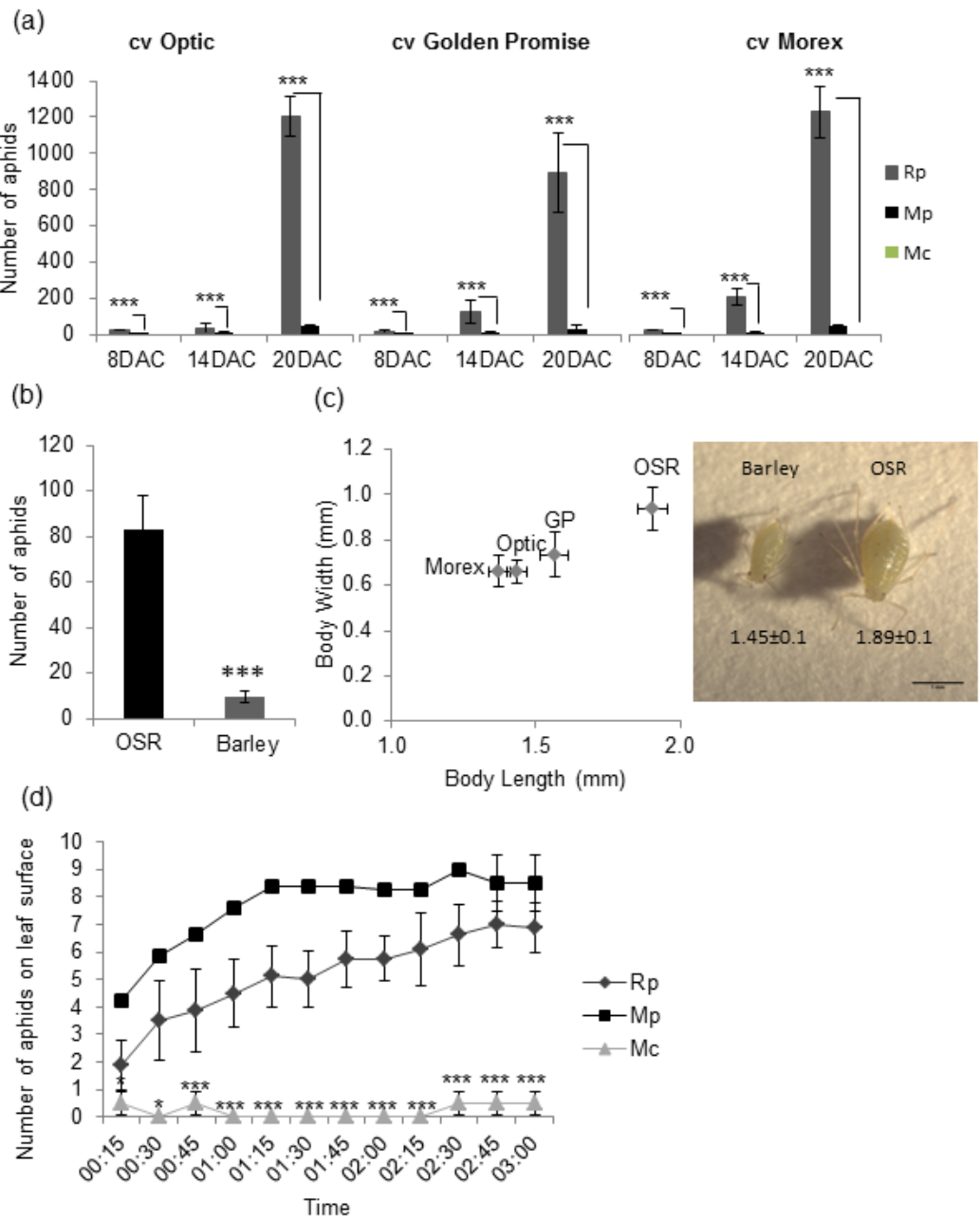
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**Figure 1. Barley colonization by aphid species *Rhopalosiphum padi*, *Myzus persicae* and *Myzus cerasi*.**

(a) Number of aphids counted on different barley cultivars (Optic, Golden Promise and Morex) 8, 14 and 20 days after challenge (DAC) of individual plants with 2 adult aphids. Error bars show the standard deviation. Triple asterisks indicate statistically significant differences, using a one-way non-

parametric test Mann-Whitney ( $p \leq 0.001$ ), between the numbers of *R. padi* (Rp) and *M. persicae* (Mp) aphids counted. *M. cerasi* (Mc) was unable to survive on barley. Seven plants per treatment and replicate were used and three independent biological replicates were performed.

