Euphytica (2018)214:236
https://doi.org/10.1007/s10681-018-2316-y

Expressional and positional candidate genes for resistance to *Peyronellaea pinodes* in pea

S. Fondevilla · M. D. Fernández-Romero · Z. Satovic · D. Rubiales

Received: 16 August 2018 / Accepted: 20 November 2018

Abstract Ascochyta blight is one of the most damaging pea diseases. Resistance to this disease in pea is quantitative, being governed by several genes with minor effect. Knowledge of the genes controlling resistance would allow their pyramiding and tracking in breeding programs. In previous studies, a number of QTLs associated with resistance to this disease have been identified. Complementarily, genes differentially expressed in resistant reactions have been identified. However, the actual genes controlling resistance, underlying these QTLs are unknown. Previously, genes with a putative involvement in defense and located into QTLs associated with resistance to *P. pinodes*, have been postulated as candidate genes. This study wanted to go a step forward, being the first report of candidate genes involved in defense that, besides being located in a genomic region controlling resistance, are also differentially expressed in resistant reactions. With this aim, in this study ten genes previously shown to be induced after infection in the resistant accession P665 were selected and mapped in the RIL population P665 × Messire, previously used to identify QTLs for resistance to this disease. In addition, another gene, that according to other pea maps, could be located into a QTL associated with resistance in this RIL population, was also mapped. Single-marker analysis revealed that five candidate genes showed a significant correlation with resistance traits, being also located in a genomic region showing an increased LOD for the corresponding trait. Furthermore, two of them were in the 2-LOD interval of QTLs associated with resistance traits.

Keywords Ascochyta blight · Pea · Candidate resistance genes · Marker-assisted selection · QTL mapping · *Pisum sativum*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10681-018-2316-y) contains supplementary material, which is available to authorized users.

S. Fondevilla (✉) · M. D. Fernández-Romero · D. Rubiales
Institute for Sustainable Agriculture, CSIC, Avda. Menéndez Pidal s/n, 14004 Córdoba, Spain
e-mail: sfondevilla@ias.csic.es

Z. Satovic
Department of Seed Science and Technology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, 10000 Zagreb, Croatia

Z. Satovic
Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), 10000 Zagreb, Croatia

Introduction

Legume cultivation is strongly hampered by the occurrence of *Ascochyta* blights. Resistances identified so far in the various legume crops against

Published online: 24 November 2018

ascochyta blight is incomplete, being mostly a polygenic traits controlled by quantitative trait loci (QTLs) (reviewed in Rubiales and Fondevilla 2012).

In pea, ascochyta blight is the most destructive foliar disease (Khan et al. 2013). This disease is caused by a complex of fungi, of which, *Peyronellaea pinodes* (synonym *Didymella pinodes*; anamorph: *Ascochyta pinodes*) is the most predominant and damaging pathogen (Tivoli and Banniza 2007).

Only varieties with moderate levels of resistance to ascochyta blight are available. Resistance to ascochyta blight in pea is quantitative, being governed by several genes with minor effects (Rubiales and Fondevilla 2012). Therefore, to obtain varieties with increased level of resistance, combining the different minor genes governing resistance into a variety is desired. However, unfortunately, in this kind of polygenic traits, the genotype of an individual can not be inferred from the phenotype, as different combinations of these minor genes can produce the same phenotype. This makes difficult pea breeding for resistance to this important disease. Knowledge of the genes governing resistance to *P. pinodes* would allow tracking these resistance genes in breeding programs by marker assisted selection, facilitating gene pyramiding. However, these genes are unknown.

QTLs involved in resistance to this disease have been identified in several pea crosses (Timmerman-Vaughan et al. 2004; Tar'an et al. 2003; Prioul et al. 2004; Zhang et al. 2006; Fondevilla et al. 2008; Carrillo et al. 2014; Jha et al. 2016) and several of these QTLs have been found to be common in different genetics backgrounds (Fondevilla et al. 2011a). Even the mechanisms of resistance underlying these QTLs have been revealed for some of them (Carrillo et al. 2014). However, the resistance genes underlying these QTLs are still unknown.

Several approaches have been used to identify candidate genes for resistance to ascochyta blight. Genes located into the QTLs associated with resistance with a putative involvement in defense against pathogens have been postulated as candidate genes. These "positional" candidate genes have been identified considering the putative function of the gene-based markers located into these QTLs (Carrillo et al. 2014), or selecting a series of genes with a putative function in defense and checking by mapping whether these genes are located into a QTL associated with resistance (Prioul-Gervais et al. 2007; Timmerman-Vaughan et al. 2016). However, taking into account the wide genomic regions covered by the QTLs associated with ascochyta blight identified so far, the fact that a gene with a putative function in defense is located into a QTL associated with resistance does not imply that this gene is doubtlessly the gene governing the resistance conferred by this QTL. Jha et al. (2015) studied the correlation between the alleles of five of these "positional" candidate genes and the resistance trait in a set of lines with different level of resistance to ascochyta blight, showing that an allele of each of two of the genes (*PsDof1* and *RGA-G3Ap103*) were more common in resistant lines. However, further evidences are needed for demonstrating that these candidate genes are really the genes controlling resistance, as this association may be due only to the fact that these genes are located into a QTL governing resistance and, therefore, physically linked to the real genes governing resistance.

In addition to QTL mapping, genes differentially expressed in resistant interactions with *P. pinodes* have been identified by transcriptomic studies, providing another source of candidate genes for resistance to this pathogen. Thus, cDNA from the resistant pea accession P665 and the susceptible one Messire, inoculated with *P. pinodes*, was hybridized with the microarray Mt16KOLI1Plus. This microarray contained 16,470 different 70 mer oligonucleotides from *Medicago truncatula*, that represented all tentative consensus sequences (TCs) of the TIGR *M. truncatula* Gene Index 5 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago). This study identified 346 genes that were differentially regulated between the resistant and the susceptible line (Fondevilla et al. 2011b) after infection with *P. pinodes*. A further study identified 509 pea genes induced or repressed in the resistant line P665 after inoculation with *P. pinodes* using SuperSAGE technique (Fondevilla et al. 2014). These transcriptomic studies revealed the molecular mechanisms and metabolic pathways involved in resistance to *P. pinodes* in pea and provided a set of "expressional" candidate genes for resistance to ascochyta blight. The key question is which of these hundreds of genes differentially regulated in resistant reactions are really controlling the resistance.

The objective of this study was to merge both: (1) knowledge of the genomic regions involved in the control of resistance to ascochyta blight in pea and (2)

knowledge of the genes differentially regulated in resistant reaction to *P. pinodes*, to identify genes that are both differentially expressed in resistant reactions and located into a QTL associated with resistance. These "positional" and "expressional" candidate genes will be more probably involved in the control of resistance than solely positional or expressional candidate genes. With this aim, genes differentially expressed in the resistant accession P665 during infection with *P. pinodes*, according to the microarray and/or SuperSAGE studies mentioned above, were mapped in the RIL (Recombinant Inbred Line) population P665 × Messire to check whether they co-localized with QTLs associated with resistance to *P. pinodes*.

