Phenotypic resistance diversity underpinned by a diverse repertoire of candidate NLR loci and genotype-specific expression patterns

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Abstract

High levels of phenotypic variation in resistance appears to be nearly ubiquitous across natural host populations. Molecular processes associated with this variation in nature are still poorly known, although theory predicts resistance to evolve at specific loci driven by selection associated with the response to pathogen. Nucleotide-binding leucine-rich repeat (NLR) genes play an important role in pathogen recognition, downstream defense responses and defense signaling. Identifying the natural variation in NLRs has the potential to increase our understanding of how NLR diversity is generated and maintained, and how to manage disease resistance. Here, we sequenced the transcriptomes of five different *Plantago lanceolata* genotypes when inoculated by the same strain of obligate fungal pathogen *Podosphaera plantaginis*. A *de novo* transcriptome assembly of RNA-sequencing data yielded 24,332 gene models with N50 value of 1,329 base pairs and gene space completeness of 66.5%, suggesting a high-quality assembly. The gene expression data showed highly varying responses where each plant genotype demonstrated a unique expression profile in response to the pathogen, regardless of the resistance phenotype. Analysis on the conserved NB-ARC domain demonstrated a diverse NLR repertoire in *P. lanceolata* consistent with the high phenotypic resistance diversity in this species. We find evidence of selection generating diversity at some of the NLR loci. Jointly, our results demonstrate that phenotypic resistance diversity results from a crosstalk between different defense mechanisms. In conclusion, characterizing the architecture of resistance in natural host populations may shed unprecedented light on the potential of evolution to generate variation.

Key words:

Phenotypic variation, pathogen-imposed selection, phenotypic resistance diversity, gene expression profile

INTRODUCTION

Parasitism is perhaps the most common life-style on Earth (Weinstein & Kuris, 2016), and parasitic species, including pathogens, play an important role in shaping biodiversity in natural populations (Kursar et al., 2009; Bever, Mangan, & Alexander, 2015). Despite this, relatively little is still understood of the molecular mechanisms that enable hosts and parasites to coexist in natural populations. The threats imposed by pathogens on humans and on managed food production systems have motivated research that aims to predict where pathogens will occur and how risks of infection evolve (Koff, 1992; Woolhouse, Taylor, & Haydon, 2001; Gilligan, 2002). Pathogens can only occur where they have susceptible hosts, and hence, resistance diversity is the key determinant of disease dynamics. Thus, our ability to understand how diversity in resistance is generated and maintained underlies our ability to predict and prevent disease emergence and epidemics. In agriculture increasing the diversity of crops - even from a monoculture to a mixture of two cultivars - has been shown to reduce disease levels significantly (Zhu et al., 2000; Mundt, 2002b). Natural host populations typically support diversity in resistance phenotypes (Salvaudon, Giraud, & Shykoff, 2008; Laine, Burdon, Dodds, & Thrall, 2011), and limited data available to date show that increasing resistance diversity decreases disease risk also in the wild (Jousimo et al., 2014a).

Hosts and pathogens are assumed to coevolve through Red Queen dynamics, where the pathogen overcomes host's defenses and the host in turn responds with new counter-defenses (Jaenike, 1978; Hamilton, 1980). Theory predicts such reciprocal coevolutionary selection to be a powerful mechanism for maintaining diversity in both host and parasite populations, as the selection rate for resistance depends on the frequency of parasite alleles, and vice versa, in a negative indirect frequency−dependent manner (Leonard, 1977; Bergelson, Kreitman, Stahl, & Tian, 2001). There are numerous examples of pathogens overcoming host resistance mechanisms, both from agriculture and from the wild (Mundt, 2002a, 2014). While evidence of resistance evolving under pathogen attack in

63 the wild is scarce (Laine, 2006), there is ample support for coevolution from local adaptation studies
64 where parasite/host fitness is measured in sympatry vs. allopatry (Greischar & Koskella, 2007;
65 Hoeksema & Forde, 2008). To date, a handful of ground-breaking studies have demonstrated that
66 fluctuations in resistance and infectivity in natural systems match the predictions of coevolutionary
67 selection (Decaestecker et al., 2007; Gómez & Buckling, 2011; Thrall et al., 2012).

68 The interaction between plants and their pathogens is mediated by complex defense
69 mechanisms having several layers. Thick and waxy cell walls form the first mechanical defense barrier
70 against pathogen invasion (Miedes, Vanholme, Boerjan, & Molina, 2014). Next, the pathogen-
71 associated molecular patterns (PAMPs) trigger the so-called PAMP-triggered immunity (PTI) response,
72 aimed at stopping the pathogen infection even before it begins. If the pathogen overcomes these first
73 two defense layers, effector triggered immunity (ETI) is initiated, involving either direct or indirect
74 recognition of pathogen virulence factors (effector proteins) (Jones & Dangl, 2006). After pathogen
75 recognition a multitude of different signaling pathways, including production of reactive oxygen
76 species, elevated Ca^{2+} and MAP kinases lead to activation of plant defenses. These defenses include the
77 induction of stress hormones salicylic acid, jasmonic acid and ethylene, as well as extensive
78 transcriptional re-programming ultimately resulting in the production of defensive compounds, such as
79 antimicrobial secondary metabolites, chemicals and enzymes. As the final line of defence plants may
80 activate the hyper-sensitive response, programmed cell death, to rapidly kill the cells surrounding the
81 infection, thus preventing the spread to nearby tissues (Coll, Epple, & Dangl, 2011; Egorov &
82 Odintsova, 2012).

