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Pea transcriptional and phytohormonal responses to adapted and non-adapted aphid biotypes at early stages of infestation

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Pea (*Pisum sativum* L.), a major legume crop, is affected by various parasites including the pea aphid (*Acyrthosiphon pisum* Harris). The pea aphid is composed of multiple biotypes, each one being able to feed and reproduce on one or a few legume species. To understand the pea defense mechanisms to a pea adapted and a non-adapted *A. pisum* biotype, we studied the early molecular responses of four pea genotypes with contrasted levels of resistance, which are controlled primarily by the *ApRVII* locus. We found that major defense-related phytohormones and their derivatives in pea did not show clear response to aphid infestations. Transcriptomic analyses showed that the number of differentially expressed genes (DEGs) increased over time in pea genotypes infested with pea-adapted aphids, while significantly fewer DEGs were detected in genotypes infested with non-adapted aphids. The most resistant of the four investigated pea genotypes showed the fewest DEGs to both aphid biotypes. Aphid infestation of the three other pea genotypes commonly induced down-regulation of various pathways involved in fundamental biological processes. Comparison of the transcriptional data of pea genotypes identified candidate genes potentially involved in the aphid resistance conferred by *ApRVII*.

Keywords *Pisum sativum* L., *Acyrthosiphon pisum* Harris, Plant-aphid interactions, Resistance, Transcriptomics, Phytohormones

Crops face various environmental stresses including those from pests and pathogens. In conventional agriculture, crop pests have long been controlled using pesticides that are harmful to the environment and leads to the emergence of resistances in the pest organisms[1]. Thus, it is necessary to develop alternative methods of pest control. Aphids are sap-feeding insects that cause significant damage to many crops, directly by feeding on the plant and indirectly by transmitting phytopathogenic viruses[2]. Aphids divert phloem sap by puncturing sieve elements with a protruding mouthpart, the stylets. During the various stages of feeding, aphids release saliva into the plant cells changing biological processes of the host plants[3,4]. Understanding these processes and mechanisms of plant resistance to aphids is an essential step to develop aphid control strategies[5].

Interactions between aphids and host plants are distinct from plant-microbial pathogen interactions because aphids locate and select their host plants[6]. Nevertheless, direct plant-aphid interactions can be partially explained by using the concept developed in plant-pathogen interactions[4]. Plant immunity may be triggered by recognition of herbivore-associated molecular patterns (HAMPs), such as GroEL derived from aphid bacterial endosymbionts and present in the aphid saliva[7]. Conversely, the induced defense can be suppressed by aphid salivary proteins that function like effectors of microbial pathogens. In some cases, effectors are recognized by

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specific plant proteins, triggering strong resistance reactions. These mechanisms often rely on resistance genes, such as NLR (Nucleotide-binding site and Leucine-rich Repeat) genes, which encode intracellular receptors that are involved in the recognition and signaling of specific parasite-derived effectors and often trigger a hypersensitivity reaction (HR). Finally, some effectors can suppress the defense triggered by NLRs[8,9]. Hence, the plant genes involved in the recognition of aphid proteins and the aphid effector gene repertoire are important factors determining the compatibility of plant-aphid interactions[5].

An interaction is considered incompatible when a plant prevents aphids from establishing a feeding site, developing and reproducing[5]. The mechanisms underlying this incompatibility are diverse including the presence of deterrents or the absence of feeding stimulants, the presence of toxic compounds that aphids cannot detoxify or sequester, the composition of phloem sap that lacks essential nutrients, the presence of physical barriers, and sieve-element occlusion[5]. Non-host resistance refers to such incompatibility where all genotypes of an aphid species fail to establish successful interactions across all genotypes of a given plant species[10]. In this case, the aphid is regarded as non-adapted to that plant species. Conversely, when aphids reproduce successfully, the interaction is compatible, and the aphid is considered adapted to the host plant. Thus, non-host resistance on the plant side and host adaptability on the insect side represent two interconnected aspects of the interaction. Finally, within compatible interactions, some plant genotypes exhibit higher levels of resistance than others. This is referred to as host resistance[11].

Despite an increasing number of studies, the main pathways and factors determining host and non-host resistance to aphids are often unclear. Transcriptomic studies have been conducted for different plants infested with aphids. For example, *Arabidopsis thaliana* (L.) Heynh infested with *Myzus persicae* Sulzer (host interaction), *Myzus cerasi* Fabricius (poor-host interaction) and *Rhopalosiphum padi* Linnaeus (non-host interaction) showed overlapping but distinct sets of DEGs. Notably, the host interaction induces a higher number of DEGs than the non-host or poor-host interaction. Genes involved in reactive oxygen species (ROS) metabolism were repressed more strongly in host interaction than non-host or poor-host interactions[12].

Transcriptomic studies have also examined interactions underlying host resistance. For example, an analysis of resistant and susceptible near-isogenic lines (NILs) of cowpea [*Vigna unguiculata* (L.) Walp] infested with the cowpea aphid (*Aphis craccivora* Koch) revealed a comparable number of DEGs in the susceptible and resistant NILs. However, the transcriptional reprogramming of the resistant NIL was induced slower than the susceptible one, leading to the hypothesis that the aphids actively manipulate susceptible cowpea[12]. In resistant cowpea plants, aphid infestation repressed the phenylpropanoid metabolism and abiotic stress responses, while prolonged feeding increased expression of genes related to protein phosphorylation and stimulus response and repressed processes related to lipids, carbohydrates, and photosynthesis[13]. In contrast, a study on peach [*Prunus persica* (L.) Batsch] genotypes infested with *M. persicae* showed a higher number of induced DEGs in the resistant genotype[14]. The resistant genotype exhibited a massive response, characterized as extensive activation of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). This response included production of necrotic flecks, accumulation of salicylic acid (SA) and the activation of ROS, indicative of HR. In these two studies, each interaction showed specific plant responses to the aphids indicating that the plant pathways involved in defense to aphids cannot be generalized. These findings highlight the importance of studying interactions in each crop individually.

Pea (*Pisum sativum*) is a legume crop of major interest due to its high protein content and environmental value in low-input cropping systems[15]. Yet, pests like the pea aphid (*Acyrthosiphon pisum* Harris) cause significant yield losses[16]. This aphid species forms a complex of at least 15 biotypes that are genetically distinct, and each of the biotypes develops on one or a few specific legume species[17,18]. For example, all the *A. pisum* genotypes belonging to the alfalfa biotype feed and develop well on alfalfa (*Medicago sativa* L.) but cannot develop and reproduce on pea[5,19,20]. Consequently, the resistance of certain legumes to specific *A. pisum* biotypes can be regarded as non-host resistance; in this context, pea is considered a non-host for the alfalfa biotype of *A. pisum*. Salivary effector proteins of *A. pisum* are assumed to play a key role in determining the host specificity, and their composition differs between *A. pisum* biotypes[21,22]. However, the molecular mechanisms controlling host specificity are not known[21,22].

It has been shown that aphid infestation induces the production of phytohormones such as SA and jasmonic acid (JA) in some plants[23,24]. In certain interactions, these phytohormones are linked with increased resistance to aphids[25–27], but their roles and importance in aphid resistance are system-specific. Abscisic acid (ABA) promotes early defense responses to microbial pathogens, leads to stomata closure and stimulates callose deposition[28]. Although ABA levels were shown to decrease after aphid infestation in some legumes, its role in aphid resistance remains to be clarified[24]. In a study on a single pea genotype, no discernible pattern was identified for the phytohormones regulation in response to *A. pisum* pea, alfalfa and clover-adapted biotypes[24]. In contrast, another study showed the induction of both JA and SA in pea infested with *A. pisum* pea biotype from 24 to 96 h[29]. Given these discrepancies, it is important to reassess phytohormone involvement using different pea genotypes and aphid biotypes, especially at early timepoints that have not been carefully assessed previously.