Materials and methods

Plant material and resistant traits analysed

The population used for mapping in this study consisted of 102 F6:7 RILs derived from a cross between the *P. sativum* ssp. *syriacum*. accession P665 and the *P. sativum* ssp. *sativum* cv. Messire. P665 shows incomplete resistance to *P. pinodes*, while cv. Messire is highly susceptible (Fondevilla et al. 2005). This population had been previously used to identify QTLs associated with different traits related to *P. pinodes* resistance: visual evaluation of disease rating in leaves under controlled conditions, visual evaluation of disease rating in leaves and stems and disease severity under field conditions during two growing seasons and different traits characterizing resistance microscopically (colony establishment, lesion size, host epidermal cell death and host protein cross-linking). A complete description of these traits, as well as the results of their assessment in the RIL population and parental lines can be found in Fondevilla et al. (2008) and Carrillo et al. (2014).

Candidate gene selection and polymorphism detection

Ten genes that were differentially regulated in the resistant line P665 during interaction with *P. pinodes* (Fondevilla et al. 2011b, 2014) and that could be relevant in the resistance to *P. pinodes* in pea, according to their function, were selected as "expressional" candidate genes for resistance to *P. pinodes*. In addition, the resistance gene analogous *RGA1.1* was also included in the analysis because, according to Timmerman-Vaughan et al. (2002), *RGA1.1* was in the proximity of the QTL associated with resistance to the ascochyta blight complex *Asc3.1*, which may coincide with the QTL *MpIII.2* identified in our map P665 × Messire (Fondevilla et al. 2008).

These eleven genes were surveyed in the RIL population P665 × Messire. The list of markers analysed, amplification conditions, primers used for the amplifications and the method used to score the polymorphism are included in Table 1. For some genes, primers to amplify the genes had been already reported by other authors and were used to amplify the genes in this study. In other cases, primers were designed in this study using the corresponding pea gene sequence. In the case of the genes *JERF1* and *EREBP*, for which no clear homologous pea genes were identified in the databases, the primers were designed using the *Medicago truncatula* sequence corresponding to the Mt16KOLI1Plus microarray probes found to be up-regulated in P665 inoculated plants compared to Messire in Fondevilla et al. (2011b): MT015261 and MT009598, respectively. While the primers designed for *EREBP* amplified in pea a clear unique band, the primers designed for *JERF1* showed some inconsistencies in the amplifications, amplifying sometimes a weak band in addition to the main strong band. Therefore, to increase the specificity and accuracy of the amplification, the main consistent band was sequenced and new primers were designed using the resulting pea sequence as template.

PCR amplifications were performed in a total volume of 15ul containing 500 ng of genomic DNA, buffer 10 × NH_4 reaction buffer (670 mM Tris–HCl (pH 8.8 at 25 °C), 160 mM $(NH_4)_2SO_4$, 100 mM KCl, 10, 0.1% stabilizer), 125 µM of each dNTP, from 1.5 to 2.5 mM $MgCl_2$ depending on the primer (Table 1), 0.3 uM of each specific primer and 0.375 unit of BIOTAQ DNA polymerase (Bioline). Annealing temperatures and $MgCl_2$ concentration were optimized for each pair of primers in order to obtain a unique consistent band in the amplifications. The thermal profile for PCR consisted of an initial denaturalization at 95 °C for 5 min followed by 30 cycles of 95 °C for1 min, the required annealing temperature for 1 min, 72 °C for 1 min with a final

Table 1 List of markers analysed, primers used for the amplification, amplification conditions and method used to score the polymorphism

Marker	Genbank accession	Function	Primers (5′-3′)	Annealing temperature	Cl_2Mg (mM)	Polymorphism detection
Blec4	L11745	Lectin	Fw: AATTTCCCCAAGATCACTCCT Rv: AGGACCATGAATGGATGTTGT	65 °C	2	MSE digestion
cOMT	CD858528	Caffeic acid O-methyltransferase	Aubert et al. (2006)	59 °C	1,5	Fragment analysis
DRR206-d	U11716	Disease resistance response protein 206-d	Prioul-Gervais et al. (2007)	59 °C	1,5	RsaI digestion
DRR230-b	L01579	Disease resistance response protein 39	Fw: GAGAAGAAATCACTAGCTG Rv: TTTTGAGTGCAGAAACATTTCCA	65 °C	2	Fragment analysis
DRR49a	U31669	*Pisum sativum* disease resistance response protein PR10 (DRR49a)	Fw: CTAGGCAAGCAATTTCTTAGTT Rv: AGACCATCCCCCTTAGCTTT	59 °C	1,5	TaqI digestion
EREBP	MH521778	Putative ethylene responsive transcription factor	Fw: ATCTCATACGCCATCATCTCT Rv: GCTTTCTGACCCCTCATTCT	59 °C	1,5	TaiI digestion
GST	AB087837	Glutathione S-transferase	Fw: GTTCGTCCTCCTCCGCTAACT Rv: CATCAATCTTGTTCAGCTCCTC	59 °C	1,5	TaqI digestion
JERF1	MH521780	Putative ethylene responsive transcription factor	Fw: TGCTTCCTTGGCGTCTTTG Rv: CATAATGCAAAAACAACCAAGT	59 °C	1,5	MboI digestion
PsPOXII	AB193816	Peroxidase	Fw: CTTGGAGGACCCACATGGAT Rv: TCTAATCTCCCCATTAGTCCC	59 °C	2	MboI digestion
RGA1.1	AF123695	Putative NBS-LRR type disease resistance protein	Prioul-Gervais et al. (2007)	65 °C	2,5	Hpy188II digestion
OPR2	AB095739	12-oxophytodienoic acid 10,11-reductase	Matsui et al. (2004)	59 °C	1,5	Monomorphyc

extension at 72 °C for 5 min. Amplification products were first subjected to electrophoresis performed in a 2.5% agarose gels in TBE Buffer. Gel green-stained gels were visualized on an ultraviolet light transilluminator and photographed. When slight different in band size was observed, polymorphism was scored using fragment analysis technique. In this case forward primers were labeled with fluorophores 6FAM (in case of cOMT gene) or HEX (in the case of *DRR230-b* gene) (Sigma-Genosys Ltd.). Amplification products were separated in an automated capillary sequencer (ABI 3130 Genetic Analyzer, Applied Biosystems/HITACHI) at the Genomics Unit of the Central Service of Research Support at the University of Córdoba (Spain). The size of the amplified bands was calculated based on an internal standard DNA (400HD-ROX, Applied Biosystems) with GeneScan software (v 3.x Applied Biosystems) and the results analysed using the Genotyper software (v 3.7, Applied Biosystems). When no band size polymorphism was detected, PCR products amplified in each parental line were purified for direct sequencing using the QIAquick PCR purification Kit (Quiagen GmbH, Hilden Germany). PCR products were sequenced in both directions and the resulting sequences aligned using the Geneious Pro 5.5.6 software (Biomatters Ltd.) in order to detect polymorphisms. Restriction endonucleases sites and the

restriction endonucleases suitable for visualizing the polymorphisms were also identified using Geneious Pro 5.5.6 software.

