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Identification of potential candidate genes controlling pea aphid tolerance in a *Pisum fulvum* high-density integrated DArTseq SNP-based genetic map

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RUNNING TITLE: Identification of candidate genes controlling pea aphid resistance in *P. fulvum*.

KEY WORDS: DArTseq; SNP; QTL mapping; aphid tolerance; wild pea (*Pisum fulvum*); pea aphid (*Acyrthosiphon pisum*).

Abstract

BACKGROUND: Pea (*Pisum sativum*) is one of the most important temperate grain legumes in the world, whose production is severely constrained by the pea aphid (*Acyrthosiphon pisum*). Wild relatives, such as *P. fulvum*, are valuable sources of allelic diversity to improve the genetic resistance of cultivated pea species against *A. pisum* attack. To unravel the genetic control underlying resistance to the pea aphid attack, a quantitative trait loci (QTL) analysis was performed using the previously developed high density integrated genetic linkage map originated from an intraspecific recombinant inbred line (RIL) population (*P. fulvum*: IFPI3260 x IFPI3251).

RESULTS: We accurately evaluated specific resistance responses to pea aphid that allowed the identification, for the first time, of genomic regions that control plant damage and aphid reproduction. Eight QTLs associated with tolerance to pea aphid were identified in LGs I, II, III, IV and V, which individually explained from 17.0 to 51.2 % of the phenotypic variation depending on the trait scored, and as a whole from 17.0 to 88.6 %. The high density integrated genetic linkage map also allowed the identification of potential candidate genes co-located with the QTLs identified.

CONCLUSIONS: Our work shows how the survival of *P. fulvum* after the pea aphid attack depends on the triggering of a multi-component protection strategy that implies a quantitative tolerance. The genomic regions associated with the tolerance responses of *P. fulvum* during *A. pisum* infestation have provided six potential candidate genes that could be useful in genomic assisted breeding (GAB) after functional validation in the future.

1. Introduction

The cultivated pea (*Pisum sativum* L. subsp. *sativum*) is one of the oldest domesticated crops improved for important agronomic traits. Nowadays, pea is the cool season grain legume most cultivated in Europe and the second in the world.[1] However, its yield is still relatively unstable and low due to the limited adaptability to extreme environmental conditions of available cultivars and their susceptibility to diseases and pests. The pea aphid (*Acyrthosiphon pisum* H., hereinafter PA) is an important pest for the pea crop due to the direct damage caused when the insect feeds from the phloem and the indirect damage caused by the transmission of viral diseases (PA transmits over 50% of all plant viruses), the injection of elicitors during the feeding process, as well as the development of opportunistic fungi that reproduce as a result of the accumulation of the honeydew excreted by the aphids as waste.[2-4] Due to their viviparous parthenogenetic reproduction phase, aphids have high reproduction rates and short generation times. A small initial infestation can quickly lead to large populations and cause significant damage to the plants.[5] In recent years, these damages have been accentuated due to climate change. The increase in temperatures is also leading to a boost on insect populations and, consequently, on their population's size.[6] In addition, PA has a broad range of hosts with described biotypes that affect legume species of several genera such as *Glycine*, *Lathyrus*, *Lens*, *Medicago*, *Pisum* or *Vicia*, which contributes even more to its relevance as a pest.[4]

Unfortunately, the low resistance to PA that is available in the cultivated *P. sativum* is insufficient to achieve an effective genetic control. Several studies have been performed,

ranging from the screening of pea cultivars to the assessment of the mechanisms involved in the resistance.[7-10] No complete resistance has been reported so far, but different levels of plant tolerance are available,[9,11,12] especially in the wild *P. fulvum* Sibth. & Sm.[13] The genetic nature of PA tolerance in pea has not been described so far. However, it has been widely studied in *M. truncatula* Gaertn. describing both dominant monogenic resistance and a quantitative control.[14-17] In addition, loci associated with QTLs that have additive effects associated with PA tolerance have also been described in lucerne (*M. sativa* L.).[3,18]

Wild pea species represent a useful source of genetic diversity for pea breeding programs.[19] Although with some difficulties, *P. fulvum* can be crossed with *P. sativum* allowing the introgression of its resistance genes to pests and diseases in pea cultivars.[20-22] Nonetheless, efficient exploitation of the full potential of *P. fulvum* resistance requires the application of modern breeding tools. DNA-based genetic markers provide powerful tools for the identification and localization of genes of traits of agronomic importance and their subsequent selection for introgression in breeding programs. Several *P. sativum* linkage maps have been constructed based on different types of markers including morphological markers, isoenzymes, RFLPs, RAPDs, SSRs, and SNPs.[10,23-27] So far, the high costs of technology development have made the application of molecular markers directly in wild species impractical.

In such scenario, Diversity Arrays Technology (DArT) in combination with next-generation sequencing platforms,[28,29] known as DArTseq™, offers a good choice as a cost-effective, high throughput genotyping platform that can detect a relatively large number of polymorphic markers for the construction of dense genetic maps for virtually any genome.[30] DArTseq-derived markers that are currently used in approximately 500 species (http://www.diversityarrays.com) were recently used in our research group to develop the

genetic map of *P. fulvum*.[31] The aim of this work was to dissect the response of *P. fulvum* against the PA infestation to allow the first mapping of the genomic regions involved in PA tolerance in a population derived from an intraspecific cross of *P. fulvum*.