83 Many of the proteins involved in intracellular pathogen recognition belong to nucleotide-
84 binding–leucine-rich repeat (NLR) protein family (Monteiro & Nishimura, 2018). They are involved in
85 recognition of the pathogen's effector proteins both directly and indirectly, as well as in triggering the
86 plant immune responses (Meunier & Broz, 2017). NLRs have also been shown to be involved in

signaling and transcript regulation (Chisholm, Coaker, Day, & Staskawicz, 2006; Jones & Dangl, 2006). Moreover, NLRs play an important role in local adaptation and habitat expansion of plants (Thrall et al., 2012; Stam, Silva-Arias, & Tellier, 2019). The antagonistic interaction between plant NLR and pathogen effector proteins is considered to have a profound effect on the evolution of both organisms, shaping their genomes and gene repertoire (Upson, Zess, Bialas, Wu, & Kamoun, 2018). NLRs usually form large tandemly arrayed gene families and hence questions regarding their origins and evolutionary history have been under active research in both plants and animals (Borrelli et al., 2018; Andolfo et al., 2019). The numbers of identified NLRs differ substantially within and between plant families (Baggs, Dagdas, & Krasileva, 2017), for example *Arabidopsis thaliana* (Arabidopsis) contains between 165 to 251 NLRs (Shao et al., 2016; Van de Weyer et al., 2019) and crop species such as wheat, barley, rice, tomato and potato contain 1560, 224, 438,137 and 309 NLRs, respectively (Sarris, Cevik, Dagdas, Jones, & Krasileva, 2016; Steuernagel et al., 2020). In *A. thaliana* there is evidence of widespread positive selection in the core NLRs shared among accessions, especially in the canonical NLR domains (Van de Weyer et al., 2019), while a pioneering study on wild tomato revealed high NLR diversity with a small subset of NLRs driving local adaptation to pathogens (Stam, Scheikl, & Tellier, 2016; Stam et al., 2019). *In planta* and bioinformatics studies have assigned specific functions to plant NLR domains. The NB-ARC domain is present in all NLRs and considered a regulatory domain (Takken, Albrecht, & Tameling, 2006) determining whether the protein is active or inactive (Takken & Goverse, 2012). Other canonical domains include Toll/interleukin-1 receptor (TIR), coiled coil (CC), RPW8-like coiled-coil (CCR), and their presence defines the sub-category of the NLR (TNL, CC, or CC_R , respectively) (Van de Weyer et al., 2019). Additionally, the NLRs contain several leucine-rich repeats (LRRs) which have evolved to detect specific pathogens.

A current key challenge in molecular ecology is to understand the role of pathogen-imposed selection on generating NLR diversity. Exploring the breadth of plant NLR natural variation

111 can increase our understanding of how NLR diversity is generated and maintained, and to establish a
112 toolbox of deployable disease resistance traits (Monteiro & Nishimura, 2018). In natural plant
113 populations, neither pathogen epidemiology nor host resistance is under human management, in
114 contrast to agricultural systems where disease is managed both via resistance breeding and fungicides.
115 Hence, natural populations can offer unique insights into the processes generating NLR diversity. Our
116 study is focused on the interaction between *Plantago lanceolata* and its fungal pathogen *Podosphaera*
117 *plantaginis*. Previous studies have detected considerable phenotypic variation in *P. lanceolata*
118 resistance against *P. plantaginis* (Laine, 2004); diversity is shown to accumulate in the well-connected
119 populations across the landscape (Hockerstedt, Siren, & Laine, 2018), and has a direct negative impact
120 on disease dynamics (Jousimo et al., 2014b). Moreover, there is evidence of on-going coevolution in
121 this interaction (Laine, 2005, 2006, 2008).

122 Here, we carried out a controlled experiment where five *P. lanceolata* genotypes were
123 inoculated with the same *P. plantaginis* strain, in order to characterize the transcriptional responses and
124 regulatory pathways activated in response to the inoculation, . We assembled a *de novo* transcriptome
125 for *P. lanceolata* and used it to characterize the transcriptional responses in both resistant and
126 susceptible phenotypes. We then studied the NLR repertoire in *P. lanceolata,* looking for signs of
127 selection among the NLRs. Reliable *de novo* assembly of NLR transcripts is difficult due to highly
128 repetitive nature of the LRR domains, and we therefore limited the evolutionary analysis on the
129 conserved NB-ARC domains. Each plant genotype demonstrated a unique gene expression profile in
130 response to the pathogen, revealing a diverse NLR repertoire in *P. lanceolata,* consistent with the high
131 phenotypic resistance diversity uncovered in earlier studies.

MATERIALS AND METHODS

Study system and plant and fungal material

Ribwort plantain, *Plantago lanceola* L., is a perennial monoecious plant that reproduces both sexually by wind pollination and clonally by producing side rosettes (Sagar & Harper, 1964). *Podosphaerea plantaginis* (Castagne; U. Braun and S. Takamatsu) (*Erysiphales*, Ascomycota) is a specialist obligate biotroph infecting *P. lanceolata.* As all powdery mildews, it requires living host tissue throughout its life (Bushnell, 2002), and completes its life cycle as localized lesions on host leaves. Infected plants suffer significant stress, and infection may increase host mortality (Laine, 2004). The interaction between *P. lanceolata* and *P. plantaginis* is strain-specific suggesting gene-for-gene type control (Thompson & Burdon, 1992; Laine, 2004, 2007). In some cases the host can mitigate pathogen reproduction; the putative resistance mechanism includes two steps, recognition of the attacking pathogen and then blocking its growth (Laine, 2004) - the following infection outcome depends on both host and pathogen genotypes (Laine, 2004, 2007).

In resistant interactions no infection develops, while in susceptible interactions there is considerable variation in pathogen development, depending on both host and pathogen genotype (Laine, 2007). An inoculation protocol where conidia from small colonies or individual chains are placed on detached leaves or intact leaves of plants yields a robust characterization of resistance-susceptibility phenotype. In resistant phenotype no pathogen growth is detected following inoculation, or the plant shows rapid cell death around inoculum source, whereas in susceptible phenotype infection is detected following inoculation. From an earlier large inoculation study consisting of 2944 host genotype–pathogen genotype combinations (Hockerstedt et al., 2018), we selected three genotypes (IDs 193_2, 2818_3 and 2818_6, named Res1, Res2 and Res3 here after) that were resistant against all tested pathogen strains, and two genotypes (IDs 313_6, 1553_5, named Sus1 and Sus2 here after) that

were susceptible to all tested pathogen strains. The selected genotypes were cloned into six plants each as described in Laine (2004).