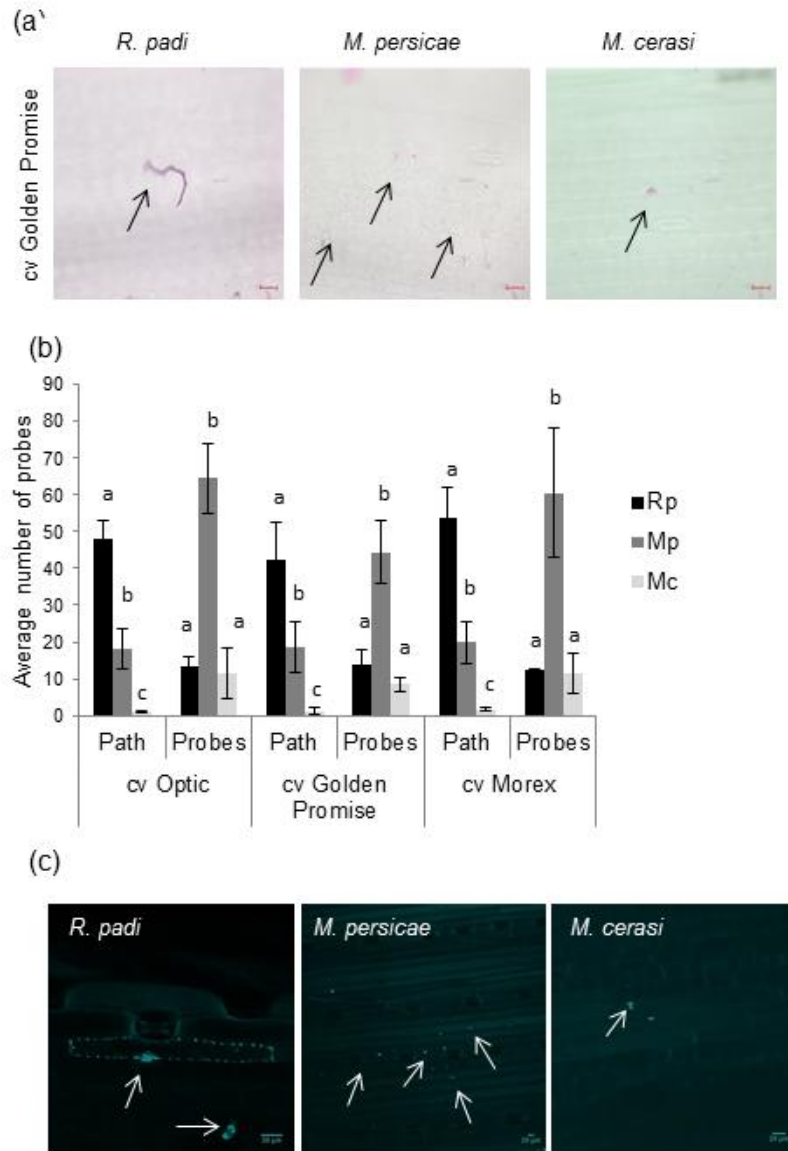
(b) Number of *M. persicae* (Mp) aphids counted on host plant oil seed rape and barley cv Golden Promise 14 days after placing 2 adult aphids on individual plants in parallel. Error bars show the standard deviation. The asterisk indicates significant differences ( $p \leq 0.01$ ) using a Mann-Whitney test. Three plants per treatment and replicate were used and three independent biological replicates were performed.

(c) Plot of the *M. persicae* (Mp) body length and width when raised on oil seed rape (OSR) and on barley cultivars Golden Promise (GP), Optic and Morex. Error bars indicate standard error. Differences were assessed by one-way ANOVA. The image on the right shows a representative individual aphid taken from the population raised on oil seed rape (OSR) and barley (cv. Golden Promise) and was taken 7 days after placing 1<sup>st</sup> instar nymphs on the different plant species. Ten 1<sup>st</sup> instar nymphs per treatment were used in each replicate and three independent biological replicates were performed.