2. Materials and Methods

2.1 Plant material and aphid rearing

A recombinant inbred line (RIL) population of 84 F_7 derived from the cross between *P. fulvum* IFPI3260 (sensitive to PA damage) and *P. fulvum* IFPI3251 (tolerant to PA damage) was used to construct an integrated DArT + SNP + SSR + STS based linkage map[31] and was screened for PA resistance under semi-controlled conditions.

To ensure germination of the seeds of the RIL population and their parental lines, the seeds were scarified and transferred to a Petri dish coated with blotting paper irrigated with sterile water. The seeds were kept in the dark at 4 °C for 48 h and then kept at room temperature (approximately 20 °C) for another 48 h. Then, the germinated seeds were sown in January 2013 under semi-controlled conditions in a shade house with insect-proof mesh according to a randomized complete block design with three independent blocks. In each block, each RIL and parent line were represented by a single row of 0.5 m in length, which contained 10 plants, and with a row spacing of 0.7 m.

The pea aphids used in this study were derived from an asexual parthenogenetic strain collected from field-infested pea plants at Córdoba, Spain.[10] All the PA used in the experiment derived from a single aphid isolate and were reared in a growth chamber at 20 °C with a photoperiod of 12 h light/12 h dark on susceptible faba bean (*Vicia faba* L.) plants.

2.2 Aphid Infestation

Thirty wingless adult aphids per infested plant were collected in individual tubes. The aphids were released on the plot in early March 2013 by placing the open tubes along the row at the base of each experimental plant. Adult aphids and their progeny were allowed to move freely through the experimental plot. The development of PA population and feeding damage caused by the attack were assessed 17, 20 and 25 days after the infestation (dai). On each date, the following traits were assessed: 1) percentage of the plant with chlorosis (*Chlor*), 2) percentage of the plant damaged by PA attack (*Dam*), 3) percentage of the tip with damage (*TipDam*), 4) percentage of the plant that presents wilting (*Wilt*), and 5) number of nymphs present at the apical part of the plant (apex + 1st and 2nd leaflets) (*Reprod*). This allowed the calculation for each trait of the area under the disease progress curve (AUDPC) according to Wilcoxson et al.[32] Pearson's linear correlations between parameters were performed using Statistix (version 8.0; Analytical Software, Tallahassee, USA).

2.3 DNA extraction and quantification

Leaves for later DNA extraction were sampled from the F_7 RIL population which was sown and grown for this purpose under controlled conditions as described by Barilli et al.[31] Around 12 days after sowing, 1 g of young leaves tissue from the 3rd to 4th node of each seedling was excised and immediately frozen in liquid nitrogen. Genomic DNA was isolated from the fresh and young material using a modified cetyltrimethylammonium bromide (CTAB)/chloroform/isoamyl alcohol method[33] and then it was quantified for the following marker analysis.[31]

157 **2.4 Genotyping of individual DNA samples using DArTseq array**

158 A high-throughput genotyping method using the DArTseq™ technology was performed at
159 the Diversity Arrays Technology Pty Ltd laboratory in Canberra (Australia). Genomic
160 complexity reduction with *PstI–MseI* enzymes and the generation, labelling and
161 hybridization of targets were previously described in Barilli et al. (2018)
162 (http://www.diversityarrays.com/dart-application-dartseq-data-types).[31]

163

164 **2.5 Genotyping with Simple Sequence Repeats (SSRs), Sequence Tagged Site (STS)**
165 **and Single-nucleotide polymorphism (SNP) markers**

166 A set of 37 previously described markers (including genic and genomic SSRs, STS_s and
167 SNP_s) were also surveyed for polymorphism in both parental lines and the derived RIL
168 population. Specific amplification conditions were followed depending on the marker
169 type.[34-36] The purpose of their inclusion for the construction of the genetic linkage map
170 depends on their well-known position on the *P. sativum* chromosomes, acting as a bridge
171 between the two species. So, their positions on our map help us to find the correspondence
172 between the linkage groups of *P. fulvum* with the *P. sativum* chromosomes,[31] as "anchor"
173 markers.

174

175 **2.6 Linkage map and QTL mapping**

176 The scores of all polymorphic markers were converted into genotype codes ("A" or "B")
177 according to the scores of the parents and linkage groups (LGs) were obtained using the
178 software JoinMap version 4.1.[37] The maximum likelihood mapping algorithm, which was
179 optimized for constructing a dense genetic map using this software,[38] was first used for
180 grouping all of the polymorphic markers. Then, the method of regression mapping[39] was

used for map construction with appropriate genetic distance and marker position. The Kosambi mapping function[40] was used to convert recombination frequencies into map distances. The LGs of the map corresponding to each of the 6 chromosomes of pea were drawn and aligned using MapChart v2.3.[41] Linkage groups were separated using a logarithm of the odds (LOD) score of ≥ 3.0. Markers with a mean Chi-Squared value of recombination frequency > 4.0 were discarded. DArT markers were named with the numbers corresponding to unique clone ID following Kilian et al.[28]