Analysis of expression profile of *JERF1* gene by qRT-PCR (Quantitative real time PCR)

While the primers developed for the other candidate genes yielded a clear band, the primers initially designed for *JERF1*, based on *M. truncatula* sequence, amplified sometimes a weak band in addition to the main strong band. Therefore, to check that the gene we mapped was really the gene over-expressed in the resistant accession P665, compared to the susceptible Messire, the expression of this gene was analysed by qRT-PCR in control and inoculated plants of P665 and Messire. For this analysis we used cDNA obtained from leaves of non-inoculated and inoculated plants (16 h after inoculation) obtained from the microarray experiment previously mentioned (Fondevilla et al. 2011b). qRT-PCR analysis was carried out as described in Fondevilla et al. (2011b), using "cgtctttgctcggtactgatt" and "tcaaagctccagaggctcat" as forward and reverse primers, respectively, for amplifying *JERF1* gene. These primers were designed using the *Pisum sativum* sequence obtained after sequencing the main amplicon obtained using the *M. truncatula* based primers. The genes TUB, histone H3 and GAPDH were used as reference genes for normalization. The PCR efficiency of each primer pair in each individual reaction was calculated using LingRegPCR 7.5 software and used to calculate an average efficiency (E) per primer pair. This average efficiency was used to calculate the expression in each reaction using the formula Expression = E^{CT}. A normalization index was calculated as the geometric mean of the expression of the reference genes TUB, GAPDH and histone H3 and a relative expression was calculated for each reaction as the ratio of the gene expression of the gene of interest in each reaction against the normalization index.

Map construction and QTL analysis

The framework genetic map (Carrillo et al. 2014) was constructed by MAPMAKER Version 3.0b (Lander et al. 1987) using a LOD score of 5.0 as the threshold for significant linkage. The marker orders were established using MSTMap (Wu et al. 2008) by finding the minimum spanning tree of a graph for each linkage group. MAPMAKER was used to confirm marker order determined by MSTMap. The markers representing candidate genes were added to the map using the 'try' command and the final order of markers on each linkage group was confirmed using the 'ripple' command in MAPMAKER. Map distances were calculated using the Kosambi mapping function (Kosambi 1944). Windows QTL Cartographer V2.5 (Wang et al. 2012) was used to perform single-marker analyses as well as composite interval mapping (CIM) and multiple interval mapping (MIM). The markers used as cofactors in the model were selected by forward–backward stepwise regression. To determine an empirical threshold significance levels for QTL detection 1,000 permutations were performed as described in by Churchill and Doerge (1994). Multiple interval mapping (MIM) (Kao et al. 1999; Zeng et al. 1999) was used to obtain more precise information of QTL effects and positions and to evaluate the presence of digenic epistatic interactions across the QTL pairwise combinations. The initial CIM-derived QTL model was subjected to a search for significant epistatic interactions among QTLs and both main additive effects and their epistatic interactions were tested for significance using the Bayesian information criterion (BIC). One- and two-LOD support intervals for the position of each QTL were calculated as described by Darvasi and Soller (1997). The markers flanking the LOD peaks as well as the 2-LOD confidence intervals were identified.

Results

Gene mapping

After optimizing amplification conditions, the eleven genes selected were successfully amplified in both Messire and P665. For two markers (DRR230-b and cOMT) a slight difference in the size of the band amplified in Messire compared to P665 was observed. Therefore, to score these polymorphisms in the P665 × Messire RIL population, fragment analysis technique was applied. For *RGA1.1*, *DRR206-d*, *GST*, *DRR49a, PsPOXII*, *OPR2, Blec4* and *EREBP* no direct polymorphism was detected and the bands amplified in both parents were sequenced and aligned in order to identify SNPs. In the case of *OPR2*, no SNP

was identified between Messire and P665 and therefore, this gene could not be mapped. By contrast, for the other genes, SNPs between Messire and P665 were identified and used to score the polymorphisms detected using CAPS (cleaved amplified polymorphic sequence) markers (Table 1). In the case of *JERF1*, the primers initially designed to amplify the gene using the *Medicago truncatula* sequence corresponding to the Mt16KOLI1Plus probe MT015261 as template, produced sometimes, in addition to a strong main band, a slight band. In order to increase the specificity of the primers, the strong clear consistent band observed was sequenced and new primers were designed using the corresponding pea sequence as template. The sequence amplified by these new primers yielded a unique clear band in both parents, that was sequenced and used to design a CAPS marker.

In the case of the genes amplified using primers corresponding to pea gene sequences, sequencing of the resulting bands confirmed that the primers used amplified the expected genes. In the case of *EREBP*, amplified using primers designed based on a *M. truncatula* sequence, the sequence of the band amplified in pea (GenBank Accessions No: MH521778 and MH521779) was blasted (BlastX) against UniProtKB/Swiss-Prot, indicating that the gene amplified was, as expected, a putative ethylene responsive transcription factor. The BLASTN of this sequence against TSA (Transcriptome Shotgun Assembly) data base, specifying *Pisum* as hit organism, identified accession JI902274.1 as the most similar sequence,

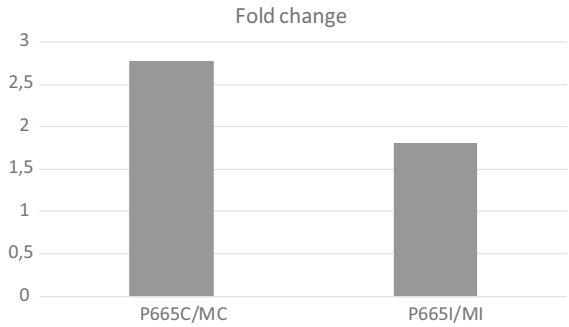


Fig. 1 Fold change of the average of the normalized expression of *JERF1* gene for the treatment "P665 non-inoculated plants (P665C)" with respect to "Messire non-inoculated plants (MC)", and "P665 inoculated plants (P665I)" with respect to "Messire inoculated plants (MI)". Gene expression was analysed by qRT-PCR using TUB, histone H3 and GAPDH housekeeping genes for normalization