Previously, we have screened 240 *Pisum* genotypes and showed different levels of resistance to *A. pisum* pea and alfalfa biotypes[30]. The *A. pisum* pea biotype reproduced with varying performances on all tested pea genotypes, but partial resistance (host resistance) was observed on a subset of them. On the contrary, the alfalfa biotype did not reproduce on most of the pea genotypes (non-host resistance), but partial susceptibility was observed on some of them. The resistance levels of the pea genotypes to the two different aphid biotypes were correlated, and a Genome-Wide Association Study (GWAS) identified the *ApRVII* locus controlling pea resistance against both biotypes[30]. Consistent with the absence of NLRs at *ApRVII*, no HR-like reaction was observed in the resistant genotypes carrying the *ApRVII*. A behavioral study of the aphids on pea genotypes showed that the alfalfa biotype inserted its stylets into pea plants, spent comparable time as the pea biotype trying to reach phloem, and some individuals managed to reach the phloem of pea and salivate. However, it

spent much shorter time on feeding compared to the pea biotype[20]. Pea resistance conferred by *ApRVII* did not affect the feeding behavior of the pea biotype but reduced the probing time of the alfalfa biotype, suggesting that *ApRVII*-mediated pea resistance mechanisms to these two biotypes may be different[20].

This study investigates early pea responses to adapted and non-adapted aphid biotypes by analyzing transcriptomic and hormonal changes in four pea genotypes with contrasting level of resistance. We also aimed to identify the molecular responses that might lead to the resistance conferred by *ApRVII* against the two biotypes. We hypothesized that the pea genotypes would show different transcriptional and hormonal responses to the *A. pisum* biotypes and that the *ApRVII* locus may control the activation of specific defense pathways.

Results

No clear pattern emerged in phytohormone production in peas infested with aphids

Four pea genotypes with contrasting aphid resistance phenotypes[30] were chosen for this study. Those were AeD99OSW-49-5-7, CE101 = FP (hereafter, AeD and FP, respectively), Cameor and Cherokee. To independently confirm their resistance or susceptibility, we conducted a new fecundity test for the two aphid biotypes, with a high number of biological replicates ($n \geq 27$). The analyses showed that AeD and Cameor remained partially resistant to the *A. pisum* pea biotype P123 while Cherokee and FP remained susceptible (Supplementary Figure S1). Regarding the resistance to the alfalfa biotype LSR1, AeD and Cameor were completely resistant while Cherokee and FP were partially susceptible (Supplementary Figure S1). The data confirmed the results of the previous study[30].

To investigate defense-related signaling response, we quantified the levels of three phytohormones and their derivatives, SA, salicylic acid glucose conjugate (SA-Glu), ABA, JA-Ile and hydroxylated jasmonic acid (OH-JA), reported to be involved in responses to aphid infestation in previous studies[23–27,29] (Supplementary Figure S2, Supplementary Table S1). While some genotype-specific and timepoint-specific differences were statistically significant (Supplementary Table S1), no consistent pattern of phytohormone accumulation was observed across genotypes or aphid biotypes (Supplementary Figure S2, Supplementary Table S1).

The pea adapted aphids induced higher number of DEGs than the non-adapted aphids

To investigate the pea's transcriptional response to *A. pisum* infestation, an RNA sequencing analysis was conducted using three of the six biological replicates used for the phytohormone quantification. We generated 108 libraries with a median of 49.5 million reads and high base-call quality (Phred score > 36.16%; Supplementary Table S2). On average, 93.93% of the paired reads mapped to the pea reference genome, Cameor[31], and 50.46% of these paired reads mapped once to the reference genome, while 43.47% were multi-mapped and 6.07% were unmapped (Supplementary Table S2). Only once-mapped paired reads were analyzed further. The samples from the AeD, Cherokee, and FP genotypes had a mean of 93.84%, 92.86% and 93.32% of paired reads mapping to the Cameor genome, respectively, while samples from Cameor showed a mean of 95.70%. Mapping efficiency was consistent across genotypes, supporting the continued use of the Cameor reference genome. Principal component analysis revealed a strong clustering of the samples by pea genotype, indicating genotype-specific gene expression patterns (Fig. 1). Partial least squares discriminant analysis, followed by permutation tests, showed significant differences between genotypes at each timepoint (p-values = 0.001). For each timepoint, a comparison was conducted between conditions of infestations, and significant differences were only observed at 72 hpi between non-infested control and P123-infested samples (p-value = 0.001) and between LSR1-infested and P123-infested samples (p-value = 0.003). When focusing on the interaction Genotype: Condition at 72 hpi, significant differences (p-values = 0.1) were observed in the comparisons between non-infested and P123-infested samples and between LSR1-infested and P123-infested samples, for the genotypes Cameor, FP and Cherokee (Supplementary Table S3).

After filtering, 23,093 genes were defined as expressed. Across all contrasts (control vs. infested per genotype and timepoint), 6,044 genes were identified as differentially expressed (Supplementary Table S4). Infestation with P123 induced a markedly stronger transcriptional response (6,038 DEGs) than infestation with LSR1 (22 DEGs) (Table 1). At 3 hpi (hours post infestation), one DEG was detected during infestation with LSR1 and none with P123. At 24 hpi, 283 and three DEGs were identified during the infestation with P123 and LSR1, respectively. At 72 hpi, 5,986 and 18 DEGs were detected during the infestation with P123 and LSR1, respectively. Genotype AeD showed very little transcriptomic response to the infestation of both aphids, while Cameor, FP, and Cherokee responded strongly to P123 infestation compared to LSR1.

The weak pea transcriptional response to the non-adapted aphids constitutes a core transcriptional response of pea to *A. pisum* infestation

In total, 22 DEGs were identified in the four pea genotypes infested with LSR1 (Table 1; Fig. 2). The pea response increased slightly at 24 and 72 hpi (Table 1), although no DEGs were shared across timepoints. The gene encoding an O-methyltransferase (*Psat0s1479g0040.1*) was the only gene commonly up-regulated in two genotypes after LSR1 infestation. Among the 22 DEGs, six genes were specifically regulated after LSR1 infestation, while the remaining 16 genes were differentially regulated in response to both LSR1 and P123 infestations and had the same direction (up or down) of regulation (Fig. 2). This overlap indicates that these genes represent a core transcriptional response of pea to *A. pisum* infestation, regardless of the aphid biotype.