Sequences from DArTseq-derived markers were aligned with *Medicago truncatula* genome reference by using Phytozome v.12 (http://phytozome.jgi.doe.gov/pz/portal.htlm) to perform a synteny analysis.[42] This analysis allowed searching for sequence similarity-based homology between legume species providing an alternative approach to finding correspondence between linkage groups. The genetic map was constructed as described by Barilli et al., 2018.[31]

QTLs for PA tolerance were identified using the MapQTL 6.0 package.[37] First, composite interval mapping (CIM) and multiple interval mapping (MIM) analyses were performed to find regions with potential QTL effects. Second, markers to be used as cofactors for CIM were selected by forward–backward stepwise regression. Significance thresholds of log of odds (LOD) corresponding to a genome-wide confidence level of $P < 0.05$ were determined for each trait using the permutation test of MapQTL 6.0 with 1000 iterations. The QTL graphs were performed with MapChart v2.3.[41] The coefficient of determination (R^2) for the marker most tightly linked to a QTL was used to estimate the proportion of the total phenotypic variation explained by the QTL. Broad sense heritability (h^2), that represents the part of genetic variance in the total phenotypic variance, was calculated as described by Barilli et al. (2018).[31]

The databases Unigene (http://bios.dijon.inra.fr/FATAL/cgi/pscam.cgi)[43] and Uniprot (http://www.uniprot.org) were used to identify potential candidate genes linked to the QTLs based on functional annotation.

3. Results

3.1 Aphid resistance

The parental lines showed contrasting responses to *A. pisum* for all the traits evaluated. The susceptible parent IFPI3260 showed a higher plant damage (*Dam*) with more severe chlorosis (*Chlor*) and wilting (*Wilt*) concentrated mainly on the tips (*TipDam*) than the tolerant parent IFPI3251 (Fig. 1). In addition, the tolerant parent showed a faster population growth at the apex (*Reprod*) but a lower final reproduction rate (number of nymphs on the apical part of the plant after 25 days of infestation, $Reprod_{25}$) than IFPI3260 (Fig. 1). The reproduction rate was severe 25 days after the infestation in the susceptible parent and lines but not in the tolerant ones. In addition, the damage to the susceptible accessions was very serious, which subsequently turned the plants to wilt.

The frequency distribution of *Chlor*, *TipDam*, *Dam*, *Reprod* and $Reprod_{25}$ in the RIL population followed a normal distribution (Lynch and Walsh normality test, $P > 0.01$)[44], with *Chlor* values ranking between 250 and 890, *TipDam* between 5.25 and 30, *Dam* between 215 and 1470, *Reprod* between 168 and 498 and $Reprod_{25}$ between 1.7 and 29.7 (Fig. 1). By contrast, the assessment of *Wilt* did not follow a normal distribution and values were between 3 and 800 (Fig. 1). Several transgressive RILs showing higher sensitivity or tolerance compared to the parent lines were found for the traits assessed (Fig.1). The coefficient of Skewness was negative for *TipDam* and positive for the other parameters assessed, indicating that the distribution of the population tends in general to tolerance. The

broad-sense heritability values for the traits scored were of 0.71, 0.68, 0.79, 0.72, 0.67 and 0.65 for *Chlor*, *TipDam*, *Dam*, *Wilt*, *Reprod* and $Reprod_{25}$, respectively. Pearson's linear correlations between the evaluated traits are shown in Table 1. We observed a positively strong significant linear correlation between *Chlor* and *Dam* ($r = 0.89$; $P < 0.001$) and a moderately positive significant linear correlation between the evaluated traits *Reprod-Reprod25* ($r = 0.71$; $P < 0.001$), *Reprod-TipDam* ($r = 0.70$; $P < 0.05$) and *TipDam-Wilt* ($r = 0.70$; $P < 0.05$) (Table 1).

3.2 Genotyping and Linkage Mapping

The mapping dataset consisted of 12,021 DArTseq derived markers and 37 "anchor" markers of different types which were distributed across 7 linkage groups (LG), as previously described in Barilli et al.[31] Authors reported that the genetic linkage map of *P. fulvum* covers a total length of 1877.45 cM with an average density of 1.19 markers cM^{-1}. Map distances between two consecutive markers varied from 0 to 13.04 cM, while the gap average between markers varied from 1.66 cM on LGVII and 2.05 cM on LGVI (Fig. 2; Table 2).

"Anchor" markers, as well as 89 sequences from *P. fulvum* DArTseq-derived markers that were BLASTed to *Medicago truncatula* genome (http://www.medicagogenome.org/) defined the correspondence between *P. fulvum* and *P. sativum* linkage groups and pea chromosome assignment, as follows: *P. fulvum* linkage group I, II, III, IV, V, VI and VII are related to *P. sativum* LGs 7, 5, 3, 2, 6, 1 and 4, respectively (Table 3) (see Barilli et al.[31] for more information).