(d) Number of aphids counted on barley leaves (cv Optic) during the first 3 hours after placing 10 aphids in clip cages to the underside of leaves. Four plants (1 clip cage per plant) were used per treatment per replicate, and three independent biological replicates were performed. Error bars indicate standard deviation of three independent replicates. Asterisks

indicate significant differences in ANOVA with Fisher's protected least significant difference post-hoc test per timepoint (single asterisk  $p > 0.05$ , triple asterisk  $p > 0.01$ ). Rp stands for *R. padi*, Mp stands for *M. persicae* and Mc stands for *M. cerasi*.



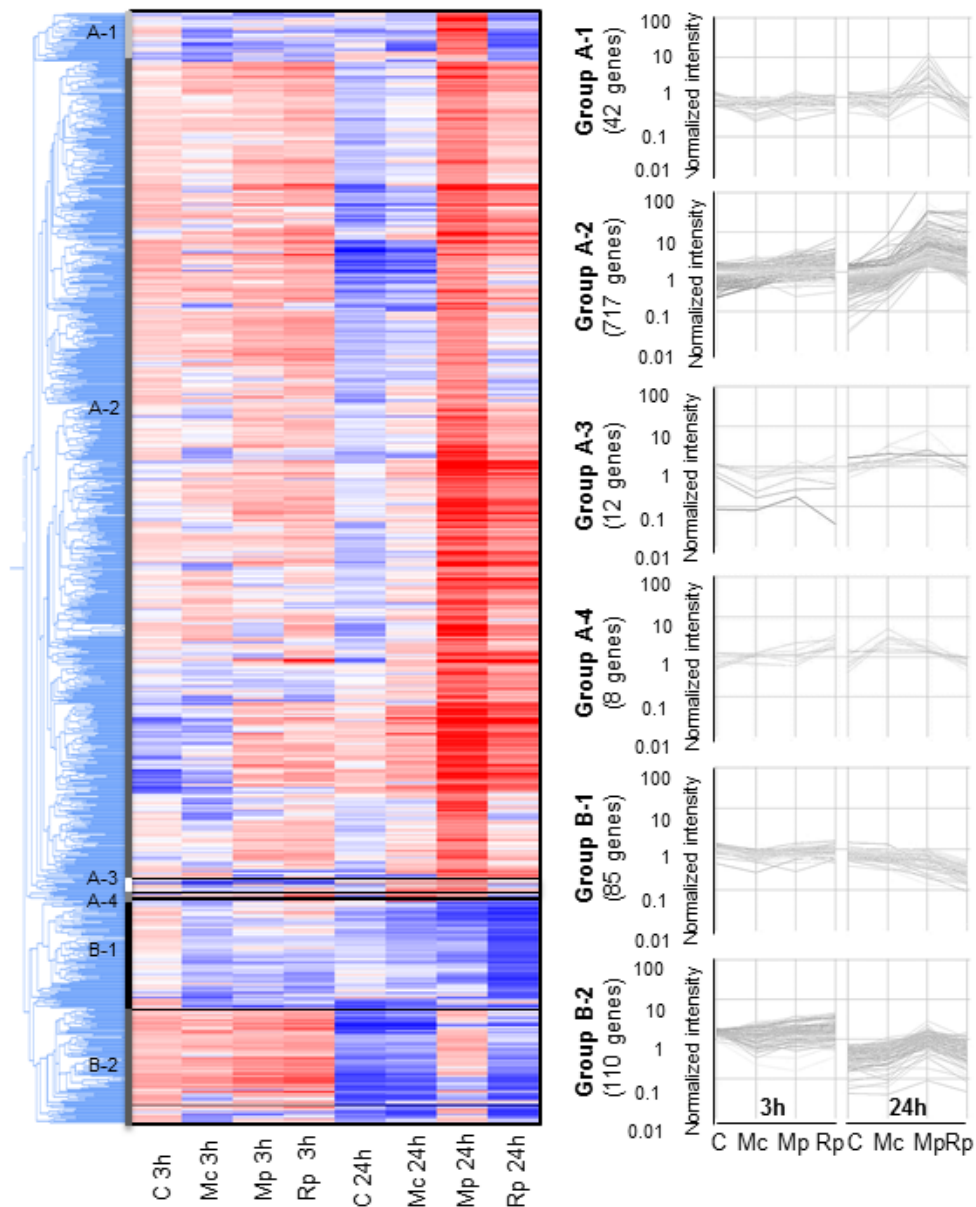


**Figure 2. Probing of barley leaf tissue by aphid species *Rhopalosiphum padi*, *Myzus persicae* and *Myzus cerasi*.**

(a) Images showing aphid probes and stylet pathways in barley cv Golden Promise two days after aphid challenge visualized by staining with acid fuchsine. Images were taken with a light microscope. The probes and stylet pathways are indicated by arrows. Scale bars are 20  $\mu$ m.