The adapted aphids induced parasite response pathways and repressed various biological processes in pea

At 3 hpi, no DEG was identified in the pea genotypes infested with P123. However, the pea response increased sharply at 24 and 72 hpi (Table 1; Fig. 3). Many of these DEGs were pea genotype-specific, but some were shared among genotypes (Fig. 3). Only three DEGs were identified as commonly down-regulated in all four

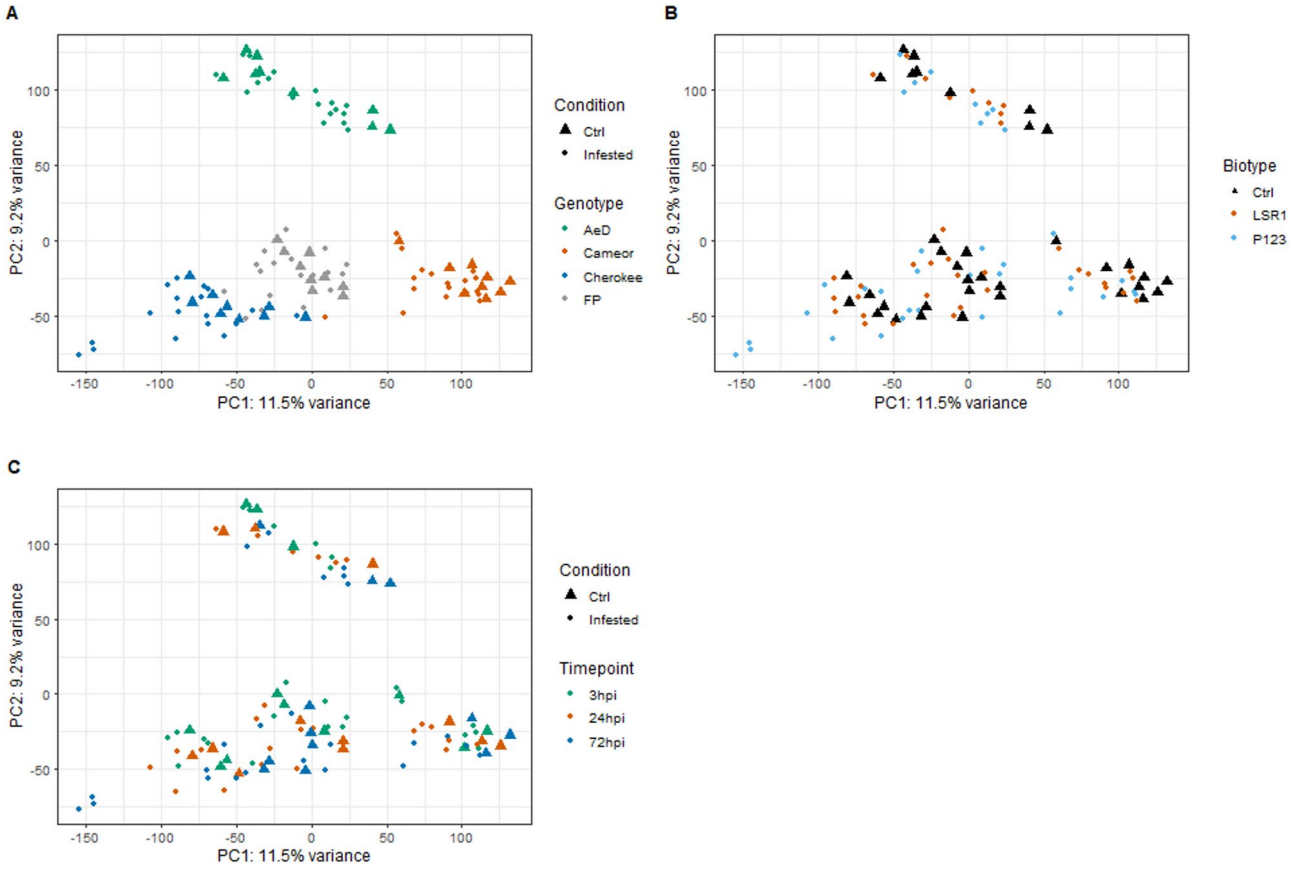


Fig. 1. Principal component analysis (PCA) of the 108 libraries, displaying the two first dimensions. The samples are coloured according to (**A**) the pea genotype (AeD, Cameor, Cherokee and FP) and the conditions of infestation (non-infested control [Ctrl] and infested), (**B**) the conditions of infestation (Ctrl, LSR1 and P123 infestations) and (**C**) the sampling timepoints (3, 24 and 72 hpi) and the conditions of infestation. In all the panels, non-infested control samples are represented by triangles, whereas infested samples are represented by circles.

Pea genotype	Resistance to P123	Resistance to LSR1	Timepoint	P123 (pea biotype)		LSR1 (alfalfa biotype)	
				Down-regulated	Up-regulated	Down-regulated	Up-regulated
AeD	Partially resistant	Resistant	3h	0	0	0	0
			24h	1	1	0	1
			72h	7	1	1	0
Cameor	Partially resistant	Resistant	3h	0	0	0	0
			24h	4	14	0	0
			72h	1,381	579	0	1
FP	Susceptible	Partially Susceptible	3h	0	0	1	0
			24h	5	3	0	2
			72h	923	171	2	1
Cherokee	Susceptible	Partially Susceptible	3h	0	0	0	0
			24h	219	43	0	0
			72h	3,609	1,917	13	1

Table 1. Number of differentially expressed genes in each condition of infestation in the four pea genotypes. DEGs were identified using three replicates per condition and based on comparisons between infested and non-infested conditions.

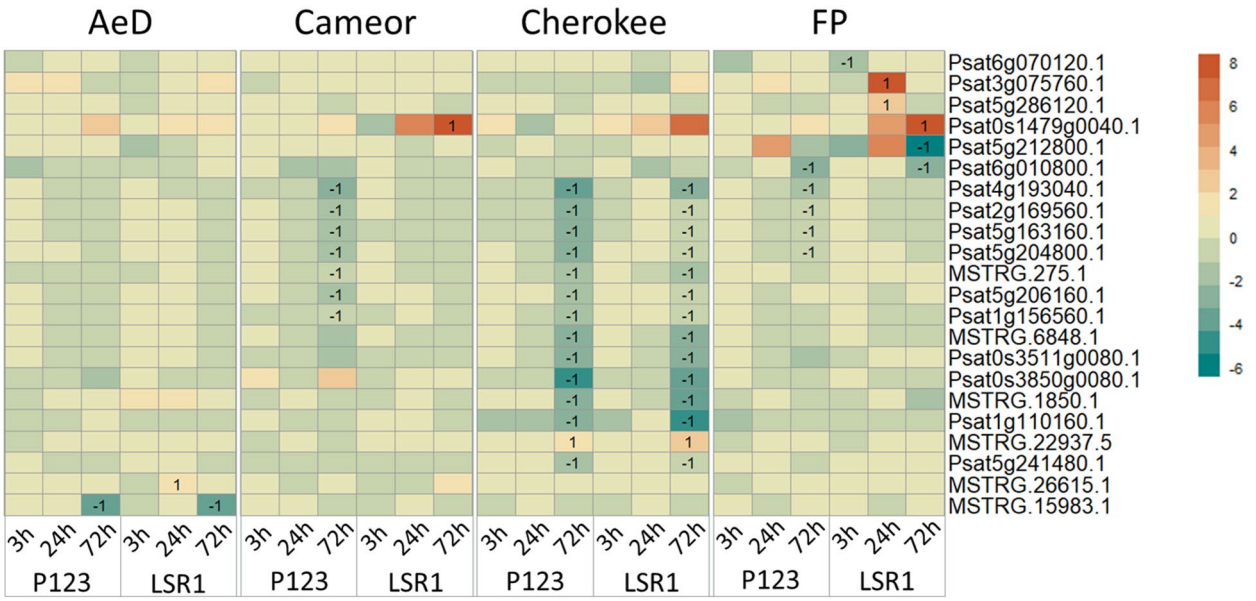


Fig. 2. Expression patterns of all differentially expressed genes (22 genes) upon LSR1 infestation in four pea genotypes. The color scale represents the intensity of differential expression in Log2(fold change) upon LSR1 and P123 infestation. Cells containing "1" and "−1" represent, respectively, significantly up- and down-regulated genes (FDR p-value < 0.05). Some of the DEGs were differentially regulated upon infestation with the two aphid clones and showed the same direction of regulation. AeD and Cameor are partially resistant to P123, while Cherokee and FP are susceptible to P123. AeD and Cameor are completely resistant to LSR1, while Cherokee and FP are partially susceptible to LSR1.