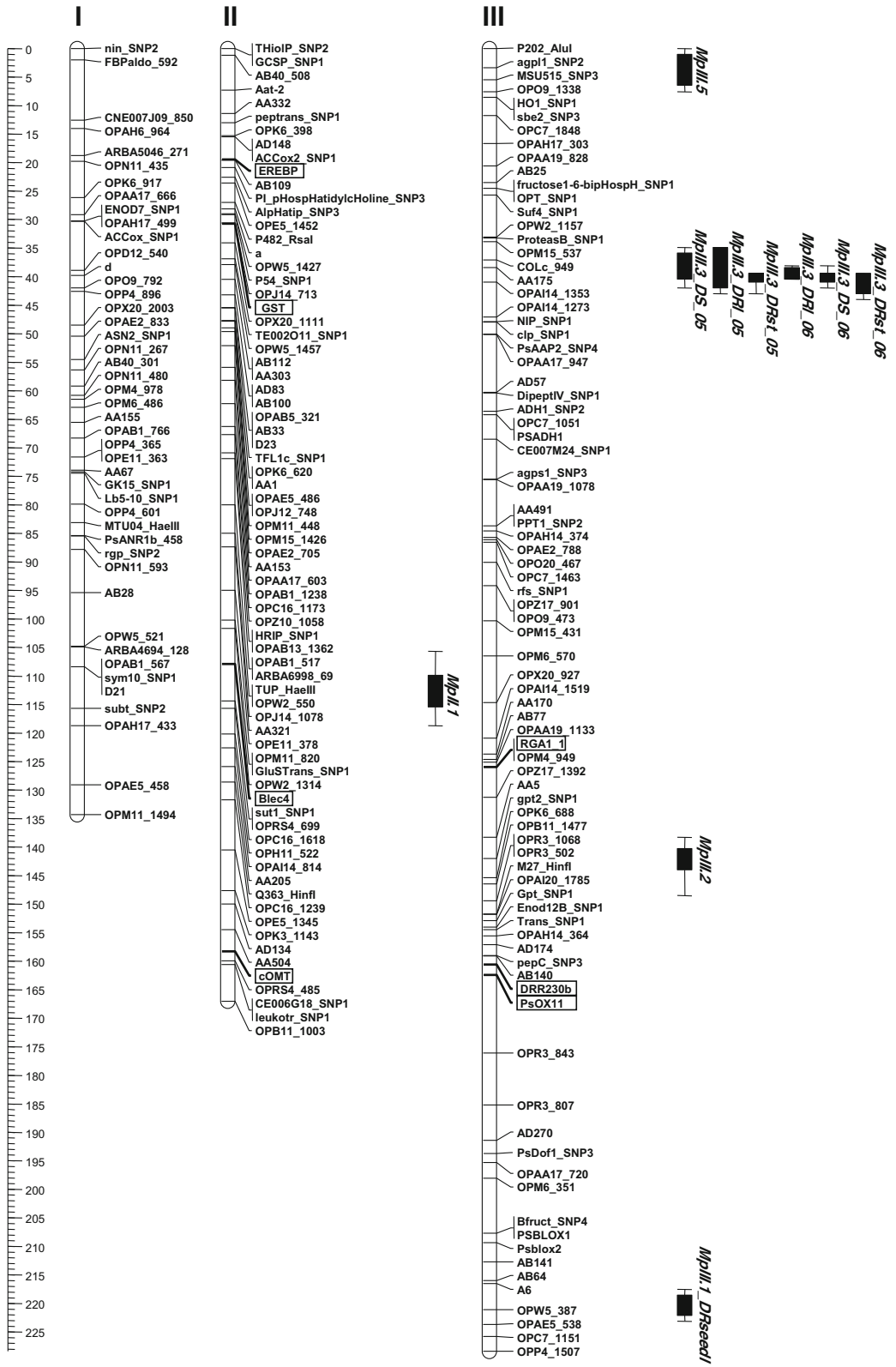
Fig. 2 Pea genetic linkage map constructed from a population ▶ formed by 102 F6:7 recombinant inbred lines (RILs) derived from the cross between the *P. sativum* subsp. *syriacum* accession P665 and the *P. sativum* subsp. *sativum* cv. Messire. Bar positions indicate locations of quantitative trait loci: outer and inner interval corresponding to 1-LOD and 2-LOD support interval are indicated as a full box and a single line, respectively. Candidate genes mapped are shown in bold and framed

showing a 93% sequence cover and 99% identity. For *JERF1*, using a similar approach, BlastX (against RefSeq_protein) showed that the sequence amplified by the primers designed on pea (GenBank Accessions No: MH521780 and MH521781) corresponded also probably to a "ethylene responsive transcription factor". By BLASTN the most similar sequence identified in pea was GAMJ0101029777 (59% sequence cover, 96% sequence identity). According to the qRT-PCR analysis, pea *JERF1* gene was 1.8 times more expressed in P665 plants inoculated with *P. pinodes* than in Messire inoculated plants and 2.76 times more expressed in P665 non-inoculated plants than in Messire non-inoculated plants (Fig. 1).

Markers EREBP, GST, Blec4 and cOMT mapped in pea linkage group (LG) II, RGA1.1, DRR230b and PsOX11 on LGIII, JERF1 on LGIV, DRR49a on LGIV and DRR206-d on LGVII (Fig. 2). Of those, cOMT, RGA1.1, DRR230-b and DRR49a had been previously mapped by other authors. Marker cOMT mapped in the same LG as in Aubert et al. (2006). However, on the map developed by Aubert et al. (2006), cOMT was located between the SSRs AA504 and AD134, while in our map this marker was located after AA504 but in the other direction. RGA1.1, DRR230-b and DRR49a were also located in the same LG as previously reported (Timmerman-Vaughan et al. 2000, Prioul-Gervais et al. 2007). However, the lack of enough common markers between our map and these previous maps hamper a more detailed comparison of the position of the genes.

Relation between candidate genes, QTLs for resistance to *P. pinodes* and resistance traits

Single-marker analysis revealed that five candidate genes showed a significant correlation with resistance traits (Table 2). Thus, *Blec4* and *EREBP* were significantly associated with "percentage of germinated spores causing protein cross-linking in epidermal cells", *DRR49a* was associated with "disease rating


I
II
III
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130
135
140
145
150
155
160
165
170
175
180
185
190
195
200
205
210
215
220
225
nin_SNP2
FBPaldo_592
CNE007J09_850
OPAH6_964
ARBA5046_271
OPN11_435
OPK6_917
OPAA17_666
ENOD7_SNP1
OPAH17_499
ACCox_SNP1
OPD12_540
d
OPO9_792
OPP4_896
OPX20_2003
OPAE2_833
ASN2_SNP1
OPN11_267
AB40_301
OPN11_480
OPM4_978
OPM6_486
AA155
OPAB1_766
OPP4_365
OPE11_363
AA67
GK15_SNP1
Lb5-10_SNP1
OPP4_601
MTU04_HaeIII
PsANR1b_458
rgp_SNP2
OPN11_593
AB28
OPW5_521
ARBA4694_128
OPAB1_567
sym10_SNP1
D21
subt_SNP2
OPAH17_433
OPAE5_458
OPM11_1494
THiolP_SNP2
GCSP_SNP1
AB40_508
Aat-2
AA332
peptrans_SNP1
OPK6_398
AD148
ACCox2_SNP1
EREBP
AB109
PI_pHospHatidylcHoline_SNP3
AlpHatip_SNP3
OPE5_1452
P482_RsaI
a
OPW5_1427
P54_SNP1
OPJ14_713
GST
OPX20_1111
TE002O11_SNP1
OPW5_1457
AB112
AA303
AD83
AB100
OPAB5_321
AB33
D23
TFL1c_SNP1
OPK6_620
AA1
OPAE5_486
OPJ12_748
OPM11_448
OPM15_1426
OPAE2_705
AA153
OPAA17_603
OPAB1_1238
OPC16_1173
OPZ10_1058
HRIP_SNP1
OPAB13_1362
OPAB1_517
ARBA6998_69
TUP_HaeIII
OPW2_550
OPJ14_1078
AA321
OPE11_378
OPM11_820
GluSTrans_SNP1
OPW2_1314
Blec4
sut1_SNP1
OPRS4_699
OPC16_1618
OPH11_522
OPAI14_814
AA205
Q363_HinfI
OPC16_1239
OPE5_1345
OPK3_1143
AD134
AA504
cOMT
OPRS4_485
CE006G18_SNP1
leukotr_SNP1
OPB11_1003
MpIII.1
P202_AluI
agpl1_SNP2
MSU515_SNP3
OPO9_1338
HO1_SNP1
sbe2_SNP3
OPC7_1848
OPAH17_303
OPAA19_828
AB25
fructose1-6-bipHospH_SNP1
OPT_SNP1
Suf4_SNP1
OPW2_1157
ProteasB_SNP1
OPM15_537
COLc_949
AA175
OPAI14_1353
OPAI14_1273
NIP_SNP1
clp_SNP1
PsAAP2_SNP4
OPAA17_947
AD57
DipeptIV_SNP1
ADH1_SNP2
OPC7_1051
PSADH1
CE007M24_SNP1
agps1_SNP3
OPAA19_1078
AA491
PPT1_SNP2
OPAH14_374
OPAE2_788
OPO20_467
OPC7_1463
rfs_SNP1
OPZ17_901
OPO9_473
OPM15_431
OPM6_570
OPX20_927
OPAI14_1519
AA170
AB77
OPAA19_1133
RGA1_1
OPM4_949
OPZ17_1392
AA5
gpt2_SNP1
OPK6_688
OPB11_1477
OPR3_1068
OPR3_502
M27_HinfI
OPAI20_1785
Gpt_SNP1
Enod12B_SNP1
Trans_SNP1
OPAH14_364
AD174
pepC_SNP3
AB140
DRR230b
PsOX11
OPR3_843
OPR3_807
AD270
PsDof1_SNP3
OPAA17_720
OPM6_351
Bfruct_SNP4
PSBLOX1
Psblox2
AB141
AB64
A6
OPW5_387
OPAE5_538
OPC7_1151
OPP4_1507
MpIII.5
MpIII.3_DS_05
MpIII.3_DRl_05
MpIII.3_DRst_05
MpIII.3_DRl_06
MpIII.3_DS_06
MpIII.3_DRst_06
MpIII.2
MpIII.1_DRseed