Inoculation experiment

Two-month old plantlets (five genotypes with three replicates, total of 15 plants) were inoculated with *P. plantaginis* strain Lammi_3 by brushing spores gently with a fine paintbrush onto six test leaves and two positive control leaves. In the control set the genotypes were mock inoculated by brushing leaves without mildew spores. The treated leaves were marked with a piece of masking tape. Inoculated and mock-inoculated plant clones were placed in two separate growth chambers (Panasonic MLR-352) at 20 ± 2 °C (day) and 16 ± 2 °C (night) with 16:8 light-darkness (L:D) photoperiod, and were randomly organized to minimize potential variation in microclimatic conditions. Two inoculated or mock-inoculated leaves were collected from every plant at 24, 48 and 72 hours post inoculation (hpi), snap frozen in liquid nitrogen, and stored in glassine bags in -80 °C until RNA extraction. Positive control leaves were screened until 14 days post inoculation to confirm the plant phenotype, resistant or susceptible. Viability of spores used in the experiment was confirmed by inoculating detached leaves of a susceptible genotype.

RNA extraction

Altogether 0.2 g of frozen leaf material was ground in lysing buffer (2% CTAB, 2% PVP K-30, 100 mM Tris-HCl pH 8.0, 2 M NaCl, 25 mM EDTA), with β-MeOH (200 µl/10ml) added in prior to use (Chang, Puryear, & Cairney, 1993). Thoroughly vortexed solution was extracted twice with equal volume of acid phenol-chloroform-isoamyl-OH (ph 4.5). Prior to precipitation, 160 µl of 10M LiCl was added and samples were kept on ice overnight, followed by 30 min centrifugation (10000 rpm) in +4 °C. Pellets were dissolved in 500 µl of 65 °C SSTE (1M NaCl, 0.5% SDS, 10 mmTris-HCl pH 8.0,

1mM EDTA) and RNA was extracted twice with chloroform-Isoamyl alcohol (24:1). After EtOH precipitation and 70% wash, the pellets were dissolved in 40 µl H_2O and RNA quantity and quality were checked using NanoDrop (Thermo Fischer Scientific). Potential contamination of genomic DNA was removed using DNase I (Thermo Fischer Scientific) and samples were then reverse-transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

Selecting the time point for RNA-Seq using Quantitative real-time PCR (qPCR)

The most informative time point for RNA sequencing was selected by studying the expression of selected marker genes using qPCR. Three inoculated and mock-inoculated clones of two plant genotypes (resistant 193_2.1 and susceptible 1553_5.1) were sampled at three time points (24, 48 and 72 hours post inoculation), resulting in 12 samples. The time points were decided based on the literature, and taking into consideration that the development time of *P. plantaginis* is relatively slow compared to agricultural powdery mildews (Green, Carver, & Gurr, 2002; Laine, 2007). Primers were designed with Primer3 (Rozen & Skaletsky, 1999) based on previously *in situ* sequenced transcriptome of *P. lanceolata* (unpublished data) and known disease-induced genes in Arabidopsis. We tested seven putative disease-induced genes (Supplementary File 1). Amplification efficiencies (E) of the primer pairs were determined with five dilutions (1 : 1, 1 : 4, 1 : 24, 1 : 124, 1 : 624) of template cDNA, where $E = 10^{-1}/\text{slope}$. Three technical replicates, one water control and a plate control sample were included in a 384-well plate with 10 µL volume, using C1000™ Thermal Cycler (Bio-Rad). All samples were tested for genomic DNA contamination with -RT controls prior to qPCR. Each reaction had 1 µL of the 1:4 diluted cDNA, 5 µL of SYBR® Green containing master mix (iQ™ SYBR® Green Supermix for qPCR; Bio-Rad), 3 µL of nuclease-free water and 0.5 µL (10 µm) of each primer. The cycle conditions were one cycle at 95°C for 3 min, 40 cycles at 95°C for 10 s, 60°C 30 s, and ending with melting curve analysis. From the candidate set, Elongation factor_CL4, GADPH_28221 and Actin_34737 displayed a

stable expression across the samples with geNorm and were selected as reference genes for normalization (Supplementary File 1). Relative expression (CNRQ) and normalization was calculated in qBase+ 3.2.

RNA sequencing (RNA-Seq)

Several studies of gene expression induced by powdery mildew in host plants have found the highest number of differentially expressed genes in later time points (Li et al., 2016; Li, Dong, et al., 2019; Polonio et al., 2019). Accordingly, the qPCR demonstrated elevated levels of marker genes at time point 72 h post inoculation (hpi; Supplementary Figure 1) and was selected for RNA sequencing. Illumina paired-end sequencing (NextSeq 500) was carried out in the Institute of Biotechnology of the University of Helsinki with 78-base forward reads and 74-base reverse reads, with library insert size of 200 bases. The reads were trimmed and low quality reads were removed using Trimmomatic (Bolger, Lohse, & Usadel, 2014), resulting in an average library size of 14.6 million reads.

Transcriptome assembly

After combining all libraries, a *de novo* assembly was carried out using Trinity (Grabherr et al., 2011), SOAPdenovo-Trans (k-mer sizes 39, 41) (Xie et al., 2014) and Oases (k-mer sizes 39, 43 and 47) (Schulz, Zerbino, Vingron, & Birney, 2012). The contigs were filtered with EvidentialGene (Gilbert, 2013), and the okayset and okayalt outputs were combined and clustered using RapClust (Srivastava, Sarkar, Malik, & Patro, 2016). A representative transcript for each cluster was obtained using Lace (Davidson, Hawkins, & Oshlack, 2017). To remove contamination, the resulting contigs were queried against NCBI non-redundant protein database (Pruitt, Tatusova, & Maglott, 2007) using BLAST; only the transcripts with a best hit in plant kingdom were retained. The transcripts mapping to ribosomal

genes and having ambiguous sites (Ns) were removed. Minimum read coverage of three was used for all the assemblies.

Differential gene expression analysis

All libraries (30 in total) were mapped to the transcriptome assembly using kallisto (Bray, Pimentel, Melsted, & Pachter, 2016) with 100 bootstrap replicates. The averages of bootstrap replicates of Transcript per Million (TPM) (Li, Ruotti, Stewart, Thomson, & Dewey, 2010) values were used as counts. The count tables were imported to R by tximport package (Soneson, Love, & Robinson, 2015). Principal Component Analysis was carried out using DESeq2 and visualized with rgl package (Adler, Nenadic, & Zucchini, 2017) for 3D plot and ggplot2 (Wickham H, 2016) for 2D plots. DESeq2 package (Love, Huber, & Anders, 2014) was used for differential expression analysis at genotype and phenotype levels (Res, Sus and Res_vs_Sus), with adjusted p-value of 0.1 as a threshold for significant differential expression, as also recommended by DESeq2. To maximize the number of true positive transcripts, no fold change cut-off was used.