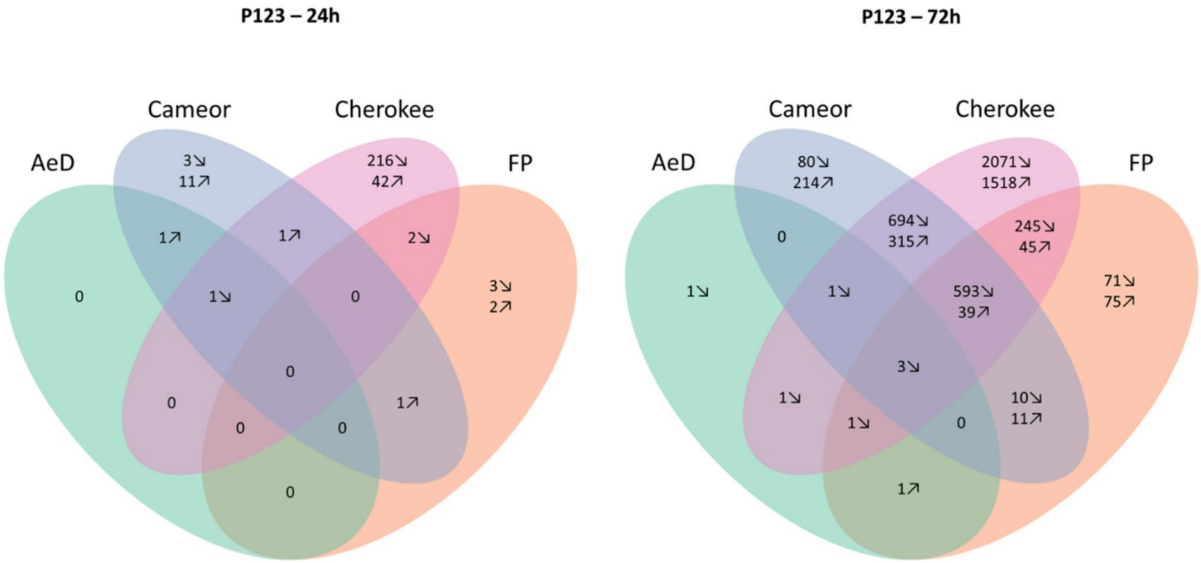


Fig. 3. Venn diagrams displaying differentially expressed genes, in four pea genotypes, during infestation with P123. The numbers of down-regulated genes are shown with downward arrows and up-regulated genes with upward arrows at 24 and 72 hpi. Small number of genes were identified in the intersections, indicating that the gene expression patterns strongly depend on the pea genotypes. AeD and Cameor are partially resistant to P123, while Cherokee and FP are susceptible to P123.

pea genotypes at 72 hpi. In each pea genotype, all the DEGs showed consistent expression direction with an increased fold change from 24 to 72 hpi (Supplementary Table S4), indicating enhanced gene regulation with a prolonged aphid infestation. To identify the biological processes that were differentially regulated in response to P123 infestation in pea, we performed a GO term (biological process) enrichment analysis. The pea genotype AeD showed a much weaker response compared to the other three pea genotypes, with no enriched GO terms.

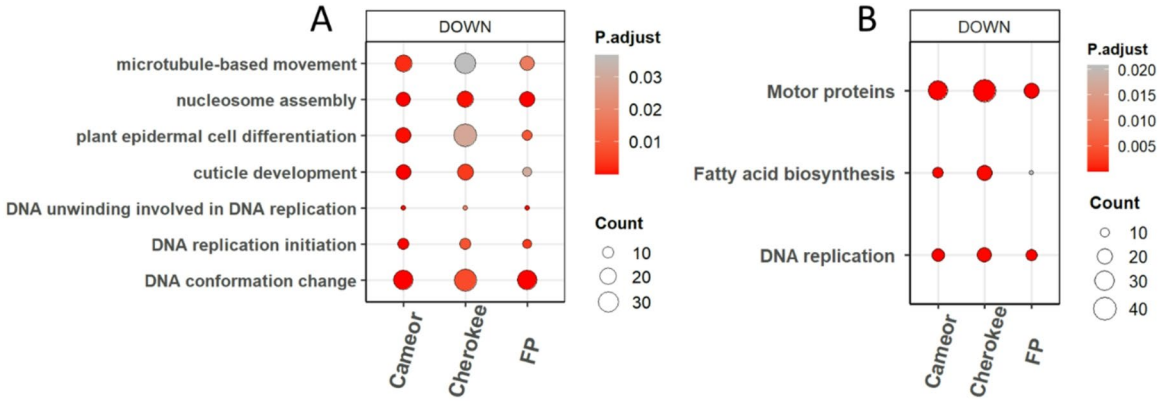


Fig. 4. Pathway enrichment analyses of the differentially regulated genes in pea, after 72 h of-infestation with P123 aphids. (**A**) Significantly enriched GO terms shared by the three genotypes Cameor, Cherokee and FP (**B**) significantly enriched KEGG pathways identified for three genotypes. The genes assigned to these pathways were commonly down-regulated in the three genotypes. AeD did not show significant enrichment of GO terms or KEGG pathways and is not included in the figures. The color and the size of the circles indicate the statistical significance (adjusted *p*-values), and the number of genes identified for each GO term or KEGG pathway according to the legend on the right. AeD and Cameor are partially resistant to P123, while Cherokee and FP are susceptible to P123.

The common pea response was therefore determined by comparing the responses of the three other genotypes, Cameor, Cherokee, and FP. At 24 hpi, only Cherokee showed enriched GO terms and KEGG pathways, specifically among down-regulated genes (Table S5 and S6), with functions related to cell division and chromatin organization. No pathways were enriched among up-regulated genes at this stage.

At 72 hpi, Cameor, Cherokee and FP had 52 significantly enriched GO terms for up and down-regulated genes (Supplementary Table S5). Seven terms were commonly down-regulated in the three pea genotypes and these terms mainly belonged to "nucleosome assembly" and "microtubule-based movement" and "DNA associated processes" (Fig. 4, Supplementary Table S5). Additional down-regulated terms shared by the three pea genotypes were related to diverse plant developmental processes such as the plant epidermal cell differentiation and the cuticle development. Seven GO terms for down-regulated genes were commonly identified in two genotypes. In FP and Cherokee, the GO term related to translation processes was commonly identified as down-regulated. The GO terms related to phospholipid transport processes and regulation of cutin biosynthetic process were enriched in both Cameor and FP. Finally, Cameor and Cherokee shared significantly enriched GO terms related to cell division and cell wall development and wax production (Supplementary Table S5). Among up-regulated genes, 14 GO terms were enriched in Cherokee and Cameor, including two shared terms, "response to chitin" and "secondary metabolite biosynthetic process", while the remaining were unique to Cherokee (Supplementary Table S5).