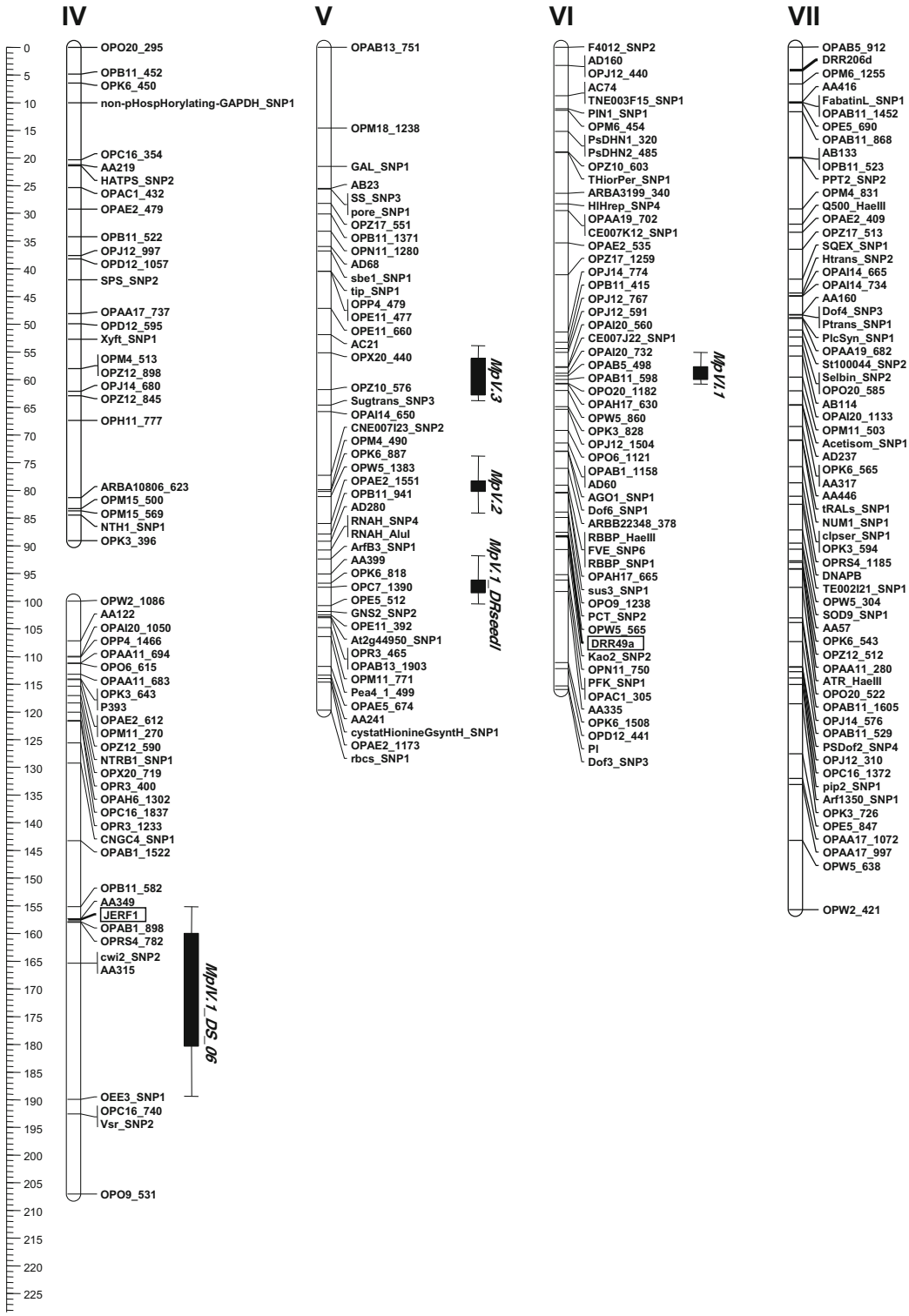


Fig. 2 continued

Table 2 Results of the single-marker analysis of the 10 candidate genes analysed showing the association between the markers and different resistance traits

Marker	LG	P (F)[a]										
		EstC[b]	DeadC	ProtC	LesS	DRseedl	DRl2005	DRst2005	DS2005	DRl2006	DRst2006	DS2006
Blec4	II	0.774	0.874	**0.021**	0.417	0.822	0.799	0.416	0.772	0.407	0.473	0.829
cOMT	II	0.790	0.528	0.868	0.916	0.432	0.067	0.058	0.114	0.819	0.439	0.500
DRR206d	VII	0.932	0.798	0.407	0.444	0.076	0.086	0.377	0.066	0.445	0.177	0.407
DRR230b	III	0.998	0.068	0.972	0.907	0.590	0.452	0.110	0.566	0.559	0.817	0.632
DRR49a	VI	0.992	0.748	0.680	0.766	**0.000**	0.458	0.134	0.681	0.161	0.122	0.433
EREBP	II	0.080	0.504	**0.046**	0.708	0.645	0.666	0.355	0.968	0.553	0.365	0.842
GST	II	0.496	0.807	0.064	0.106	0.634	0.735	0.605	0.718	0.996	0.604	0.6473
JERF1	IVB	0.765	0.864	0.477	**0.039**	0.089	0.102	0.075	0.476	**0.006**	**0.013**	**0.003**
PsOX11	III	0.700	0.060	0.594	0.529	0.536	0.512	0.093	0.778	0.892	0.473	0.934
RGA1_1	III	0.647	**0.009**	0.230	0.926	0.101	0.158	0.160	0.181	0.984	0.867	0.829

Values < 0.05 are shown in bold

[a]P(F): Probability of the F-statistics comparing the hypothesis H_0 (marker not linked to a QTL) to an alternative H_1 (marker linked to a QTL)

[b]Traits: *EstC* percentage of germinated spores that established a colony, *DeadC* percentage of germinated spores causing host epidermal cell death, *ProtC* percentage of germinated spores causing protein cross-linking in epidermal cells, *LesS* lesion size (mm^2), *DRseedl* resistance to *P. pinodes* under controlled conditions, *DRl2005* disease rating on leaves (leaflets and stipules) under field conditions during 2004–2005 season, *DRst2005* disease rating on stems under field conditions during 2004–2005 season, *DS2005* disease severity under field conditions during 2004–2005 season, *DRl2006* disease rating on leaves under field conditions during 2005–2006 season, *DRst2006* disease rating on stems under field conditions during 2005–2006 season, *DS2006* disease severity under field conditions during 2005–2006 season

in leaves of seedlings scored under growth chamber conditions", *JERF1* was associated with four resistance traits: "disease rating on leaves scored under field conditions", "disease rating on stems scored under field conditions", "disease severity estimated under field conditions" and "lesion size" and *RGA1.1* was associated with "percentage of germinated spores causing host epidermal cell death".