3.3 QTL Mapping

QTLs controlling the tolerant response to PA in *P. fulvum* are first reported in this study. Quantitative trait loci analysis with composite interval mapping (CIM) and multiple interval mapping (MIM) methods revealed several genomic regions associated with adult plant tolerance to PA under semi-controlled conditions. A total of eight QTLs were identified along LGs I, II, III, IV and V. QTLs involved in PA tolerance explained individually from 17.0 to 51.2% of the phenotypic variation, depending on the trait scored, and together from 17.0 to 88.6% (Table 4). A QTL named *ApI* was identified at 208.8 cM at LGI with a LOD value of 3.08 and flanked by the DArT markers "3568590" and "3569349". It was associated with a reduced percentage of tip damaged (*TipDam*) and explained 17.2% of the phenotypic variation (Table 4, Fig. 3A). The QTL *ApII* associated with reduced aphid damage (*Dam*) and a LOD score of 3.0 presented a peak at 94 cM in LGII of *P. fulvum* (Table 4, Fig. 3C). It accounted for 17.9% of the phenotypic variation and was flanked by the DArT markers "3535012" and "3536533". A third QTL named *ApIII* was located in LGIII with a peak at 194.1 cM between the DArT markers "3535795" and "3537104". *ApIII* presented a LOD score of 3.55 and 4.01 and explained 19.6% and 23% of the variation associated with reduced *Dam* and chlorotic area (*Chlor*), respectively (Table 4, Fig. 3C). Two QTLs explaining 17.0% of the phenotypic variation associated with the number of nymphs present at the apical part of the plant at 25 dai ($Reprod_{25}$) (*ApIV.1*) and 17.5% of the variation associated with the percentage of wilting (*Wilt*) (*ApIV.2*) were located in nearby regions of LGIV with a LOD score of 3.33 and 3.13, respectively (Table 4, Fig. 3D). *ApIV.1* presented a peak at 78.8 cM while for *ApIV.2* the peak was at 90.2 cM. *ApIV.1* was flanked by the DArT markers "3568629" and "3536355" while *ApIV.2* was flanked by markers "3535628" and "3535628". Finally, a consistent region involved in *P. fulvum* tolerance against PA was found in LGV (*ApV*) including 1) a

277 QTL named *ApV.1* with a peak at 151.9 cM explaining 47.7 and 40.6% of the phenotypic
278 variation associated with *Dam* (LOD 9.86) and *Wilt* (LOD 8.48), respectively, flanked by
279 the derived DArT markers "3538656" and "35375107" (Table 4, Fig. 3E); 2) the QTL
280 *ApV.2* (LOD 11.67) explaining 51.2% of the variation of *TipDam* flanked by "3537510"
281 and "3534526" with a peak at 154.6 cM; 3) the QTL *ApV.3* with a peak at 156.1 cM (LOD
282 of 5.11) explaining 26.9% of the phenotypic variation associated with *Chlor* and located
283 between the DArT markers "3537754" and "3534511".

284 The sequences of the markers linked to the QTLs identified were checked in the pea
285 transcriptome assembly available online[43] revealing six transcripts (Supplementary Table 1)
286 linked to potential candidate genes within the genomic regions controlling PA tolerance in
287 *P. fulvum*.

288 The desirable alleles involved in coping with PA attack derived from both parents.
289 In the case of the QTLs *ApII* (LGII, for *Dam*), *ApIV.1* (LGIV, for *Reprod25*), *ApIV.2*
290 (LGIV, for *Wilt*), *ApV.1* (LGV, for *Wilt* and *Dam*), *ApV.2* (LGV, for *TipDam*) and *ApV.3*
291 (LGV, for *Chlor*) these were derived from the tolerant parent IFPI3251 while for the QTLs
292 *ApI* (LGI, for *TipDam*) and *ApIII* (LGIII, for *Dam* and *Chlor*) the alleles responsible for
293 less damage due to PA attack derived from the sensitive parent IFPI3260 (Table 4).
294 Epistatic interactions among QTLs were not significant according to MIM for any of the
295 analyzed traits.

296

297 **4. Discussion**

298 Plant-aphid interaction is responsible for damages (wounds, sap sucking and virus
299 transmission) that produce large yield losses with a significant impact on agriculture.
300 Previous studies on pea resistance to *A. pisum* infestation have ranged from the screening of

301 pea genotypes[5,7,9,13] to the evaluation of the mechanisms involved in resistance to PA
302 attack.[10,13] No complete resistance has been reported so far, but several levels of incomplete
303 resistance have been reported in wild *Pisum* accessions that could be used to develop pea
304 cultivars with increased resistance.[13] With this objective, in this study we have analyzed
305 the damages that PA cause to plants and their reproductive and development capacity to
306 map the genetic loci involved in the resistance/tolerance to infestation by using the high-
307 density genetic map of *Pisum fulvum* previously developed by Barilli et al.[31] This
308 population was generated from a cross between two parental lines belonging to the species
309 *P. fulvum* with a wide genetic diversity and high frequency of polymorphisms of great
310 utility for the construction of the linkage map and the detection of QTLs. This newly
311 integrated genetic map, which contains SilicoDArT, SSRs, STSs and SNPs markers, has
312 been used previously to study the resistance of wild peas to rust (*Uromyces pisi*).[31]