KEGG pathway analysis revealed three pathways commonly down-regulated in all three genotypes, "fatty acid biosynthesis", "DNA replication" and "motor proteins" (Fig. 4, Supplementary Table S6), supporting the results of GO analysis. The expression patterns of the genes involved in these three KEGG categories were then examined in more details. Key steps and enzymes of the fatty acid biosynthesis pathway are shown in Supplementary Figure S3. Most of the 23 DEGs in this pathway were down-regulated in all three genotypes infested with P123 (Supplementary Table S7), including genes involved in initiation, elongation, and termination of long-chain acyl-CoA synthesis indicating the repression of the overall pathway.

In the DNA replication pathway, all but one DEG were down-regulated, with 11 genes consistently repressed across all three genotypes (Supplementary Table S8), suggesting strong inhibition of DNA replication during infestation with P123. All DEGs identified as encoding "motor proteins" were down-regulated in at least one of the three genotypes after P123 infestation. Nineteen of these 45 DEGs were commonly down-regulated in the three pea genotypes and predicted to encode kinesins, tubulins and one actin (Supplementary Table S9). Therefore, the cellular development was commonly repressed in pea genotypes by the P123 infestation. Despite some genotype-specific variations, a conserved transcriptional response to P123 infestation was observed across the three pea genotypes. The most prominent and conserved response was the down-regulation of genes involved in the pathways that are central to cell division, intracellular transport, chromatin dynamics, and biogenesis of cell membranes or secondary cell wall. A smaller set of defense-related processes was up-regulated in the two genotypes, Cherokee and Cameor.

Expression patterns of phytohormone-related genes did not suggest any clear phytohormone response during aphid infestation

No enrichment of GO terms or genes involved in the KEGG phytohormone response pathways was observed in any of the pea genotypes infested with LSR1 (Supplementary Table S5 and S6). The GO terms "regulation of salicylic acid biosynthetic process" and "response to cytokinin" were enriched in the up-regulated and down-regulated genes, respectively, of Cherokee during infestation with P123. However, these significant GO

terms were observed in this genotype only, and analysis of KEGG pathways did not show any enrichment of phytohormone-related pathways. Therefore, these hormonal responses appeared to be subtle, genotype-specific and do not seem to be involved in pea resistance to the aphids.

Constitutive differential expression of some genes in *ApRVII* and differential production of phytohormones were observed in aphid-resistant and susceptible pea genotypes

The *ApRVII* locus confers resistance to both pea-adapted and non-adapted aphid biotypes, though it may act differently against the two pea aphid biotypes[20,30]. The pea genotypes AeD and FP contain the respective haplotype sequences at *ApRVII* that are clearly associated with resistance or susceptibility[30]. Cameor and Cherokee were selected based on their phenotype, but subsequent analyses showed a less clear association between their haplotype at *ApRVII* and phenotype[30].

Therefore, to investigate the role of *ApRVII*, we compared expression patterns of the genes between AeD and FP. Because AeD showed little transcriptional response to aphid infestation at the selected timepoints, and its DEGs were not encoded by genes located at the *ApRVII* locus, we focused on the constitutive transcriptional differences between these two genotypes. Non-infested control samples of each pea genotype at the three timepoints were used for this comparison.

Out of the 65 DEGs identified in the two genotypes, 21 were encoded in *ApRVII* and showed strong expression difference (FC > 5) (Supplementary Table S10). Two genes (*Psat7g085120.1* and *MSTRG.24683.1*) out of the 21 were more than 50-fold up-regulated in the resistant pea genotype AeD, and predicted to encode an unknown protein and a ncRNA. Other AeD-up-regulated genes encoded proteins that may contribute to plant resistance, such as a phosphatase (*Psat7g079080.1*), an ethylene-responsive transcription factor (*Psat7g092760.1*) and a cytochrome P450 (*Psat7g087960.1*)[32–34]. Conversely, two genes (*MSTRG.24705.1* and *Psat7g091200.1*) were more than 50-fold down-regulated in AeD compared to FP and encoded a transposon Ty3-I Gag-Pol polyprotein and a protein of unknown function (Supplementary Table S10).

Discussion

Weak evidence for phytohormone involvement in early response of peas to aphid infestation

Quantification of phytohormones in four pea genotypes infested with either P123 or LSR1 indicated slight genotype-specific fluctuations. However, none of the phytohormones showed a clear change in quantity in relation to aphid infestation at the studied timepoints, a result consistent with previous results of Sanchez-Arcos et al.[24]. The phytohormone quantification was performed on the entire above-ground parts of the plants, which may mask local changes occurring at aphid feeding sites. The GO term enrichment analysis indicated the up-regulation of the SA biosynthetic process and the down-regulation of the response to cytokinin (CK) in the genotype Cherokee only (Supplementary Table S5). This pattern suggests a potential hormonal crosstalk influencing defense regulation in Cherokee. As previously reported, the up-regulation of the SA biosynthetic process alongside the down-regulation of CK response may reflect an interaction between SA and CK in promoting immunity against biotrophic pathogens[35]. While CK can enhance SA-mediated defense responses, SA has also been shown to suppress CK-driven growth signals, indicating a finely balanced trade-off between defense and development[35]. In Cherokee, this regulation might contribute to prioritizing defense over growth upon aphid attack.

Plant responses to aphid infestation are highly specific to plant and aphid genotypes. In addition, models of phytohormone signaling pathways and response genes have been mostly developed based on experimental results from Arabidopsis and may not be applicable to other plant species like pea[36,37]. While the most prominent defense-associated phytohormones have been investigated in this study and showed no clear involvement in pea response to the pea aphids, other molecules may also be involved in the signal transduction upon aphid infestation. Therefore, we cannot completely rule out the involvement of phytohormone pathways in pea responses to *A. pisum* biotypes. Though technically challenging, additional studies, focusing on local feeding sites could help identify phytohormone changes with greater accuracy.

Weak inducible defense against the non-adapted *A. pisum* biotype in pea

Transcriptional analysis of four pea genotypes showed a very weak gene expression response to the infestation of LSR1. A subset of DEGs was commonly identified in host and non-host interactions with the same direction of regulation (Fig. 2), indicating that the differential expression of those genes was a general pea response to *A. pisum* infestation. Furthermore, these commonly identified DEGs tended to show higher fold changes after infestation with P123 compared to LSR1 (Supplementary Table S4). Transcriptional studies of *A. thaliana* and *Brassica juncea* (L.) Czern upon aphid infestation also identified a subset of genes that was differentially regulated by both adapted and non-adapted aphids[12,38]. These common responses to adapted and non-adapted aphid might be triggered by the recognition of aphid HAMPs in different plant-aphid interactions.

Stronger transcriptional reprograming upon infestation with adapted aphids was seen in some previous studies[12,38], but in our case, pea response to LSR1 was strikingly little compared to these studies, suggesting the involvement of non-transcriptional or preformed defense mechanisms[38–41]. Analysis of the feeding behaviour of LSR1 on several pea genotypes, including the four genotypes tested in this study, showed that individuals of this clone spent less time inserting their stylets into the pea plants than pea-adapted aphids and failed in establishing prolonged phloem feeding in all the pea genotypes tested[20]. This suggests that the pea plants may lack stimulants that help LSR1 to establish phloem feeding or may contain, in the sieve elements or companion cells, the molecules that deter LSR1 to maintain a prolonged feeding. Also, phloem proteins, such as forisomes that mediate phloem occlusion upon injury[39–42] may prevent the feeding by LSR1. Post-transcriptional regulation may also influence LSR1 feeding by altering the production of specific secondary metabolites. Finally,

the relatively small number of differentially expressed genes in response to LSR1 may also be explained by the short feeding duration of this biotype on pea[20].