After including the candidate genes, a new QTL analysis was carried out. The results of this analysis are summarized in Table 3. QTLs identified were in general the same, they were located in similar positions and explained similar percentages of the phenotypic variation of the resistance traits as in Carrillo et al. (2014). However, two QTLs previously identified in Carrillo et al. (2014), associated with the trait "percentage of germinated spores causing host epidermal cell death", *MpIII4* and *MpIII5*, did not reach the required LOD threshold for this trait in the new QTL analysis. By contrast, *MpIII.5* reached the required LOD threshold, for the trait "percentage of germinated spores causing protein cross-linking in epidermal cells". This QTL explained the 13.56% of the phenotypic variation of this trait (Table 3). The candidate gene *Blec4* was in the 2-LOD interval of the QTL *MpII.1* and *JERF1* in that of *MpV.1_DS_06*. The other genes associated with the resistance traits were not located into the 2-LOD interval of any declared QTL, but they were all included in a genomic region showing and increase in LOD for their corresponding associated trait that did not reached the required LOD threshold (Online Resource 1). Interestingly, RGA1.1 was located into the previous QTL *MpIII4* (Carrillo et al. 2014) that was associated with "percentage of germinated spores causing host epidermal cell death", although this region in the new QTL analysis reached a LOD value of 2.81 while the LOD threshold was 3.28. No epistatic interactions among QTLs were found for any of the analysed traits using multiple-interval mapping (MIM) as in Carrillo et al. (2014).

Discussion

Previous studies aiming to identify candidate genes involved in resistance to *P. pinodes* reported genes

Table 3 Quantitative trait loci (QTL) for resistance to *Peyronellaea pinodes* detected by composite interval mapping (CIM) and multiple interval mapping (MIM) in the RIL population derived from the cross P665 × Messire

Trait[a]	LG[b]	QTL	Peak-flanking markers	2-LOD interval-flanking markers	Peak[c]	LOD[d]	TLOD[e]	Add[f]	R^{2g}
EstC	V	MpV.2	OPM4_490/OPK6_887	OPAI14_650/OPAE2_1551	79.95	3.78	3.22	− 0.06	15.05
Total									15.05
ProtC	III	MpIII.5	agpl1_SNP2/ MSU515_SNP3	P202_AluI/OPO9_1338	5.45	3.78	3.07	0.06	13.56
Total									13.56
DRseedl	III	MpIII.1_DRseedl	OPW5_387/OPAE5_538	A6/OPAE5_538	221.06	5.59	3.14	− 0.29	13.96
DRseedl	V	MpV.1_DRseedl	OPK6_818/OPC7_1390	RNAH_AluI/OPE5_512	96.83	4.74		− 0.27	14.74
DRseedl	V	MpV.3	OPZ10_576/ Sugtrans_SNP3	AC21/Sugtrans_SNP3	61.84	3.86		− 0.21	10.94
Total									39.64
DRl2005	III	MpIII.3_DRl_05	AA175/OPAI14_1353	OPM15_537/OPAI14_1273	39.43	10.40	3.08	− 0.31	29.54
Total									29.54
DRst2005	III	MpIII.3_DRst_05	AA175/OPAI14_1353	AA175/OPAI14_1273	40.43	13.07	3.14	− 0.41	39.09
DRst2005	III	MpIII.2	gpt2_SNP1/OPK6_688	AA5/OPR3_1068	141.96	4.40		− 0.22	7.12
DRst2005	VI	MpVI.1	OPAB5_498/ OPAB11_598	OPJ12_591/OPAH17_630	59.46	4.05		− 0.22	7.76
DRst2005	II	MpII.1	OPRS4_699/ OPC16_1618	OPW2_1314/OPH11_522	114.41	3.35		0.13	1.84
Total									55.81
DS2005	III	MpIII.3_DS_05	COLc_949/AA175	OPM15_537/OPAI14_1273	37.11	11.43	3.08	− 4.80	37.75
Total									37.75
DRl2006	III	MpIII.3_DRl_06	AA175/OPAI14_1353	COLc_949/OPAI14_1353	40.43	16.20	3.06	− 0.37	45.58
Total									*45.58*
DRst2006	III	MpIII.3_DRst_06	OPAI14_1353/ OPAI14_1273	AA175/OPAI14_1273	41.96	13.38	3.77	− 0.34	46.09
Total									46.09
DS2006	III	MpIII.3_DS_06	AA175/OPAI14_1353	COLc_949/OPAI14_1273	40.43	15.47	3.06	− 4.98	52.34
DS2006	IVB	MpIV.1_DS_06	cwi2_SNP2/AA315	OPB11_582/OEE3_SNP1	65.40	4.11		1.70	7.39
Total									59.73

[a]Traits: *EstC* percentage of established colonies, *ProtC* percentage of germinated spores causing protein cross-linking in epidermal cells, *DRseedl* disease rating in leaves of seedlings scored under growth chamber conditions, *DRl* disease rating on leaves scored under field conditions, *DRst* disease rating on stems scored under field conditions, *DS* disease severity (percentage of the plant area covered by symptoms) estimated under field conditions

[b]*LG* Linkage group

[c]Peak QTL position (cM)

[d]*LOD* the peak LOD score

[e]*TLOD* LOD threshold derived from 1000 permutations at p = 0.05

[f]*Add* the additive effect

[g]R^2 proportion of phenotypic variance explained by the respective QTL (%)

with a putative function in defense and located into the confidence interval of a QTL associated with resistance to this pathogen, or genes differentially expressed in resistant reactions. This is the first study reporting genes that are, located in a genomic region associated with resistance traits, have also a putative function in defense and are, in addition, differentially expressed during resistance. Therefore, although further evidences, as functional analysis in transgenic knock-out or over-expression lines are needed to confirm that these genes are really the genes underlying the resistance conferred by these genomic regions, the identification of the "positional" and "expressional" candidate genes reported in this study represent a step forward toward the identification of

the genes that are controlling resistance to ascochyta blight in pea.

Five of the candidate genes tested, *Blec4*, *EREBP*, *DRR49a*, *JERF1* and *RGA1.1* were associated with resistance traits by single-marker analysis. In addition, although only two of them, *Blec4* and *JERF1* were in the 2-LOD confidence interval of declared QTLs that reached the required LOD threshold, all of these four candidate genes were located in a genomic region showing an increase in LOD for the corresponding resistance traits. Therefore, these genes are postulated as candidate genes for resistance to *P. pinodes* in pea.