313 To the best of our knowledge, the present study is the first to identify genomic
314 regions that control PA resistance in wild peas. Significant differences were found in the
315 tolerance to infestation between the parents of the evaluated cross, noting that the sensitive
316 parent (IFPI3260) suffered greater damages than the tolerant parent IFPI3251. The tips
317 were the plant tissue in which *A. pisum* showed the highest proliferation and the symptoms
318 appeared quickly and clearly. The apex is a region with an active development in plants,
319 which imply a local over accumulation of photo-assimilates that allow the growth and the
320 plant development. PA physiology determines that the insects move through the plant in
321 search of the most nutritious feeding areas, among which the apexes are particularly
322 attractive.[10,45] In addition, the architecture of this part of the plant provides hiding places
323 among the new leaflets. PA population growth (*Reprod*) was faster at the tips of the tolerant
324 parent IFPI3251 than in the sensitive IFPI3260, although the final reproduction rate

(*Reprod*$_{25}$) was lower on the tolerant parent. The damage caused by the PA was less important in the tolerant parent in both the apexes and in the rest of the plant with respect to the damage observed in the sensitive parent. The presence of resistance/tolerance mechanisms acting on IFPI3251 to cope with the attack could force PAs to group around the apexes (since it is the most nutritious part of the plant) to feed properly, not without suffering the effect of the defense mechanisms. This hypothesis could explain the fact that a large number of aphids concentrated in the apexes of the tolerant parent and not in the rest of the plant. In fact, the aphids observed on IFPI3251 had small sizes and took longer to mature (adults could be mistaken for late-stage nymphs due to their small size) (Carrillo-Perdomo, personal communication). As expected, the values scored for the traits evaluated related with plant damage caused by PA infestation (*Chlor*, *Dam*, *TipDam* and *Wilt*) were positively correlated with each other (Table 1). In the case of *Chlor-Dam* and *Tip-Dam,* the correlation was significantly strong and moderate, respectively (Table 1). The appearance of chlorosis in the infested areas is one of the first symptoms that can be visualized after phloem suction by aphids. This is followed by a decrease in turgor and wilting of the affected parts and, finally, the wilting of the whole plant. With this work, we wanted to collect as much information as possible about the effects that the attack have on peas and that is why the variables evaluated follow the natural timeline of the infestation. In the same way, the traits evaluated related to the ability of PAs to reproduce and, therefore, to fast develop on the plant (*Reprod* and *Reprod25*) were positively and significantly correlated (Table 1). In addition, a significant positive correlation was also observed between *Reprod* and *TipDam* (Table 1). This is logical because the preferred places for aphids to feed are the apexes of the plants due to their nutritional richness and their intricate architecture that offers them refuge.

The traits assessed in the RIL population have allowed the identification of eight QTLs, confirming the complex nature of the resistance. The major QTLs named *ApV* (*ApV.1, ApV.2* and *ApV.3*) were located in the same region of LGV (peaks at 151.9, 154.6 and 156.1 cM, respectively) and explained a high percentage of the phenotypic variation of *Chlor, Dam, TipDam* and *Wilt*, highlighting its importance in *P. fulvum* tolerance against pea aphid (Table 4, Fig. 3e). We found that LGV of our *P. fulvum* genetic map corresponds to LG6 of the *P. sativum* genetic map.[4,34,46,47] The search of potential candidate genes by aligning the sequence of the molecular markers linked to the identified *ApV* QTLs with the Unigene database[43] highlighted the transcript "PsCam025546" (IPR001940: Peptidase S1C, HrtA/DegP2/Q/S; IPR009003: Serine/cysteine peptidase, trypsin-like; 153.3 cM) (Suppl. Table 1) that corresponded to the DArT marker "3539215". This serine-type peptidase expressed in the common bean is related with the disease resistance (R) gene cluster B4 highly expressed in pea leaves and shoots, which is one of the largest R clusters known in *Phaseolus vulgaris* L.[48] The *ApV* QTLs identified in LGV of *P. fulvum* were also closely located to three resistance genes: *er1* that confers pre-penetration resistance to *Erysiphe pisi* D.C.,[49] *sbm-1* that provides resistance against seed-borne mosaic virus[50] and *ppi1* that confers resistance to *Pseudomonas syringae* pv. *pisi* (S.) Y.[51] It is noteworthy to mention the potential relation between the QTLs identified in LGV of *P. fulvum*, which is syntenic to LG6 of *P. sativum* and consequently to chromosome (Chr) 2 and 6 of *M. truncatula*,[26] with the resistance QTLs to cowpea aphid (*Aphis craccivora* K.) and spotted alfalfa aphid[52,53] mapped in Chr 2 and Chr 6 of *M. truncatula*, respectively. However, the detection of several QTLs separated from each other by such a small distance could be due to inaccuracies in phenotyping. Therefore, more in-depth analyzes are needed to corroborate the existence of several clustered genes or the action of a single gene.