Infestation of pea adapted aphids suppressed various pathways in pea

The infestation with P123 induced significant transcriptional changes in three out of the four analyzed pea genotypes, starting at 24 hpi that continued to increase toward 72 hpi. Previous transcriptional analysis of other plant-aphid interactions showed differential gene expression as early as 3 hpi[12,43]. However, we detected almost no differential expression induced by P123 at this timepoint. Furthermore, more genes were down-regulated rather than up-regulated upon P123 infestation in the three pea genotypes (Table 1). The genotype AeD showed little transcriptional changes, however, a direct relationship between the magnitude of transcriptional response and resistance appears unlikely as another resistant genotype, Cameor, displayed a large number of DEGs.

The GO enrichment analyses of 72 hpi showed 14 terms over-representing the up-regulated genes. Twelve of these GO terms are specific to the pea genotype Cherokee and two are shared with the pea genotype Cameor. Most of these GO terms are related to the plant's general defense response to the aphid. However, since Cherokee is susceptible to pea aphids, induction of these genes seems to have little effect against aphids. Conversely, the GO term "perturbation of innate immune response" is enriched among the up-regulated genes in Cherokee (Supplementary Table S5). This could indicate a host manipulation by the pea-adapted aphid biotype, which weaken the plant's defenses.

The down-regulation of genes from seven GO terms that seem to be involved in DNA associated processes and cell wall development was commonly observed in the three pea genotypes Cameor, Cherokee and FP (Fig. 4). Other GO terms related to photosynthesis, translation and other biological processes were identified in one or two genotypes (Supplementary Table S5). The analysis of KEGG pathways identified three categories of genes (DNA replication, motor proteins and fatty acid biosynthesis) that were commonly down-regulated in the three pea genotypes. These categories overlapped with the GO terms that were identified (Fig. 4). The down-regulation of DNA replication and other pathways involved in fundamental pea biology upon aphid infestation may be explained by a reallocation of resources to defense reactions or for survival while the aphids restrict the availability of plant nutrients. The "motor proteins" category, including 19 genes encoding several kinesins, tubulins and one actin, was down-regulated. These proteins contribute to key cellular functions such as DNA replication but also contribute to transport of cellular components[44]. As the cytoskeleton network plays a role in plant defense to pathogens[45–47], its down-regulation may weaken pea resistance to aphids.

Three KEGG pathways involving fatty acids (biosynthesis, elongation and metabolism) were enriched in two to three of the four pea genotypes, except for genotype AeD (Supplementary Table S6). Fatty acids are involved in the composition of cell membranes and secondary cell walls and have also important roles in plant defense[48–50]. In line with this, the "cutin, suberine and wax biosynthesis" pathway, which is related to physical barriers, was detected for the down-regulated category in two genotypes (Supplementary Table S6). Although the role of these genes in aphid resistance has not been investigated, a cell wall-based defense mechanism against aphids seems highly plausible given that the aphids regularly insert their stylet into the plant cell wall[51,52].

Our previous study showed that co-infestation with P123 on pea induced the pea susceptibility to LSR1[6]. We hypothesize that the suppression of some of these pathways by P123 contributes to the observed induction of susceptibility to LSR1[6]. Since P123 and LSR1 express their salivary genes differently[22], we also hypothesize that P123 specific salivary genes may contribute to the down-regulation of the pathways. However, at this stage, it is not possible to precisely identify the mechanisms responsible for this early down-regulation.

The resistance conferred by *ApRVII* may have a constitutive component

Although we selected the four pea genotypes with different levels of resistance to P123 and LSR1 for the transcriptional profiling, subsequent genetic studies by our group revealed that only AeD and FP had haplotypic sequences at the *ApRVII* locus clearly associated with their phenotype[30]. Therefore, we compared transcription profiles of these two pea genotypes to gain insight into the mechanism of resistance conferred by *ApRVII*.

ApRVII locus does not contain any NLR resistance genes[30], and AeD carrying *ApRVII* locus did not show HR-like response. Hence, the resistance mediated by *ApRVII* likely relies on different mechanisms from classical gene-for-gene interactions triggering HR. Furthermore, the resistant AeD showed very little transcriptional change upon infestation with both *A. pisum* biotypes. This could suggest that the resistance does not involve induced transcriptional responses but may be conferred by differences in constitutively expressed genes or molecules and/or involve post-transcriptional regulatory mechanisms that were not examined in our study or induced at a later time. By comparing the constitutive expression levels of the genes encoded by *ApRVII* between the two non-infested pea genotypes, 12 genes were identified as more expressed in AeD than in the susceptible FP[32]. Constitutively different expression of those genes may cause structural or chemical differences between the resistant and the susceptible genotypes. Feeding behavior analysis using the electrical penetration graph (EPG) revealed that pea adapted aphids exhibited similar feeding behavior in pea genotypes with different resistant levels, but alfalfa biotype reduced the probing time on the resistant genotypes[20]. Hence, the quality of the phloem sap, such as the concentration of nutrients or toxic compounds, and quantity of feeding stimulants or deterrents for the alfalfa biotype may differ between resistant and susceptible pea genotypes. Further studies are needed to elucidate these mechanisms, including comparing the metabolome of different pea genotypes and investigating the role of various pea metabolites in pea-aphid interactions. To test the significance of the DEGs identified in this study, aphid fecundity assays are needed on the pea plants with altered expression of the genes or with mutations in the genes.

In conclusion (see Supplementary Figure S4 for a graphical overview), this study showed that the infestation of pea-adapted *A. pisum* biotype induced strong transcriptional reprogramming while non-adapted biotype had little effect on pea transcription. The results suggested the involvement of preformed and/or non-transcriptional

defense mechanisms of pea against the non-adapted biotype. The *ApRVII*-mediated defense mechanisms to the adapted and non-adapted aphids did not seem to involve induced transcriptional responses, but constitutively differentially expressed genes encoded in *ApRVII* locus were identified as the candidate genes involved in the resistance mechanism to *A. pisum* biotypes.

Materials and methods

Insect material

Aphid multiplication for pea infestation experiments was conducted in a growth chamber in long day conditions (18 ± 1.5 °C, 16 h day/8 h night cycle) to maintain parthenogenetic reproduction of aphids. Aphids were reared on *Vicia faba* L., which is a common host for all the biotypes of *A. pisum*[53]. We used the P123 clone (without secondary symbiont), which is adapted to pea, and the LSR1 clone, which is adapted to alfalfa. These two clones have been used in previous studies and are strongly specialized on their respective host plants[22,30].

Plant material

Four *P. sativum* genotypes, AeD99OSW-49-5-7 (P_AMS_DCG0661), CE101 = FP (P_AMS_RCG0228), Cameor (P_AMS_DCG0251) and Cherokee (P_AMS_DCG0472), were selected from the pea AMS ("Architecture and Multi-Stress") collection previously used in GWAS analysis[30]. Their origin is France and the seeds are maintained in INRAE. Pea plants were grown in pots (one plant per pot of 8 × 8 × 9 cm) in a climatic chamber, set at 18 ± 1.5 °C and 16 h day/8 h night cycle.