(b) Graph showing numbers of brief probes and stylet pathways as observed in Figure 1(a) for the different barley-aphid interactions. Four adult aphids per species were placed in 1 cm<sup>2</sup> leaf sample and after two days number of stylet paths or short probes was counted. Six leaf samples were used per aphid species per replicate and three independent biological replicates were performed. Rp stands for *R. padi*, Mp stands for *M. persicae* and Mc stands for *M. cerasi*. Error bars indicate standard deviation. Different letters indicate significant differences in ANOVA with Fisher's protected least significant difference post-hoc test ( $p > 0.05$ ).

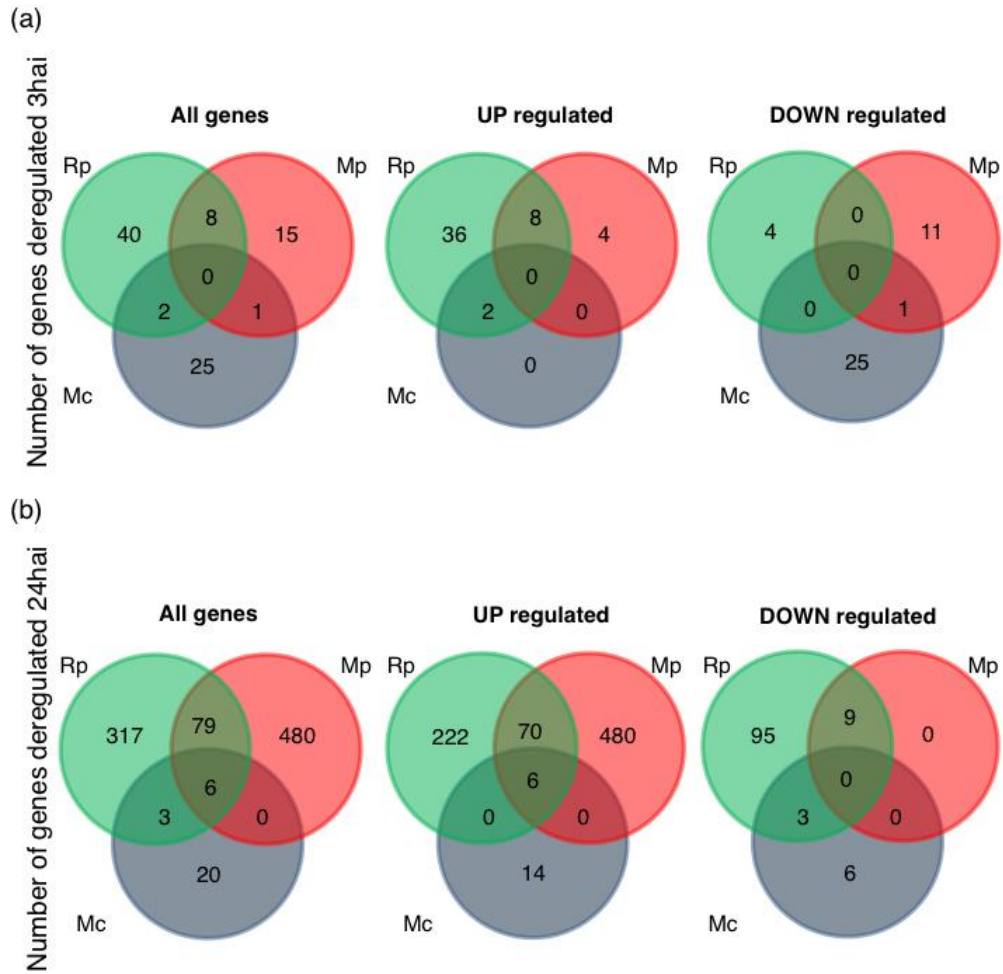
(c) Representative images showing callose deposition upon aphid probing visualized using aniline blue staining two days after aphid challenge. The sites of callose deposition after aphid stylet penetration are indicated by an arrow. Samples were visualized under the confocal microscope using a green filter (wavelength 516 nm). Six leaf samples were used per aphid species per replicate and the experiment was done in three independent biological replicates. Scale bars are 20  $\mu$ m. Additional images are shown in supplementary figure 3.