Since we have identified nearby genomic regions involved in controlling the damage produced by insects to the plant (*Chlor*, *Dam*, *TipDam* and *Wilt*) (Table 4, Fig. 3), it seems clear that this region of LGV is involved in the physiological response of wild pea to PA attack. As mentioned above, some of the variables evaluated correlated with each other (Table 1), which was expected to lead to the co-location of QTLs. However, four of the eight QTLs identified in this study did not co-locate with other QTLs (*ApI*, *ApII*, *ApIV.1* and *ApIV.2*; which will be described below) (Table 4, Fig. 3). In the case of complex inheritance traits such as resistance/tolerance, the success of breeding programs lies in the identification of a battery of candidate genes with which to confront pathogens. For this, it is important to decompose the response of the plant by identifying the different stages of the disease/attack and evaluating the symptoms in order to locate the different genomic regions involved in the triggering of the defense mechanisms and which, therefore, lead to the identification of different candidate genes that act sequentially as barriers. Of course, this must avoid duplicities but implies that parts of the evaluated variables share some level of correlation and that part of the QTLs will co-locate. This far from subtracting credibility to the identified QTLs gives them solidity. However, the most important result of this approach is that in this way we are able to identify singular genomic regions involved in particular defense mechanisms that would go unnoticed with a more generalized evaluation. The associations of many resistance genes organized into clusters have been well studied in other species such as maize[54] or barley[55-57] in which the *Rpl* cluster and *Mla* clusters have been identified, respectively. The fact that resistance genes located closely can confer resistance against different pathogens is not surprising, since members of the same gene family often maintain only partial redundancy while retaining a shared set of conserved functions (structural motifs needed to work in similar pathways, like disease resistance) but

acquiring unique specificities that allow them to respond to unique signals.[58,59] Thus, clustering of resistance genes suggests that the events of duplication, recombination and multiple rearrangements during evolution may have contributed to the development of new resistance specificities.[60] Aphid resistance genes are usually grouped into clusters in the same region of the chromosome and combined with other genes that confer resistance to other insects and pathogens.[61] This is the case of barrel medic (*M. truncatula* G.), which is one of the legume species best studied for its general resistance to aphids and especially to *A. pisum*.[62] In this species, the major *loci AKR*, *TTR* and *RAP1* confers resistance to three different aphid species: the bluegreen aphid (*A. kondoi*S.), the spotted alfalfa aphid (*Therioaphis trifolii* f.s. *maculate* M.) and the pea aphid (*A. pisum*) respectively. They have been mapped in CC-NBS-LRR-rich regions on chromosome 3 separated approximately 40 cM.[15,17] Moreover, this region houses a quantitative loci for the reduction of the dry weight of the plant after the infestation of PA and for the antibiosis against the spotted alfalfa aphid.[3,53] Other examples are found in apple (*Malus domestica* B.) in which the genes *Er1* and *Er3* for the resistance to the woolly apple aphid (*Eriosoma lanigerum* H.) are mapped in the same genomic region as a major gene for powdery mildew resistance [*Podosphaera leucotricha* (E.&E.) S.][63], or in tomato (*Solanum lycopersicum* L.) where the *Mi-1* gene for resistance to root-knot nematode [*Meloidogyne incognita* (K.&W.)] and potato aphid (*Macrosiphum euphorbiae* T.) are located in the short arm of chromosome 6 together with a cluster of resistance genes against fungi, oomycetes and nematodes.[64] In the present work, most of the identified minor QTLs were also located around regions that harbor genes involved in resistance. Thus, the search in the Unigene data set revealed the transcript "PsCam000168" highly expressed in leaves (corresponding to the DArT marker ""3559882_5:G>T") homologous to the BTB/POZ domain-containing protein SR1IP1

whose expression is controlled by the gene *SR1IP1* of *Arabidopsis thaliana* in the minor QTL *ApI* (*TipDam*) identified in LGI (peak at 208.8 cM) (Table 4, Fig.3a, Suppl. Table 1). The *SR1IP1* gene is involved in plant immunity regulation through ubiquitin-mediated modulation of Ca2+–calmodulin–AtSR1/CAMTA3 signaling.[65] Ubiquitination is a common cellular process that modifies proteins after translation and also participates in the responses of plants to stress through immune signaling pathways, playing an important role in the response to aphid attacks.[66,67] The ubiquitination of calmodulins is of great importance for intracellular signaling, since their concentration influences the immunity of the plant.[67] On the other hand, in correspondence with the sequence of the DArT marker "3567350" located in the *ApIII* QTL (*Chlor* and *Dam*) in LGIII of *P. fulvum* with a peak at 194.1 cM (correspondent to LG3 of *P. sativum*) (Table 4, Fig. 3c) were located both transcripts "PsCam035280" and "PsCam033538" (IPR000767: Disease resistance protein; 192.7 cM) that are highly expressed in the stem and peduncle of leaves (Suppl. Table 1). These sequences of transcripts are homologous to the *RGC20* gene involved in the resistance to citrus tristeza virus[68] and to the JHL06P13.14 protein of *Jatropha curcas* L. involved in the defense response.[69] In addition, the QTL region was located near to the *Er2* gene that provides post-penetration resistance to *E. pisi*[70] and the *fw* gene that confers resistance to *Fusarium oxysporum* f. sp. *pisi* S. & H. race 1.[71] Thus, our results suggest the involvement of the two subfamilies of R proteins TIR-NBS-LRR and CC-NBS-LRR in *P. fulvum* resistance against PA attack, which induces systemic acquired resistance (SAR), protecting the entire plant form further attacks.[72,73]