Aphid infestations and sample collection

Three-week-old pea plants, corresponding to the four-node stage in most cases, were infested with 100 five-day-old P123 or LSR1, enclosed in a perforated plastic bag and maintained in a climatic chamber set at 18 ± 1.5 °C, 16 h day/8 h night cycle. The aerial parts of the plants were cut at 3, 24, and 72 h post-infestation and flash frozen by liquid nitrogen. All the aphids on the frozen plants were removed by a paint brush in liquid nitrogen. Non-infested plants were treated in the same manner and served as controls. Six biological replicates were prepared for each condition of infestation. Three replicates were harvested in a first lot and the three other replicates were harvested in a second lot, one week after the first one. Plant samples were ground with mortar and pestle with liquid nitrogen. After the grinding, the samples were stored at −80 °C, divided for extraction of RNA and phytohormones, and lyophilized in Eppendorf tubes (2 ml) for 48 h for phytohormone extraction.

Phytohormone quantification and statistical analysis

All six biological replicates were used for phytohormone analysis. For the phytohormone extraction, 1 mL extraction buffer containing 80% methanol (MeOH) with 0.1% of formic acid and 4 µL of internal standard was added to 10 mg of freeze-dried plant powder. The internal standard was composed of 40 ng/mL JA-d_6, SA-d_4 (Sigma Aldrich), ABA-d_6 (Santa Cruz Biotechnology, Santa Cruz, USA); 8 ng/mL JA-Ile-d_6. A sonication by ultrasonic bath was applied for 15 min at maximum frequency (35 kHz) at 20 °C. Samples were centrifuged (−10 °C, 20 min, 7000 rpm) to conserve only the supernatant.

Phytohormone detection of SA, SA-Glu, ABA, JA-Ile and OH-JA was realized using tandem mass spectrometer in negative ionization mode in accordance to Sanchez-Arcos et al[24]. Elution gradient was carried out on the Agilent Technologies 1260 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a binary solvent system consisting of water with 0.05% formic acid (solvent A) and acetonitrile (solvent B).

The quantification method varied from the previously published method as follows: A QTRAP 6500 + tandem mass spectrometer (SCIEX, Darmstadt, Germany) was utilized in negative ionization and multiple reaction monitoring (MRM) mode and the following settings. The turbo gas was set to 650 °C, the curtain gas at 45 psi, and the ion source gas 1 and 2 set at 70 psi. The parent ion → product ion was monitored through MRM as follows: SA m/z 136.93 → 93 (collision energy (CE) −24 V; declustering potential (DP) −20 V) and SA-Glu m/z 299.128 → 136.9 (CE −18 V; DP −20 V) and their corresponding internal standard SA-d 4 m/z 140.93 → 97 (CE −24 V; DP −20 V); ABA m/z 263 → 153.2 (CE −22 V; DP −20 V) and its corresponding internal standard ABA-d 6 m/z 269 → 159.2 (CE −22 V; DP −20 V); JA-Ile m/z 322.19 → 130.1 (CE −30 V; DP −50 V) and its corresponding internal standard JA-Ile-d 6 m/z 328.19 → 130.1 and 327.19 → 130.1 (CE −30 V; DP −50 V); OH-JA m/z 225.1 → 59 (CE −24 V; DP −20 V) and its corresponding internal standard JA-d 6 m/z 215 → 59 (CE −24 V; DP −20 V). The instrument was operated through the Analyst 1.6.3 software (SCIEX) and peaks integrated with the MultiQuant™ 3.0.3 software (SCIEX). All concentrations were calculated based on the corresponding internal standards and a response factor of 1.

To investigate the potential effect of the aphid clones and the selected timepoints on the phytohormone production in each pea genotype, we applied linear mixed effects models with the lme function from the R package "nlme" version 3.1–166.1[54,55] on R version 4.3.1[56]. In each model, we included the sampling week as random effect factor. In case of unequal variances, the varIdent variance structure was included, to account for the variance heterogeneity of the residuals following the procedure explained in[57]. The optimal variance structure was selected by comparing different models integrating the variance of aphid clones, the timepoint, the combination of both factors, or no specified variance structure. The model with the smallest AIC (Akaike information criterion) and BIC (Bayesian information criterion) was selected. To find the optimal fixed effects in this model, we used F-statistics using the ANOVA function from the R package "stats" version 4.3.1[56]. To determine the difference between factor levels, a multiple comparison was conducted using the function "glht" from the R package "multcomp" version 1.4–26, with p-values adjusted using the FDR method[58,59].

To investigate the difference of phytohormone production between selected genotypes, we used the same method as described above, but using additionally the aphid infestation timepoints and the pea genotype as fixed effects, and the sampling week as random variable.

RNA sequencing and read mapping

The quantification of the phytohormone data was used to select three out of the six biological replicates per condition for RNA sequencing. To identify the three most representative replicate per condition, we identified outlier samples from the phytohormone quantification and used non-outlier samples for the RNAseq. Outliers could be the plants reacting differently from others due to environmental effect. To identify the outliers, the median was calculated for each phytohormone quantity, for each treatment. The difference between the median and each data point (x_{ij}) was scaled by the range (range = maximum value $x_{i.max}$ – minimum value $x_{i.min}$) of the data to get a relative difference. Then the relative difference was calculated ($relative\ difference = \frac{x_{ij} - median_i}{x_{i.max} - x_{i.min}}$). Outlier samples were identified by detecting the data that lie 1.5 times outside of the inter quartile range of the boxplots.

If there were more than three samples considered as outliers during the identification of outliers, then we removed the ones with values that were the most different from the median value or values with the highest relative difference. If there were less than three outliers, then we removed samples with the highest relative difference. For each treatment, at least one biological replicate was selected from one of the lots, while the two others were chosen from the second lot, to minimize potential batch-related effects.

For the preparation of the RNAseq samples, total RNAs was extracted using a Macherey-Nagel NucleoSpin RNA Plus Mini kit with DNA removal column. RNA and residual DNA quantification were realized by using a Quantus fluorometer (QuantiFluor) and RNA quality was assessed by 2100 Bioanalyzer (Agilent Technologies). The library preparation and RNA sequencing were executed by the Genewiz Company. Ribosomal RNAs were depleted with the NEB Ultra II DIRECTIONAL RNA library preparation kit and RNA sequencing was performed on an Illumina NovaSeq to generate 150-nucleotide-long paired-end reads. The libraries were reverse stranded. The chloroplastic genome was included in addition to the *P. sativum* genome v1a from the NCBI reference sequence NC_014057. In total, 108 libraries were generated.

The raw data preprocessing was conducted using the nf-core RNAseq pipeline version 3.4[60,61] encompassing different tools, using Nextflow version 21.04.0. To assess the sequence quality of the raw data, FastQC software version 0.11.9 was used[62]. Data were trimmed for low-quality and adapter sequences using TrimGalore version 0.6.7. The residual ribosomal RNAs was removed with sortMeRNA version 4.3.4. Once filtered, the reads were aligned against the *P. sativum* reference genome sequence v1a[31] using the HISAT2 version 2.0.5 alignment program[63]. The alignments were assembled into potential transcripts for each gene locus using StringTie version 2.1.7[64]. The entire process, from the quality control of raw sequencing data to the annotation of new transcripts with StringTie, was carried out using the nf-core RNAseq pipeline, using default parameters[60,61]. Across the 108 RNAseq libraries analyzed, we obtained a median sequencing depth of 49.6 million of reads, with Phred quality scores above 36 (Supplementary Table S2). StringTie version 2.1.7 was used to assemble the alignments into potential transcripts for each gene locus. Transcripts potentially expressed from transposable elements (TEs) were removed using the Bedtools intersect v2.27.1 software and samtools v1.15 software with the options "-f 0.5 --wao" to remove transcript that are overlapping with more than 50% of their sequences with TEs[65,66]. Long non-coding RNAs (lncRNA) were identified using the FEElnc version 0.1.0 software[67] with default parameters. Mapped reads were enriched with the new transcripts and were counted using FeatureCounts version 1.6.0[68] with these parameters: paired reads that mapped once and were correctly mapped on the same chromosome were counted using the corresponding options (-B -C -p).