Redundancy analysis of the count data

Vegan package (Oksanen et al., 2018) was used for redundancy analysis (RDA). Statistical significance was tested with a permutation test (permtest) with 10,000 permutations. The genes significantly contributing to the RDA axes were identified using cut-off of three standard deviations (corresponding to two-tailed p-value = 0.0027 in Z-test). The overlap among gene sets was analyzed using venn package in R (Dusa, 2018).

Annotation and gene ontology (GO) analysis

For functional annotation of the transcripts, blastp (Camacho et al., 2009) was used to find the best match among Arabidopsis representative set of proteins (Berardini et al., 2015), available at TAIR server (ftp://ftp.arabidopsis.org/home/tair/Proteins/TAIR10_protein_lists/). Due to the low sequence similarities between Plantago and Arabidopsis, the best match was selected with no similarity cut-off. The functional annotation and gene ontology (GO) category assignment of the best Arabidopsis hit (downloaded from ftp://ftp.arabidopsis.org/home/tair/Ontologies/Gene_Ontology/) was then transferred to the *P. lanceolata* query transcript.

GO enrichment analysis was carried out using piano software (Varemo, Nielsen, & Nookaew, 2013), with $\log_2$ fold changes and false discovery rate (FDR) adjusted P-values imported from DESeq2 results. For threshold-based GO enrichments, GOAtools was used (Klopfenstein et al., 2018). To focus on signaling responses, the responses and signaling sub-branches of the Biological Process category were selected. The GO enrichments were plotted using R with fold change values obtained from the piano package. The GO enrichment of RDA loadings was carried out with piano software (Varemo et al., 2013) using RDA loadings as gene level statistics and plotted in R.

Prediction of candidate NLRs

The candidate NLRs in the reference transcriptome were predicted using NLR-Parser (Steuernagel, Jupe, Witek, Jones, & Wulff, 2015). The highest scoring domain found per reading frame per transcript was picked and screened manually. The transcripts were filtered out if the ORF was too short, if the start and stop codons were missing, or if BLAST queries did not return hits to NCBI non-redundant database. To account for partial or miss-assemblies, we performed an online search for NB-ARC domain in NCBI Web CD Search Tool (Lu et al., 2020), and selected the transcripts with a complete

NB-ARC domain. Both protein and nucleotide sequences of these domains were extracted from the NLR transcripts and used in subsequent analyses.

In order to identify the NB-ARC domains contributing to the separation of the phenotypes (resistant vs. susceptible), the complete NLR transcripts were replaced with the complete NB-ARC domains in the transcriptome and the reads were remapped using kallisto. Next, RDA analysis was carried out on NB-ARC domains with vegan package, using significance cut-off of one standard deviation. The results were visualized using vegan package. Differential expression was assessed from TPM-normalized values (Wilcoxon test with Benjamini-Hochberg correction) and results were plotted using ggplot2 in R.

For evolutionary analysis of the NB-ARC domains, *Antirrhinum majus* L. (snapdragon) (Li, Zhang, et al., 2019) was used as outgroup, since it is the most recently diverged plant where full genome assembly is available. NLR transcript prediction and extraction of transcripts with complete NB-ARC domains in snapdragon was carried out using the protocol described above for *Plantago*. Multiple sequence alignment of the complete NB-ARC domains was carried out using MAFFT, and the phylogenetic tree was estimated using RAxML version 8 (Stamatakis, 2014). Confidence was assessed with 100 bootstrap trees estimated with PROTGAMMAAUTO option. The tree was cut with ClusterPicker (Ragonnet-Cronin et al., 2013) with 90 percent initial threshold and main support threshold for clusters and genetic distance of 0.2 with gap option into clusters. For an ancestral state, the most common snapdragon protein hit among BLAST queries with the cluster sequences was selected and added to the cluster. The gene tree produced by ClusterPicker was visualized with ggtree package (Yu et al., 2017) in R. For all the analyses with NB-ARC domains, the putative Arabidopsis ortholog was selected with BLAST query of the full transcript against the TAIR database.

Neutrality test (dN/dS and H statistic)

Multiple sequence alignment of the clusters from ClusterPicker was carried out using MAFFT and gene tree was estimated with FastTree, followed by reverse-transcription of the aligned sequences. For each alignment, the dN/dS ratios (ratio between non-synonymous mutations and synonymous mutations) were calculated using PAML software (Yang, 2007), ratio >1 was used as an indicator of putative positive selection. Per base dN/dS ratios were also calculated since, due to functional constraints on conserved protein domains, it is much more likely that certain regions in a gene are under selective pressure rather than the whole gene.

Fay & Wu's H statistic (Fay & Wu, 2000) was calculated by aligning the *Plantago* reads to the reference transcriptome assembly containing NB-ARC domains with BWA (Li & Durbin, 2009) and using ANGSD (Korneliussen, Albrechtsen, & Nielsen, 2014) to calculate H statistic within a sliding window of three nucleotides. H values less than -3 were chosen to signify positive selection and more than 1 purifying selection, respectively. The H statistic along NB-ARC domains were plotted in R.

The nucleotide diversity (π) and Watterson theta (θ), were calculated by averaging the per base pi and theta from ANGSD over each transcript and over the whole transcriptome.

RESULTS

Inoculation experiment and qPCR

A schematic overview of the experiment design is shown in Figure 1a. We detected powdery mildew spores growing on the inoculated, susceptible plant clone leaves on day 14 post inoculation. None of the mock-inoculated or inoculated resistant plant clones showed visible disease symptoms at that time. Gene expression of the marker genes varied considerably in the susceptible inoculated plants and

showed elevated levels only at time point 72 h post inoculation (Supplementary Figure 1). This time point was then chosen for further analysis. The qPCR-based expression values of tested NLR transcripts showed low concordance with RNAseq expression (Supplementary Table 1).