Finally, the minor QTLs *ApIV* (*ApIV.1, ApIV.2*) were identified in LGIV (Table 4, Fig. 3d) that correspond to the LG2 region of *P. sativum* where the *sbm-2* and *mo* genes, involved respectively to the resistance to pea seed-borne and bean yellow mosaic virus, are

located.[74] The transcript "PsCam036654" (IPR011333:BTB/POZ fold IPR013069:BTB/POZ IPR020683: Ankyrin repeat-containing domain) differentially expressed in the nodules was identified in *ApIV.1* (*Reprod*$_{25}$, peak at 78.8 cM), while "PsCam035644" (IPR013210: Leucine-rich repeat-containing N-terminal domain, type 2; IPR015765: Toll-like receptor 7, leucine rich repeat-containing; IPR020635: Tyrosine-protein kinase, catalytic domain; IPR020669: MAP/microtubule affinity-regulating kinase 3) differentially expressed in the root system and peduncle of leaves was identified in *ApIV.2* (*Wilt*, peak at 90.2 cM) (Suppl. Table 1). The DArTseq SNP markers "3568629" and "3560376_29:G>C" were related to the above markers, respectively (Suppl. Table 1). The transcript "PsCam036654" sequence resulted to be homologous to the *NPR4* gene of *Arabidopsis thaliana* that acts as a receptor of salicylic acid in plants in order to regulate the defense response via acquired resistance through ubiquitination.[67,75,76] Particularly, it has been reported to be involved in the early response to *Myzus persicae* S. feeding.[77] The "PsCam035644" transcript sequence resulted to be homologous to the *PEPR2* gene that is expressed locally in response to wounds.[78] *PEPR2* controls the expression of leucine-rich repeat receptor-like protein kinases that contribute to systemic defense response in *Arabidopsis*[79,80] and amplify the ethylene signal pathway to enhance the immune response.[81] Ethylene (ET), together with the signaling pathway of salicylic acid (SA) and jasmonic acid (JA) induces alterations in the expression of defense genes, which leads to specific metabolic changes that improve the innate defense responses of the plant against aphid attacks.[82-84] As an example, resistance to *M. persicae* in *Arabidopsis* has been related to an increasing level of ET and the expression of the ethylene insensitive 2 (*EIN2*) gene, a bifunctional transducer of ET and JA signal transduction, during the early response to the aphid attack.[77,85] ET-dependent responses are elicited by the oral secretion of specific

compounds that are transferred during aphid feeding and include the emission of specific volatile organic compounds such as ET, which act as an indirect defense, the accumulation of phenolic compounds, or the inhibitory activity of proteinases. Besides being ET regulated, these defenses strongly depend on the wound-hormone JA widely known for promoting defense against insects.[86] Instead of being the main elicitor of defense responses, ethylene modulates sensitivity to a second signal and its downstream responses.[87] Carrillo et al.[10] also reported on the involvement of wound signaling molecules related to the JA defense pathway in response to PA infestation in the wild relative *P. sativum* ssp. *syriacum*. Regarding the QTL *ApII* (*Dam*) with a peak at 94 cM (Table 4, Fig.3b), no potential candidate genes of interest and no previously identified genes involved in resistance were identified.

5. CONCLUSION

Our work shows how the survival of *P. fulvum* after the attack of PA depends on the triggering of a multi-component protection strategy that implies a quantitative tolerance. The genomic regions associated with the tolerance responses of *P. fulvum* to PA infestation reported in this work have provided six potential candidate genes that could be useful in genomic assisted breeding (GAB) after validation in the future. In addition, the syntenic relationships found between our map and the *P. sativum* and *M. truncatula* genetic maps[26] have allowed the identification of potentially conserved aphid resistance genes among these species and *P. fulvum*. Therefore, this work contributes significantly to the understanding of the genetic basis of pea resistance to PA attack and provides new tools that, after functional validation, could be used to overcome the susceptibility of plants.

AUTHOR CONTRIBUTION STATEMENTS

ECP and DR designed the experiments. ECP developed plant trials. EB and MC carried out the RILs DNA extractions. AK and JC performed DArTseq analysis, marker selection as well as established the synteny between *P. fulvum* and *M. truncatula*. EB and MC carried out the linkage groups and QTL analysis. EB and ECP wrote most of the manuscript. DR contributed to the interpretation of results and writing of the manuscript. AK and JC also contributed to critical reading.

COMPLIANCE WITH ETHICAL STANDARDS

Ethical standards The authors state that all experiments in the study comply with the ethical standards in EU.

Conflict of interest The authors declare that they have no conflict of interest.

ASSOCIATED CONTENT

Additional information may be found in the online version of this article

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Table 1. Pearson's linear correlation coefficient between plant responses to *Acyrthosiphon pisum* infestation.

	Chlor	*Dam*	*Reprod*	*TipDam*	*Wilt*
Dam	0.8887***				
Reprod	-0.3984***	0.3961***			
TipDam	0.3608***	0.3617***	0.7148***		
Wilt	-0.1210 ns	-0.0890 ns	-0.3201***	0.6946***	
Reprod25	-0.5708***	-0.5941***	0.6949**	-0.2135ns	0.2451*

Significant at ***P = 0.001, **P = 0.01, *P = 0.05; ns, not significant.