Statistical analysis of RNAseq data

Using AskoR pipeline[69,70], which contains some of the different R packages described below, genes were defined as expressed if the CPM value of at least three libraries was more than 1. RNAseq data were normalized with the TMM method[71]. Differential expression was analyzed with the R package edgeR version 3.42.4[72]. Genes were considered as differentially expressed when the absolute fold change was superior to 1.5 and FDR-adjusted *p*-value was inferior to 0.05. GO term enrichment analysis was conducted using the TopGO R package version 2.54.0[73], with the weight01 algorithm and Fisher test including a selection of the GO term containing at least five differentially expressed genes. Adjusted *p*-values for these GO terms were obtained with the FDR method, using the R function p.adjust from the package "stats". Gene clustering was applied using the R package "coseq" version 1.24.0[74,75]. Enrichment analysis of the KEGG pathways[76,77] was conducted using the R package "clusterProfiler" version 4.8.3 on the orthologous genes from *Medicago truncatula* Gaertn V5[78]. *P*-values were adjusted using the FDR method, implemented in the function "compareCluster", from the R package "clusterProfiler". An FDR *p*-value cutoff of 0.05 was used.

A heatmap of all differentially expressed genes during LSR1 compared to P123 infestation, based on log fold-change (log(FC)) values extracted from AskoR outputs, was generated using the R package pheatmap version 1.0.12[79]. Venn diagrams were produced using the R package "VennDiagram" version 1.7.3[80], implemented in AskoR package. Plots showing the enrichment analyses were generated using the R package "ggplot2" version 3.5.1[81]. A principal component analysis (PCA) was conducted on RNA-seq count data. Variance stabilization was achieved using the DESeq2 R package (version 1.40.2), through size factor estimation, dispersion calculations, and the application of a variance-stabilizing transformation. PCA was then performed using a custom function that utilized singular value decomposition (SVD) from the R package "base" (version 4.3.1). SVD was applied to a reduced, centered, and scaled matrix using the R function "scale." A partial least squares discriminant analysis (PLSDA) was conducted using the R package "mixOmics" (version 6.26.0) on the same data used for the PCA, and the permutations were conducted using the R package "vegan" (version 2.7-1.7).

Annotation of protein-coding genes

Protein-coding transcripts identified using FEElnc v0.1.1[67] were analyzed with TransDecoder v5.5.0[82] to detect open reading frames (ORFs) of at least 50 amino acids in length. FEELnc was run with the following parameters:

FEELnc_filter.pl -i -a, FEELnc_codplot.pl -m shuffle -g –spethres 0.93 0.93 and FEELnc_classifier.pl -i -a. The ORF prediction process included optional homology-based searches employing BlastP (BLAST v2.9.0 + [83]) with parameters: makeblastdb -in uniport_sprot.fasta -dbtype prot; blastp -query transdecoder_dir/longest_orfs.pep -db uniprot_sprot.fasta -max_target_seqs 1 -outfmt 6 -evalue 0.001. The protein sequence database used was the UniProtKB/Swiss-Prot dataset (accessed: August 2022). In addition, protein domains were identified using the Pfam-A database (accessed: August 2022) and HMMER v3.3 with the following commands: hmmpress Pfam-A.hmm, hmmscan –domtblout pfam.domtblout Pfam-A.hmm transdecoder_dir/longest_orfs.pep)[84]. Functional annotation of the predicted proteome was carried out with EggNOG-mapper v2.1.6, using the EggNOG database v5.0.2[85,86], and complemented with InterProScan5 v5.54–87.0.0[87]. Gene Ontology (GO) terms were derived from EggNOG outputs. In cases where multiple ORFs were predicted for a single gene, only the annotation associated with the first ORF was retained.

For KEGG pathway annotation, orthology was inferred using the annotated *M. truncatula* genome version V5[78]. Coding DNA sequences (CDS) from the pea genome 'Cameor'[31] were aligned to the *M. truncatula* CDS using BlastN (BLAST v2.9.0 + [83]) with parameters: makeblastdb -in MtrunA17r5.0-ANR_cds_from_genomic.fna -dbtype nucl -out DBMedicV5CDS; blastp -query CDS -db DBMedicV5CDS -max_target_seqs 1 -outfmt 6 -evalue 0.001.

Aphid fecundity assays

Pisum sativum plants were cultivated in pots (one plant per 7 × 7 × 8 cm pot) within a climate-controlled chamber, maintained at 18 ± 1.5 °C with a 16-hour light/8-hour dark cycle. To eliminate potential maternal and grand-maternal effects resulting from feeding on a generalist host (*V. faba*), P123 aphids were multiplied on the tested genotype for two generations before fecundity was assessed, following the method described in[30]. Ten days after placing a one-day-old larva (G1), a one-day-old larva from the second generation (G2) was transferred onto a fresh pea plant of the same genotype. Fifteen days after the G2 aphid installation, all offspring (G3) were gathered and counted. For assessing the fecundity of the LSR1 clone, a different protocol was used, anticipating a very low reproduction rate on pea plants. A single one-day-old larva was placed on each plant, and all G2 offspring were collected and counted three weeks later. For both experiments, the four pea genotypes were tested simultaneously, with 20 to 30 replicates per genotype per test. Plants were randomly distributed in the climate chamber, with either two or three biological replicates of the four pea genotypes in separate trays. Due to space constraint, the fecundity assessment for P123 was repeated in two experiments separated by two weeks.

Statistical analysis of phenotypic data

A Quasipoisson Generalized Linear Mixed Model (GLMM) [R package "glmmTMB" version 1.1.10[88] was applied to analyze the number of aphid offspring from P123 infestation. Pea genotype was used as fixed factor, "Experiment" (replicate of the fecundity assessment) and "Plate" (tray in which the plant was placed in the growth chamber) as random effects. For the analysis of LSR1 fecundity data, a model integrating a zero-inflated generalized Poisson parameterization was used. This model was fitted by including "Plate" as random effect and "Genotype" as fixed explanatory variable. For the genotype "AeD99OSW-49-5-7", showing a complete resistance to LSR1, we intentionally modified one of the "0" value by "1" to insert a minimal variability for this factor level. Indeed, without changing any value, the standard error for this factor level is estimated to be huge. By artificially including a minimal variability, we obtained a more realistic standard error estimate. Pairwise comparisons of Estimated Marginal Means (EMMs) with Tukey's adjustment for multiple testing ($\alpha = 5\%$) was applied with the "lsmeans" function of the R package "emmeans" version 1.10.4[89].

Data availability

The raw RNAseq sequence data reported in the paper have been deposited in NCBI as BioProject: PRJNA1008650.

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Author contributions

RO, MLPN, JCS, AS, MP, PYS, JG, GK contributed to the study conception and design. RO, SR, MG, MP, JG, GK, PYS, MLPN, JCS, AS contributed to the RNAseq data analysis. RO, MP, JG, GK, PYS, MLPN, MG, JCS, AS contributed to the phytohormone data analysis. Material preparation and data collection were performed by RO, MP, PYS, SM, JCS and AS. The manuscript was written by RO, MLPN, MG, JCS and AS.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval and consent to participate

Not applicable.

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