Transcriptome assembly and expression analysis

The pooled assembly contained altogether 1,315,458 transcript models, which were then clustered using EvidentialGene pipeline into 86,648 transcripts. The resulting transcriptome was of high quality, since the Busco score of universal single-copy genes (Seppey, Manni, & Zdobnov, 2019) was 87% (including complete and fragmented genes), but the high proportion of duplicated gene models (46.3%) suggested the presence of many splice variants and allelic variants. Subsequent careful clustering and filtering (see M&M) resulted in 24,332 high quality non-redundant transcripts with an average length of 1,858 bases. The procedure reduced the Busco score to 66.5%, but clearly removed the allelic variants, as only 2% of gene models remained duplicated (Supplementary Table 2). The filtered gene models had mostly low expression counts and therefore were of low biological significance to the experiment.

On average, 75 % of the transcriptome data mapped to the *de novo* assembly (Supplementary Table 3). The mean nucleotide diversity (π) and Watterson theta (θ) were 0.068 and 0.077, respectively, over the whole transcriptome.

Principal Component Analysis (PCA) of TPM normalized gene expression data showed a clear grouping by genotype (Figure 1g) along the first three PCs. These first three PCs explained altogether 53 percent of the total variation, illustrating that genotype is the main contributor to the variation between samples. The inoculation treatment had a smaller but marked effect, as was clearly demonstrated in genotype-specific PCA plots (Figure 1 b to f). For example, in resistant R1 and R2

genotypes the variation explained by PC1 was 65 and 52 percent, respectively, and clearly separated the inoculated and control plants (Figure 1 b and c).

Differential gene expression analysis

The PCA analyses showed marked differences between the gene expression profiles of the genotypes and their responses to the inoculation. Similarly, high genotype-specific variation was observed in the differential expression between mildew inoculated and mock-inoculated plants. The R1 genotype had the highest number of differentially expressed (DE) transcripts (3803), from which about 2000 had absolute $\log_2$ fold change greater than one. On the other hand, the S2 genotype had the lowest number of DE transcripts, 43, with only 20 having absolute $\log_2$ fold change greater than one (Table 1; Supplementary Table 4).

Redundancy Analysis (RDA)

To study the effect sizes and their statistical significance, we carried out multivariate regression using Redundancy Analysis (RDA). The genotype and phenotype (resistant versus susceptible) effects were highly significant ($P = 0.001$ and $P = 0.004$), describing 35 % and 9 % of the overall variation (Table 2). The effect of inoculation alone was not significant ($P = 0.238$), but the combined effects of genotype-by-inoculation and phenotype-by-inoculation were ($P = 0.001$ and $P = 0.048$, respectively), suggesting genotype-specific response profiles. Accordingly, the RDA plots displayed clear separation when using genotype and phenotype as a covariate but not with inoculation treatment alone (Figures 2a, b and c). Venn diagrams of the genes contributing to the separation in the RDA demonstrate that while 87 genes contribute significantly to separation according to genotype, only 7 genes contribute directly to the phenotypic variation and 109 genes to the joint effect of phenotype-by-inoculation (Figures 2 d and e).

Gene ontology (GO) analysis based on differential expression

We next looked for common pathways among the differentially expressed transcripts in all plants using GO enrichment analysis of the differentially expressed genes. The contrast in expression profiles between the genotypes was also visible in the GO analysis (Figure 3; Supplementary Figure 2; Supplementary Table 5). This may be due to differences in plant defense responses or manipulation of the plant defense mechanisms by the pathogen.

To explore molecular underpinnings between susceptible and resistant genotypes, we searched for differential activation of defense response pathways by identifying the GOs with decreased average expression levels in susceptible phenotypes and elevated levels in resistant phenotypes. In resistant phenotypes, genes encoding photosynthesis-related proteins (e.g. Photosystem II antenna complex, chloroplast photosystem I/II) and NAD(P)H dehydrogenase complex had increased transcript levels (Supplementary Figure 2). This could contribute in defense against the pathogen, as it has been shown that photosynthesis plays an important role in plant defense against biotic stress (Gohre, 2015). Genes assigned to photosynthesis functions showed elevated transcript levels in susceptible phenotypes as well but not to the same extent. Chlorosis is a hallmark sign of powdery mildew infection and biotrophic fungi are known to reduce photosynthetic rate and possibly damage chloroplast structure (Perez-Bueno, Pineda, & Baron, 2019), thus the upregulation could be either compensation, plant defense mechanism or induced by pathogen. Specifically, uroporphyrinogen decarboxylase activity (GO:0004853) was upregulated in resistant phenotypes (Supplementary Figure 2, Resistant). Involved in chlorophyll biosynthesis, it also points towards acting against the chlorosis induced by the pathogen (Mock, Keetman, Kruse, Rank, & Grimm, 1998).

In both susceptible phenotypes, the GO category with most decreased expression levels was induction of programmed cell death (GO:0012502) (Supplementary Figure 2), suggesting that as a biotrophic pathogen, *P. plantaginis* may be downregulating the programmed cell death to keep the host

cells alive. However, also the resistant phenotypes showed reduced expression levels in this category, possibly due to successful manipulation by the pathogen, and therefore the comparison between susceptible versus resistant did not identify this process as significantly different between phenotypes (P=0.0559).

In addition to the shared responses, the genotypes showed individual enrichment of various disease resistance pathways (Supplementary Figure 2). In susceptible genotype 1 (S1), the processes with most decreased average expression levels were tripeptide transporter activity (GO:0042937), tripeptide transport (GO:0042939) and delta12−fatty acid dehydrogenase activity (GO:0016720), whereas S2 demonstrated decrease in Oxazole or thiazole biosynthetic process (GO: 0018131) and Low−affinity nitrate transport (GO:0080054 & GO:0080055). Fatty acids play a direct role in modulating the plant defense response to pathogens (Kachroo & Kachroo, 2009), and thiazole or thiamine has been shown to play a crucial role in activation of the defense responses, callose/lignin deposition and stomatal closure (Zhou, Sun, & Xing, 2013).