Table 2. Map features of IFPI3260 x IFPI3251 linkage map.*

Linkage group	Markers			Unique position	Distance (cM)	Average density	Larger gap
	DArTseq derived	Others	Total				
LGI	1,790	2	1,792	163	307.41	1.89	7.14
LGII	2,058	5	2,063	181	349.48	1.93	8.45
LGIII	1,790	16	1,806	152	281.02	1.85	6.85
LGIV	1,760	4	1,764	121	219.54	1.81	6.76
LGV	1,612	4	1,616	139	265.57	1.91	13.04
LGVI	1,344	3	1,347	107	223.27	2.09	8.82
LGVII	1,667	3	1,670	131	231.15	1.76	7.25
Total	*12,021*	*37*	*12,058*	*994*	*1,877.45*	*1.19*	*8.33*

*Data were originated by authors and previously published in Barilli et al. (2018).[31]

Table 3. Summary and description of reference markers used to generate de *P. fulvum* composite map, including their linkage group assignment and position on *P. fulvum* map and their correspondence to *P. sativum* linkage groups and chromosomes.

Linkage Group (*P. fulvum*)	Marker type			Linkage Group (*P. sativum*)
	DArTseq derived	Others	Total	
LGI	20	2	22	VII
LGII	14	5	19	V
LGIII	12	16	28	III
LGIV	5	4	9	II
LGV	10	4	14	VI
LGVI	18	3	21	I
LGVII	10	3	13	IV

Table 4. Quantitative trait loci (QTL) for tolerance to *Acyrthosiphon pisum* detected by composite interval mapping (CIM) and multiple interval mapping (MIM) in the RIL population derived from the cross IFPI3260 x IFPI3251.

Trait[a]	LG[b]	QTL	Peak[c]	Flanking markers	LOD[d]	Add[e]	R^{2f}
TipDam	I	*ApI*	208.8	3568590 - 3569349	3.08	-2.42	17.2
	V	*ApV.2*	154.6	3537510 - 3534526	11.67	4.23	51.2
Total							*68.4*
Dam	II	*ApII*	94.0	3535012 - 3536533	3.00	12.9	17.9
	III	*ApIII*	194.1	3535795 - 3537104	4.01	-14.6	23.02
	V	*ApV.1*	151.9	3538656 - 3537510	9.86	21.3	47.7
Total							*88.62*
Chlor	III	*ApIII*	194.1	3535795 - 3537104	3.55	-79.1	19.6
	V	*ApV.3*	156.1	3537754 - 3534511	5.11	99.4	26.9
Total							*46.5*
Wilt	IV	*ApIV.2*	90.2	3535628 - 3535348	3.13	80.2	17.5
	V	*ApV.1*	151.9	3538656 - 3537510	8.48	12.2	40.6
Total							*58.1*
Reprod$_{25}$	IV	*ApIV.1*	78.8	3568629 - 3536355	3.33	12.7	17.0
Total							*17.0*

[a] *TipDam* tip with damage (AUDPC), *ChLsev* severity of leaf chlorosis (AUDPC), *Dam* plant damaged by aphid attack (AUDPC), *Chlor* plant with chlorosis (%), *Wilt* portion of plant presenting wilting (AUDPC), *Reprod*$_{25}$ number of instars nymphs present at the apical part of the plant 25 days after infestation.

[b] *LG* linkage group

[c] *Peak* QTL position (cM)

[d] *LOD* the peak LOD score

[e] *Add* the additive effect

[f] R^2 proportion of phenotypic variance explained by the respective QTL.

Figure legend

Figure 1. Frequency distribution of pea aphid response among F_7 RILs progenies from the *P. fulvum* cross (IFPI3260 x IFPI3251). AUDPC calculated from: (A) % of the plant with chlorosis (*Chlor*), (B) % of the tip with damage (*TipDam*), (C) % of the plant damaged by aphids attack (*Dam*), (D) % of the plant presenting wilting (*Wilt*), (E) number of aphid nymphs present at the apical part of the plant (*Reprod*). (F) Final reproduction rate calculated as the number of nymphs on the apical part of the plant 25 days after infestation ($Reprod_{25}$). Arrows indicate the means of the tolerant (IFPI3251) and the susceptible (IFPI3260) parental lines.

Figure 2. Pea genetic linkage map constructed from a population formed by 84 F_7 recombinant inbred lines (RILs) derived from the cross between *Pisum fulvum* accessions IFPI3260 and IFPI3251. The bar shows the genetic distance (cM). Anchor markers are reported in red. Results based on data previously published by Barilli et al. (2018).[31]

Figure 3. Likelihood plots of consistent quantitative trait loci (QTLs) for plant tolerance to pea aphid on linkage groups (LG) I (a), LGII (b), LGIII (c), LGIV (d) and V (e), using MapQTL in the IFPI3260 × IFPI3251 RIL population. Significant LOD thresholds were detected based on 1000 permutations. Absolute positions (in cM) of the molecular markers along LGs are shown on the vertical axes. Anchor markers are reported in red. *Dam*: % of damage severity; *Chlor*: % of plant with chlorotic area; *TipDam*: % of tips with damage; *Wilt*: % of plants presenting wilting; $Reprod_{25}$: number of nymphs on the apical part of the plant 25 days after infestation. Arrows indicate the means of the tolerant (IFPI3251) and the susceptible (IFPI3260) parental lines.

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829 **Supplementary material**

830 **Supplementary Table 1.** Sequences of the DArTseq markers linked to the genomic
831 regions controlling PA tolerance in *P. fulvum* which correspond to transcripts linked to
832 potential candidate genes, their correspondence to InterPro and UniProt databases.

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