**Figure 3. Hierarchical clustering of differentially expressed barley genes among the different aphid treatments and controls.**

Significantly changing genes were identified using paired t-test comparisons (Benjamini Hochberg correction,  $p$ -value  $\leq 0.05$ ) between the aphid treatment and the corresponding no-aphid control for each timepoint. The tree represents all 974 genes differentially expressed in comparisons to the no-aphid control. Hierarchical gene tree cluster

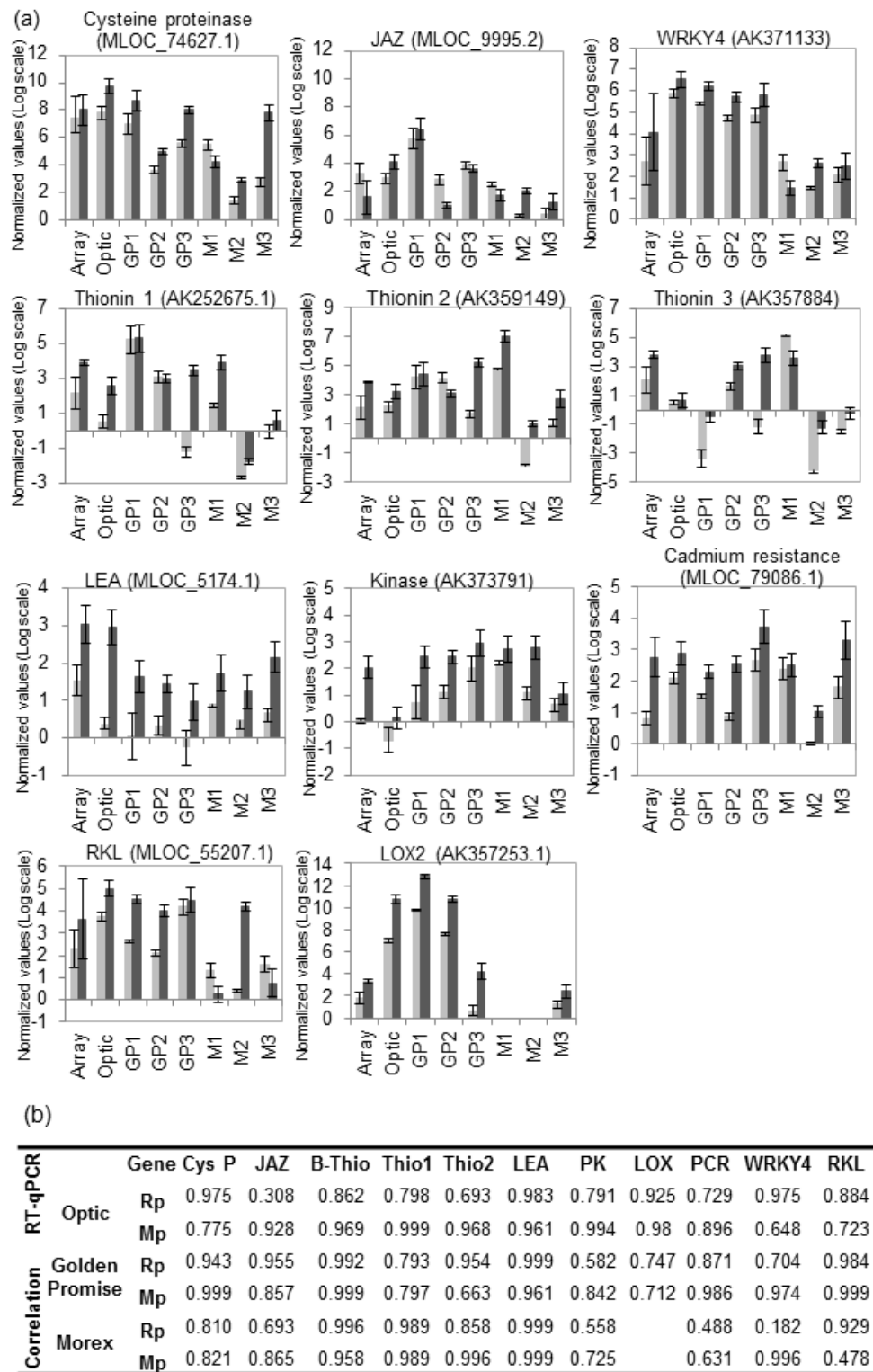
analysis of the 974 genes identified two main clusters (A and B). Cluster A was divided in 4 sub-clusters and cluster B was comprised of 2 sub-clusters. Blue color indicates low expression level and red color indicates high expression level. No-aphid control (C), *R. padi* (Rp), *M. persicae* (Mp), and *M. cerasi* (Mc) treatments are indicated.