Tripeptide transport includes also nitrate transporters. Interestingly, powdery mildew causative agent *Erysiphe necator* elevates the expression levels of nitrate transporters in grapevine and Arabidopsis (Pike et al., 2014), possibly to acquire nutrients from the host. In addition to decreased levels of the GO categories related to nitrate transport in both S1 and S2, we identified homolog of Arabidopsis nitrate transporter (AtNRT1.5) to be upregulated after inoculation in susceptible vs resistant comparison. In Arabidopsis, the protein is responsible for nitrate transport from roots to shoots, and in this context suggests towards manipulation of host nutrient distribution by the pathogen. Nitrogen, nitrates and their transport to different tissues in the plant during the pathogen infection could be the “silver bullet” of the plant defense (Mur, Simpson, Kumari, Gupta, & Gupta, 2017). In general, tripeptide transport also plays an important role for defense against biotic and abiotic stress (Karim et

al., 2007), suggesting a reason for the decreased expression of the tripeptide transporters as a whole in the susceptible phenotypes.

In resistant phenotypes, the glucosyltransferase (GO:0050284) upregulation in R1 is a possible sign of early preparation for pathogen response (Le Roy, Huss, Creach, Hawkins, & Neutelings, 2016), and in R2 genotype, the activation of NADH dehydrogenase complex assembly (GO:0010258) has been shown to be involved in defense signaling (Wallstrom et al., 2014).

Gene ontology (GO) analysis based on Redundancy Analysis (RDA)

To look for biological processes differentially activated between the phenotypes or the treatments, we calculated the average RDA loadings of the genes in each of the GO categories and tested for their statistical significance. Genes contributing to the separation between inoculation and control were enriched for ABA and cytokine signaling, primary metabolism and chloroplast activity (Supplementary Figure 3). ABA induces resistance to powdery mildew in barley (Wiese, Kranz, & Schubert, 2004), and repression of ABA biosynthesis as well as genes regulated by ABA, such as cold/dehydration/salinity responsive genes, are associated with mildew resistance in nonhost plants in general (Jensen et al., 2008). Cytokinin suppresses programmed cell death and plays a role in the synthesis and maintenance of chlorophyll (Walters & McRoberts, 2006) (Supplementary Figure 3). Additionally, cytokinin levels regulate cell division together with auxin. Interestingly, in Arabidopsis, *Golovinomyces orontii* inoculation induced cell cycle related genes and endoreduplication, possibly due to increased metabolic demands of the pathogen (Chandran, Inada, Hather, Kleindt, & Wildermuth, 2010). On the other hand, Choi, Choi, Lee, Ryu, and Hwang (2011) have shown that plant based cytokinins systematically induce plant resistance against pathogens by cytokinin and salicylic acid signaling.

Genes associated with the differences between the phenotypes showed GO enrichments for kinase activity, carbohydrate metabolism, plant cell wall organization, photosystem II and response

to cold GO categories (Supplementary Figure 3), whereas the genes contributing to the differences between genotypes were enriched for tryptophan metabolism, plant cell wall and chloroplast (Supplementary Figure 3). In Arabidopsis (Chandran et al., 2010), the expression of cold/drought responsive genes were decreased together with ABA biosynthesis after inoculation with *G. orontii*. Together with the observed induction of ABA during inoculation, this suggests that the phenotypes may differ in how strongly ABA activates its targets such as cold responsive genes.

Different responses to infection are visible in the genotype-by-inoculation effect. Overall, the enriched GOs show a clear activation of defense responses in general, and defense responses to fungi in particular (e.g. regulation of immune response, regulation of defence response; Supplementary Figure 3), illustrating that the genes in these processes differ in their transcription levels between genotypes. The GO category with highest positive average of RDA loadings (and therefore, high contribution to separation) is aldose 1−epimerase activity (GO:0004034) which, may be activated because of the mechanical damage inflicted by the pathogen and results in methanol emission and priming of the non-infected leaves (Sheshukova et al. (2017). Next, hydrogen peroxide metabolic process and salicylic acid mediated signaling pathway are both well-established pathogen-induced defense mechanisms (Kuniak & Urbanek, 2000; Hua, 2009; Niu & Liao, 2016; Sheshukova et al., 2017), further demonstrating the activation of the defense processes due to the pathogen infection. The GO category with most negative average RDA loadings is RNA splicing, via endonucleolytic cleavage and ligation (GO:0000394). It is becoming increasingly clear that plants use alternative RNA splicing extensively as a means to respond to their environment and defend against pathogens (Staiger, Korneli, Lummer, & Navarro, 2013; Shang, Cao, & Ma, 2017). Within the signaling-specific GOs (Figure 4b) the genotype-by-experiment effect showed the increased transcript levels of jasmonic acid (JA) and abscisic acid signaling (as expected, (Yang et al., 2019)), again in a genotype-specific manner. Further inspection of putative orthologs of marker genes for different hormonal signaling pathways showed

increased transcript levels of auxin biosynthesis and signaling, as well as differences in the increased transcript levels of JA signaling and NLR signaling through EDS1 ortholog (Supplementary Table 6).

The most significant contributor to phenotype-by-experiment is photosystem II activity (Supplementary Figure 3), as several GO terms from this category showed significant enrichments. The GO category with highest average RDA loadings for phenotype-by-inoculation is oligopeptide transmembrane transporter activity (GO:0035673). The perception and transduction of fungal oligopeptides will trigger multiple defense responses (Nürnberger et al., 1994; Hahlbrock et al., 1995). Multitude of photosynthetic processes were also enriched; their role in defense was discussed above. The categories with most negative average loadings were response to fungus, and cytokinin biosynthetic process (GO:0009691).

NLR transcripts

To look for the variation in the plant defense arsenal we carried out an in-depth study of the resistance NLR genes induced in the experiment. Due to highly repetitive nature of the LRR domain that causes problems in *de novo* assembly from short-read RNA sequencing data, we focused the analysis on the conserved NB-ARC domains. From the 543 candidate NLR transcripts in the full transcriptome, 210 had a complete NB-ARC domain. The inoculation did not have a significant effect on expression levels in RDA analysis of NB-ARC domains ($p = 0.13$), but the genotype and phenotype both contributed significantly ($p = 9.999e-05$ and $P = 4e-04$), explaining 55% and 15% of the variation, respectively (Supplementary Figure 4). Based on RDA loadings, the NLR transcript with highest contribution to resistance phenotype was transcript2322. A BLAST query against Arabidopsis revealed this to be homolog of AtRPP13 gene. RPP13 has the highest amount of amino acid diversity in Arabidopsis and is involved in defense against *Peronospora parasitica* (Rose et al., 2004; Hall et al., 2009), an oomycete causing downy mildew in Brassicaceae. Gene expression analysis of transcript2322 in

resistant versus susceptible phenotypes revealed that the transcript is not differentially expressed due to inoculation, but it has significantly higher base expression level (p-value = 0.0003996) in resistant phenotypes (Supplementary Figure 4).