Gene *Blec4* codifies a lectin type gene. BlastX of this gene against Refseq_proteins data base showed that the protein corresponding to this gene contained the legume lectins, the arcelin family of lectin-like defense proteins, the LecRK family of lectin-like receptor kinases, concanavalinA (ConA), and an alpha-amylase inhibitor motifs, being a putative member of the Lectin_L_type superfamily. *Blec4* was associated with an increase in protein cross-linking in epidermal cells attached by *P. pinodes*, being located in our study into a QTL controlling this trait. In the case of necrotrophic pathogens plants usually recognize non-specific elicitors that activate a battery of basal defense responses that act against a wide range of pathogens (Fondevilla et al. 2011b). The presence of a LecRK (lectin-like receptor kinases) motif in *Blec4* suggests that this gene could be involved in this kind of recognition. Plants perceive structurally conserved molecules produced by challenging microbes, collectively known as microbe-associated molecular patterns (MAMPs). The perception of MAMPs is carried out by pattern recognition receptors (PRRs) and induces immune response (Desaki et al. 2018). Lectins are a major group of PRRs and consist of structurally heterogeneous proteins with one or more characteristic carbohydrate recognition domains (CRDs) that bind reversibly and specifically to cognate carbohydrate residues present on the pathogen membrane (Kugapreethan et al. 2018). The recognition of *P. pinodes* carbohydrate residues by *Blec4* could led to the induction of mechanisms of defense as covalent reticular links formation between cell walls proteins, called protein cross-linking. This mechanism has proven to be an effective and fast defensive response against pathogens improving cell wall resistance after pathogen attack, stopping or delaying the spread of pathogens, and contributes to the reduction on the size of the lesions caused by *P. pinodes* in pea accession P665 (Carrillo et al. 2014).

Two other candidate genes identified in this study, *JERF1* and *EREBP*, are putative transcription factors belonging to the APETALA2/Ethylene Responsive Factor (AP2/ERF) family, mediators of stress responses and developmental programs (Licausi et al. 2013). The ERF genes examined to date have been shown to be induced by biotic and abiotic stresses, including pathogen infection, salt stress, osmotic stress, wounding, drought, hypoxia, temperature stress and the stress-related hormones ethylene, jasmonic acid and ABA (Licausi et al. 2013). This is in agreement with the induction of these genes after *P. pinodes* infection in our previous transcriptomic studies and with the conclusions obtained from these studies suggesting that ethylene and jasmonic acid pathways are key factor on the resistance against *P. pinodes* in pea (Fondevilla et al. 2011b, 2014). Previously, another gene involved in ethylene pathway, an ethylene receptor-like sequence, was postulated as a candidate gene for resistance to ascochyta blight in chickpea (Madrid et al. 2012).

EREBP was amplified using primers designed on the *M. truncatula* probe found to be differentially regulated in P665 *vs* Messire plants inoculated with *P. pinodes* in our previous microarray experiment (Fondevilla et al. 2011b). Interestingly, the sequencing of the amplicon produced in pea using these primers, showed that this gene showed a 99% identity with the pea sequence accession JI902274.1, a gene that was also found to be up-regulated in P665 after inoculation with *P. pinodes* in our previous SuperSAGE experiment (Fondevilla et al. 2014), giving further evidences of the involvement of this gene in the resistance to *P. pinodes*.

Regarding *JERF1*, qRT-PCR confirmed that the gene we mapped was differentially expressed in the resistant accession P665 compared to the susceptible cultivar Messire, giving similar results as our previous microarray experiment (Fondevilla et al. 2011b). Thus, in both experiments (qRT-PCR and microarray) this gene was around 1.8 times more expressed in P665 than in Messire after inoculation with *P. pinodes*. Furthermore, qRT-PCR showed that this gene was constitutively 2.76 times more expressed in P665 than in Messire. Interestingly, constitutive expression of ETHYLENE RESPONSE FACTOR1 (ERF1)

increases *Arabidopsis* resistance to the pathogens *B. cinerea* and *P. cucumerina* (Lorenzo et al. 2003). The constitutive resistance present in P665 had been previously detected in Fondevilla et al. (2011b), where several genes involved in defense were constitutively at a higher concentration in P665 than in the susceptible cultivar Messire.

JERF1 gene showed 82% identity with the *M. truncatula* accession Medtr8g022820, similarly as the "ethylene responsive transcription factor RAPD2-12" (sequence name 2686586), analysed as a putative candidate gene for resistance to *P. pinodes* by Timmerman-Vaughan et al. (2016), that showed a 85% identity with this *M. truncatula* sequence. In addition, both, the gene analysed by us and that studied by Timmerman-Vaughan et al. (2016), mapped in pea LG IV. Therefore, these two genes may be the same gene, although in Timmerman-Vaughan et al. (2016) this gene was not located into any QTL associated with resistance, while, in our case, *JERF1* was located into a QTL associated with disease severity estimated under field conditions. The amplification of two bands in pea using the primer designed according to the *M. truncatula* sequence corresponding to this gene open also the possibility of the presence of tandem duplication for this gene in pea. By single marker analysis *JERF1* gene was associated with other field resistance traits and also to a reduced size of the necrotic lesions produced by *P. pinodes* on the mesophyll. These evidences suggest a role of this gene in resistance to *P. pinodes* in pea.

Gene *DRR49a* was strongly related to resistance to *P. pinodes* in seedlings under controlled conditions in our study. The protein encoded by this gene, disease resistance response protein Pi49, has been also shown to be up-regulated in response to other pathogens such as *Fusarium solani* f. sp. *phaseoli* (Riggleman et al. 1985), *F. solani* f. sp. *pisi* (Fristensky et al. 1985) and *Pseudomonas syringae* pv. *pisi* (Daniels et al. 1987). In addition, expression of this protein in potato conferred resistance to early dying disease (Chang et al. 1993). This protein is a PR10 protein. This kind of proteins are involved in plant defense responses and have antimicrobial activity and in vitro ribonuclease activity (Park et al. 2004; Liu and Ekramoddoullah 2006). Therefore, the involvement of *DRR49a* gene in resistance to *P. pinodes* in pea is plausible.

Regarding *RGA1.1*, this gene is putative NBS-LRR type disease resistance protein. Although we do not know whether this gene is differentially expressed in P665 resistant reaction, interestingly, *RGA1.1* was located into a QTL, *MpIII.4*, previously found to be associated with an increased epidermal cell death in the cells penetrated by *P. pinodes*. This gene was also associated with this resistance trait by single marker analysis (Carrillo et al. 2014). An increased epidermal cell death is one of the mechanism characterizing P665 resistance against *P. pinodes* (Carrillo et al. 2013), and as pathogen recognition mediated by NBS-LRR type disease resistance proteins usually trigger hypersensitive response, a kind of induced cell death, *RGA1.1* gene can be considered a good candidate gene for *MpIII.4* QTL.

Acknowledgements This research was supported by Projects AGL2014-52871-R and AGL2017-82907-R co-financed by FEDER.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

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

References

Aubert G, Morin J, Jacquin F, Lorindon K, Quillet MC, Petit A, Rameau C, Lejeune-Hénaut I, Huguet T, Burstin J (2006) Functional mapping in pea, as an aid to the candidate gene selection and for investigating synteny with the model legume *Medicago truncatula*. Theor Appl Genet 112:1024–1041

Carrillo E, Rubiales D, Pérez-de-Luque A, Fondevilla S (2013) Characterization of mechanisms of resistance against *Didymella pinodes* in *Pisum* spp. Eur J Plant Pathol 135:761–769

Carrillo E, Satovic Z, Aubert G, Boucherot K, Rubiales D, Fondevilla S (2014) Identification of quantitative trait loci and candidate genes for specific cellular resistance responses against *Didymella pinodes* in pea. Plant Cell Rep 33:1133–1145. https://doi.org/10.1007/s00299-014-1603-x

Chang MM, Chiang CC, Martin WM, Hadwiger LA (1993) Expression of a pea disease resistance response gene in the potato cultivar Shepody. Amer Potato J 70:635–647

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Daniels CH, Fristensky B, Wagoner W, Hadwiger LA (1987) Pea genes associated with non-host disease resistance to