**Figure 4. Venn diagrams showing the overlap between differentially expressed genes among different aphid interactions and timepoints.**

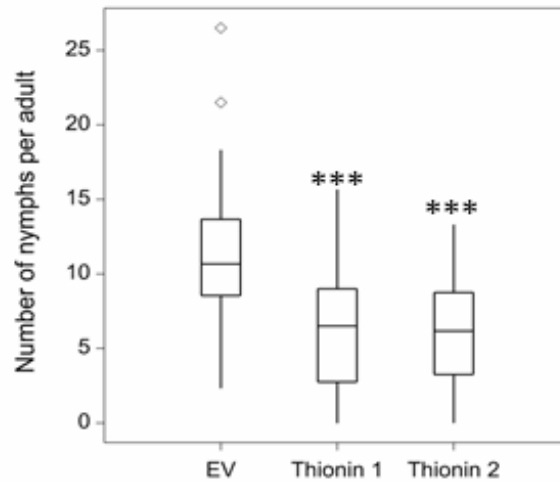
(a) Venn diagrams showing the numbers of genes differentially expressed from the no-aphid control at 3 hours after aphid challenge (paired t-test,  $p \leq 0.05$ ). Rp indicates *R. padi*, Mp indicates *M. persicae* and Mc indicates *M. cerasi*.

(b) Venn diagrams showing the numbers of genes differentially expressed from the control at 24 hours after aphid challenge (paired t-test,  $p \leq 0.05$ ). Rp indicates *R. padi*, Mp indicates *M. persicae* and Mc indicates *M. cerasi*.



**Figure 5. Validation of microarray data for selected genes using qRT-PCR across different barley cultivars.** Gene expression profiles of selected genes for validation of microarray results by qRT-PCR. (a) The

values represent the average of the Log2 (ratio =  $E_{\text{sample}}^{\Delta Ct_{\text{sample}}} / E_{\text{reference}}^{\Delta Ct_{\text{reference}}}$ , where reference genes were Actin 2, Ubiquitin and Pentatricopeptide). Array indicates the average intensity of the three replicates according the microarray results. Optic indicates the expression level within the pool of three independent biological replicates used for microarray hybridization as determined by qRT-PCR. GP1, GP2 and GP3 indicate the expression level in three independent biological replicates of barley cv Golden Promise challenged with aphids. Morex 1, Morex 2, and Morex 3 indicate the expression level in three independent biological replicates of barley cv Morex challenged with aphids. Light grey bars represent the Log2 intensity during *Rhopalosiphum padi* interaction and dark grey bars represent the Log2 intensity during *Myzus persicae* interaction. Error bars indicate standard error. (b) Correlations between the expression values obtained in the microarray experiment and the values obtained by qRT-PCR for the different barley cultivars (Optic, Golden Promise and Morex) upon interaction with *R. padi* (Rp) and *M. persicae* (Mp).



**Figure 6. Ectopic expression of two barley thionin genes in *Nicotiana benthamiana* reduces *Myzus persicae* reproduction.** Box plots showing the number of nymphs produced per adult aphid 14 days after infestation of leaf areas transiently expressing thionins or the vector control (EV). Differences between treatments were assessed by one-way ANOVA and post-hoc Fisher's test ( $p \leq 0.01$ ). EV represents the empty vector, whereas thionin 1 (AK252675.1) and thionin 2 (AK359149) indicate the selected barley thionins.