Clustering of the NB-ARC domains resulted in 47 clusters containing 179 sequences and 31 singletons (Figure 5). Cluster 4 with 12 sequences had the highest number of sequences. BLAST query against Arabidopsis protein database for the longest transcript in this cluster returned a hit to AT3G14460, a leucine rich repeat protein that also contains an adenylate cyclase catalytic core motif. This gene is involved in adenylyl cyclase activity and signaling and its knockouts in Arabidopsis have compromised immune responses to the biotrophic fungus *Golovinomyces orontii* (Bianchet et al., 2019).

Neutrality test (dN/dS and H statistic)

To look for NLR clusters under positive selection, we analysed dN/dS, the ratio between non-synonymous (amino acid changing) to synonymous mutations (Figure 5). None of the NLR transcript clusters had an ω value greater than one, which would indicate positive selection. However, site-wise analysis of dN/dS revealed that 25 of the clusters contained a varying number of one to 58 amino acid positions under positive selection, based on Bayes Empirical Bayes (BEB) analysis (P>95%). Cluster 14 with the highest number of loci under selection returned Arabidopsis NLR protein AT1G50180 (CAR1) as the best BLAST hit, an immune receptor which recognizes the conserved effectors AvrE and HopAA1 (Laflamme et al., 2020).

In order to investigate potential selection pressure by a complementary method, considering the shortcomings of within population dN/dS analysis (Kryazhimskiy & Plotkin, 2008), we also calculated Fay & Wu's H statistics on the NLRs using NB-ARC domains for mapping the reads. A

positive value of H indicates balancing or purifying selection, whereas high negative values indicate positive selection in the form of selective sweeps, or drift, for example from population bottlenecks.

We identified 27 NLR transcripts with regions having H statistics less than -3 (Figure 5; Supplementary Figure 6; Supplementary Table 7). This set included one gene from the cluster with the highest number of loci under selection based on dN/dS analysis, as well as the transcript2322 having significantly elevated expression levels in the resistant vs susceptible comparison. BLAST query of the NB-ARC domains under selection against TAIR database resulted in 16 hits to RPP13 and 3 hits to CAR1 (Supplementary Table 7).

DISCUSSION

Given that pathogens are prevalent across all ecosystems, an individual's reproductive success and survival depend on its ability to resist infection. Natural host populations have been shown to support considerable diversity in resistance (Salvaudon et al., 2008; Laine et al., 2011), and theory predicts that this variation is maintained by pathogen-imposed selection. However, empirical support for the role of selection in generating resistance diversity still scarce. With recent advances uncovering the molecular underpinnings of resistance, it is becoming increasingly feasible to study resistance also in non-model systems.

Gene and pathway expression patterns reveal genotype specific responses to pathogen inoculation

Here, we established a high-quality *de novo* transcriptome assembly of *P. lanceolata* to investigate the gene expression and processes activated in different plant genotypes in response to inoculation of the same pathogen strain. In our study, all five plant genotypes showed unique gene expression patterns. This was clearly demonstrated in the principal component analysis showing clustering by genotype,

while the inoculated and mock-inoculated replicates remained in the same cluster. Significant variation in gene expression patterns among plant genotypes has also been discovered in other studies (Burghardt et al., 2017; Muller, Kersten, Fladung, & Schroeder, 2019). In the redundancy analysis (RDA), inoculation explains only 4 % of the total variation, while genotype-by-inoculation interactions contribute 46 %, suggesting that the genotypes have highly unique responses to pathogen attack. While such genotype specificity may be expected between susceptible and resistant genotypes, the split to resistant versus susceptible phenotypes explains only 9 % of the variance, with considerable expression pattern differences between phenotypes. Overall, the plant genotypes differ by the number, fold change and the function of the transcripts differentially expressed in response to the pathogen. Furthermore, even though the gene expression shows the known induction of JA, SA and ABA signaling pathways, they also show highly varying activation patterns with JA and ABA significantly contributing to the genotype by experiment differences. This suggests that plant genotypes have different strategies in response to the same pathogen and have variation in the extent of activation of signaling pathways, which could be an important mechanism generating phenotypic resistance diversity. One possible explanation for the diverse responses is the extremely high genetic variation within the species; overall, the transcriptome had very high Watterson θ=0.068 and nucleotide diversity π=0.077, suggesting effective population sizes in the order of millions. The high genetic diversity where, on average, eight nucleotides out of 100 differ between any two individuals, is likely manifested also in the diverse responses. The experimental take home message is that including multiple genotypes in experiments and avoiding pooling for RNA-Seq is essential to uncover variation relevant for phenotypic differentiation.

Despite genotype-specific responses, the pathways commonly induced by the pathogen were visible in the gene expression data, including the induction of specific nitrate transport genes in

susceptible phenotypes as well as elevated expression of photosynthesis-associated genes and related biological processes taking part in chloroplast in all genotypes. Powdery mildew fungi have a contracted carbohydrate metabolism, for example they are not able to degrade pectin, an essential component of plant cell walls (Liang et al., 2018), whereas the lipid metabolism is intact, suggesting that their main source of energy is from lipids. Chlorosis is another hallmark sign of a successful pathogen attack. In our results, elevated expression of specific nitrate transporters as well as chloroplast processes in general suggests elevated chlorophyll biosynthesis. Together, this suggests that, at least at the early stage of infection, *P. plantaginis* may target the chloroplast lipids of *Plantago* to obtain its nutrients. However, more molecular work is needed to truly understand the photosynthetic response of the *Plantago* when infected by *P. plantaginis.*

Discovery of a diverse repertoire of NLRs in *P. lanceolata*

NLRs play an important role in pathogen recognition and downstream defense responses, defense signaling, as well as activation of hyper sensitive response (Monteiro & Nishimura, 2018). In our study, a combined transcriptome of five different *Plantago* genotype NLR repertoires contained 543 NLR isoforms, out of which 210 transcripts contained a complete NB-ARC domain. A majority of these transcripts were expressed to some extent in all five plant genotypes. Presence-absence polymorphism in a subset of NLRs has been demonstrated across Arabidopsis accessions (MacQueen et al., 2019; Van de Weyer et al., 2019), and hence it could contribute to the slight differences in the numbers of NLRs detected in the genotypes.