Fusarium are also active in race-specific disease resistance to *Pseudomonas*. Plant Mol Biol 8:309–316

Darvasi A, Soller M (1997) A simple method to calculate resolving power and confidence interval of QTL map location. Behav Genet 27:125–132

Desaki Y, Kouzai Y, Ninomiya Y, Iwase R, Shimizu Y, Seko K, Molinaro A, Minami E, Shibuya N, Kaku H, Nishizawa Y (2018) OsCERK1 plays a crucial role in the lipopolysaccharide-induced immune response of rice. New Phytol 217:1042–1049

Fondevilla S, Ávila CM, Cubero JI, Rubiales D (2005) Response to *Mycosphaerella pinodes* in a germplasm collection of *Pisum* spp. Plant Breed 124:313–315

Fondevilla S, Satovic Z, Rubiales D, Moreno MT, Torres AM (2008) Mapping of quantitative trait loci for resistance to *Mycosphaerella pinodes* in *Pisum sativum* subsp *syriacum*. Mol Breed 21:439–454

Fondevilla S, Almeida NF, Satovic Z, Rubiales D, Patto MCV, Cubero JI, Torres AM (2011a) Identification of common genomic regions controlling resistance to *Mycosphaerella pinodes*, earliness and architectural traits in different pea genetic backgrounds. Euphytica 182:43–52. https://doi.org/10.1007/s10681-011-0460-8

Fondevilla S, Küster H, Krajinski F, Cubero JI, Rubiales D (2011b) Identification of genes differentially expressed in a resistant reaction to *Mycosphaerella pinodes* in pea using microarray technology. BMC Genom 12:28

Fondevilla S, Rotter B, Krezdorn N, Jüngling R, Winter P, Rubiales D (2014) Identification of genes involved in resistance to *Didymella pinodes* in pea by deepSuperSAGE transcriptome profiling. Plant Mol Biol Rep 32:258–269

Fristensky B, Riggleman RC, Wagoner W, Hadwiger LA (1985) Gene expression in susceptible and disease resistant interactions of peas induced with *Fusarium solani* pathogens and chitosan. Physiol Plant Pathol 27:15–28

Jha AB, Tar'an B, Diapari M, Sindhu A, Shunmugam A, Bett K et al (2015) Allele diversity analysis to identify SNPs associated with ascochyta blight resistance in pea. Euphytica 202:189–197. https://doi.org/10.1007/s10681-014-1254-6

Jha AB, Tar'an B, Stonehouse R, Warkentin TD (2016) Identification of QTLs associated with improved resistance to ascochyta blight in an interspecific pea recombinant inbred line population. Crop Sci 56:2926–2939. https://doi.org/10.2135/cropsci2016.01.0001

Kao CH, Zeng ZB, Teasdale RD (1999) Multiple interval mapping for quantitative trait loci. Genetics 152:1203–1216

Khan TN, Timmerman-Vaughan GM, Rubiales D, Warkentin TD, Siddique KHM, Erskine W, Barbetti MJ (2013) *Didymella pinodes* and its management in field pea: challenges and opportunities. Field Crops Res 148:61–77

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Kugapreethan R, Wan Q, Nilojan J, Lee J (2018) Identification and characterization of a calcium-dependent lily-type lectin from black rockfish (*Sebastes schlegelii*): molecular antennas are involved in host defense via pathogen recognition. Dev Comp Immunol 81:54–62

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Licausi F, Ohme-Takagi M, Perata P (2013) APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytol 199:639–649

Liu JJ, Ekramoddoullah AKM (2006) The family of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. Physiol Mol Plant Pathol 68:3–13

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15:165–178

Madrid E, Rajesh PN, Rubio J, Gil J, Millán T, Chen W (2012) Characterization and genetic analysis of an *EIN4*-like sequence (*CaETR-1*) located in QTL_{AR1} implicated in ascochyta blight resistance in chickpea. Plant Cell Rep 31:1033–1042

Matsui H, Nakamura G, Ishiga Y, Toshima H, Inagaki Y et al (2004) Structure and expression of 12-oxophytodienoate reductase (subgroup I) genes in pea, and characterization of the oxidoreductase activities of their recombinant products. Mol Gen Genom 271:1–10

Park CJ, Kim KJ, Shin R, Park JM, Shin YC, Paek KH (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J 37:186–198

Prioul S, Frankewitz A, Deniot G, Morin G, Baranger A (2004) Mapping of quantitative trait loci for partial resistance to *Mycosphaerella pinodes* in pea (*Pisum sativum* L.), at the seedling and adult plant stages. Theor Appl Genet 108:1322–1334

Prioul-Gervais S, Deniot G, Receveur EM, Frankewitz A, Fourmann M, Rameau C et al (2007) Candidate genes for quantitative resistance to *Ascochyta pinodes* in pea (*Pisum sativum* L.). Theor Appl Genet 114:971–984. https://doi.org/10.1007/s00122-016-2669-3

Riggleman RC, Fristensky B, Hadwiger LA (1985) The disease resistance response in pea is associated with increased levels of specific mRNAs. Plant Mol Biol 4(81):86

Rubiales D, Fondevilla S (2012) Future prospects for ascochyta blight resistance breeding in cool season food legumes. Front Plant Sci 3:27

Tar'an B, Warkentin T, Somers DJ, Miranda D, Vandenberg A, Blade S, Penner G (2003) Quantitative trait loci for lodging resistance, plant height and partial resistance to *Mycosphaerella* blight in field pea (*Pisum sativum* L.). Theor Appl Genet 107:1482–1491

Timmerman-Vaughan GM, Frew TJ, Weeden NF (2000) Characterization and linkage mapping of R-gene analogous DNA sequences in pea (*Pisum sativum* L.). Theor Appl Genet 101:241–247

Timmerman-Vaughan GM, Frew TJ, Russell AC, Khan T, Butler R, Gilpin M, Murray S, Falloon K (2002) QTL mapping of partial resistance to field epidemics of Ascochyta blight of pea. Crop Sci 42:2100–2111

Timmerman-Vaughan GM, Frew TJ, Butler R, Murray S, Gilpin M, Falloon K, Khan T (2004) Validation of quantitative trait loci for Ascochyta blight resistance in pea (*Pisum*

sativum L.) using populations from two crosses. Theor Appl Genet 109:1620–1631

Timmerman-Vaughan GM, Moya L, Frew TJ, Murray SR, Crowhurst R (2016) Ascochyta blight disease of pea (*Pisum sativum* L.): defence-related candidate genes associated with QTL regions and identification of epistatic QTL. Theor Appl Genet 129:879–896. https://doi.org/10.1007/s00122-016-2669-3

Tivoli B, Banniza S (2007) Comparison of the epidemiology of ascochyta blights on grain legumes. Eur J Plant Pathol 119:59–76

Wang S, Basten CJ, Zeng ZB (2012) Windows QTL Cartographer 2.5, Department of Statistics, North Carolina State University, Raleigh, NC (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm)

Wu Y, Bhat PR, Close TJ, Lonardi S (2008) Efficient and accurate construction of genetic linkage maps from the minimum spanning tree of a graph. PLoS Genet 4:e1000212

Zeng ZB, Kao CH, Basten CJ (1999) Estimating the genetic architecture of quantitative traits. Genetics 74:279–289

Zhang R, Hwang SF, Chang KF, Gossen BD, Strelkov SE, Turnbull GD, Blade SF (2006) Genetic resistance to in 558 field pea accessions. Crop Sci 46:2409–2414