The NLR transcripts with a complete NB-ARC domain divided into 47 clusters of varying sizes, with 12 transcripts in the largest cluster. We found considerable variation in the branch lengths among clusters, which could indicate different evolutionary rates (Tucker, Ackerman, Eads, Xu, & Lynch, 2013). Indeed, NLR genes are among the fastest evolving gene families in plants. They

often form tandemly arrayed gene clusters, and this is believed to be critical for the fast pace of their structural and functional diversification (Michelmore & Meyers, 1998; Meyers, 2003). Frequent homologous recombination events and errors produced during the process, followed by diversifying selection, may generate the structural diversity needed to match high effector evolution rates in the pathogens (McDowell & Simon, 2006; Jacob, Vernaldi, & Maekawa, 2013). NLR genes are also under evolutionary pressure resulting from inappropriate activation of cell death. If the plant cannot control NLR-activated cell death, it leads to decreased fitness (Phadnis & Malik, 2014). In particular, we found multiplication in the number of homologs of Arabidopsis RPP13, a gene which is involved in defense against downy mildew (*Peronospora parasitica*) in Arabidopsis, as well as other defense processes and signaling (Bittner-Eddy, Crute, Holub, & Beynon, 2000; Rentel, Leonelli, Dahlbeck, Zhao, & Staskawicz, 2008), and one of these homologs showed different expression patterns in resistant vs susceptible comparison. While none of the clusters had significant dN/dS values, we found between one and 58 loci under selective pressure in 25 of the clusters. The cluster with the highest number of loci under selection, cluster 14, has been suggested to be involved in recognition of the conserved effectors AvrE and HopAA1 (Laflamme et al., 2020) based on Arabidopsis orthologues. The H statistic identifies the same transcripts as the dN/dS analysis (18 transcripts), plus four other NLR transcripts that may have been under putative selection pressure. Again, the homolog of RPP13 showing high expression values in resistant phenotypes was among the genes putatively under selective sweeps.

Overall, we find that the NLR transcripts are differentially expressed in response to the pathogen treatment, and that this response varies according to genotype. Transcripts of many NLR genes are known to accumulate in response to defense induction or related stimuli (Lai & Eulgem, 2018). For example, 75 of the 124 studied Arabidopsis NLR genes were found to exhibit at least two-fold higher transcript levels in response to one or more of the 15 implemented defence-related

580 treatments (Mohr et al., 2010). Up−regulation of NLR transcripts after defence induction has also been
581 observed in other plant species, such as wheat, *Brassica rapa*, soybean and rice (Ribot et al., 2008;
582 Brechenmacher et al., 2015; Chen, Pang, Chen, Zhang, & Piao, 2015; Steuernagel et al., 2020). While
583 we were more likely to observe up-regulation of NLR expression levels in response to the pathogen
584 treatment, this was not consistent across transcripts and genotypes. This is in line with recent studies on
585 crop plants testing different genotypes in response to pathogen infection (Sari, Bhadauria, Vandenberg,
586 & Banniza, 2017; Sari et al., 2018; Cruz-Miralles, Cabedo-Lopez, Perez-Hedo, Flors, & Jaques, 2019).
587 Plants have evolved mechanisms to stabilize their basal expression levels, and to reduce the fitness
588 costs of an overexpressed immune response that could have more deleterious effects on plant fitness
589 than the infection (Fei, Xia, & Meyers, 2013). This may explain the down-regulation of some of the
590 NLR transcripts we observe in both susceptible and resistant phenotypes. Future studies are needed to
591 determine how sensitive the detection of NLRs, and their expression patterns are to the sampling time
592 which in our study was 72 h post inoculation.
593

594 **Conclusions**

595 Our results are well in line with the extensive phenotypic variation and highly strain-specific disease
596 resistance measured in *P. lanceolata* in earlier studies (Jousimo et al., 2014a; Hockerstedt et al., 2018).
597 High levels of variation in resistance seems to be nearly ubiquitous across natural host populations that
598 experience pathogen-imposed selection without any human interference, in contrast to agricultural
599 systems (Salvaudon et al., 2008; Laine et al., 2011). We show that phenotypic resistance may be
600 generated by different mechanisms. First, we discovered a large repertoire of candidate NLRs in *P.*
601 *lanceolata.* We also find evidence of selection generating diversity in a subset of the identified NLRs.
602 Moreover, we discovered that the genotypes have unique expression profiles in response to pathogens,

603 a mechanism which may further contribute to phenotypic variation. Indeed, this high level of genetic
604 and expression profile diversity may be the key to successful defense against pathogens in sessile
605 plants that lack a long lasting immune memory (Hall et al., 2009; Roux & Bergelson, 2016). Finding
606 different mechanisms that contribute to phenotypic resistance is nontrivial, given how effectively this
607 variation may be utilized to predict and control disease epidemics (Mundt, 2002a). Moreover,
608 resistance in agricultural crops is highly prone to breakdown following pathogen adaptation, and for
609 many commercially important pathogens, the known effective resistance genes are becoming limited.
610 Wild plant populations are currently identified as the most promising source of genes required for
611 development of sustainable agriculture (Fu et al., 2019). In conclusion, characterizing the architecture
612 of resistance in natural host populations may yield unprecedented light on the potential of evolution to
613 generate variation, and it can have broad and long-lasting impacts in our food production environments.

614

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Data Accessibility

We have submitted the raw reads and the transcriptome in NCBI under the bioproject "Phenotypic resistance diversity underpinned by a diverse repertoire of candidate NLR loci and genotype-specific expression patterns" Accession: PRJNA636383 ID: 636383.

Author Contribution

A-LL, JS, PS and LH conceived the ideas and JS, PS, LH, MB and A-LL designed experiment. LH conducted the experimental work and PS, JS and LH analyzed the data. PS, JS and A-LL led the writing of the manuscript; all the authors contributed to the drafts and gave final approval for publication.