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Identification of quantitative trait *loci* and candidate genes for specific cellular resistance responses against *Didymella pinodes* in pea

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Abstract

Key message **Phenotyping of specific cellular resistance responses and improvement of previous genetic map allowed the identification of novel genomic regions controlling cellular mechanisms involved in pea resistance to ascochyta blight and provided candidate genes suitable for MAS.**

Abstract *Didymella pinodes*, causing ascochyta blight, is a major pathogen of the pea crop and is responsible for serious damage and yield losses. Resistance is inherited polygenically and several quantitative trait *loci* (QTLs) have been already identified. However, the position of these QTLs should be further refined to identify molecular markers more closely linked to the resistance genes. In previous works, resistance was scored visually estimating the final disease symptoms; in this study, we have conducted a more precise phenotyping of resistance evaluating specific cellular resistance responses at the histological level to perform a more accurate QTL analysis. In addition, P665 × Messire genetic map used to identify the QTLs was improved by adding 117 SNP markers located in genes. This combined approach has allowed the identification, for the first time, of genomic regions controlling cellular mechanisms directly involved in pea resistance to ascochyta blight. Furthermore, the inclusion of the gene-based SNP markers has allowed the identification of candidate genes co-located with QTLs and has provided robust markers for marker-assisted selection.

Keywords Quantitative trait *loci* · *Didymella pinodes* · Pea · Epidermal cell death · Candidate genes · SNP markers · Cellular resistance

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Introduction

Dry pea (*Pisum sativum* ssp. *sativum* L.) is an important crop, being the most produced grain legume in Europe and the second most in the world (FAOSTAT 2012; http://faostat.fao.org/). Legumes are a cheap source of high-quality vegetable proteins and are able to fix atmospheric nitrogen symbiotically, which improves soil fertility and reduces the need for nitrogen fertilizers. However, pea yield is severely affected by diseases. *Didymella pinodes* (Berk & Blox) is the most common and damaging pathogen causing ascochyta blight (Bretag et al. 2006; Tivoli and Banniza 2007). It is widespread throughout temperate regions (Wallen 1965; Lawyer 1984) and together with the broomrape (*Orobanche crenata* Forsk) constitutes the major constraint for pea production in the Mediterranean basin (Rubiales et al. 2003, 2009). Current control practices are uneconomic and

inefficient (Schoeny et al. 2008; Fernández-Aparicio et al. 2010; McMurray et al. 2011). The use of resistant cultivars is the most desired method to control the disease. However, only moderate levels of resistance have been reported in cultivated pea (Stuckey 1940; Bretag 1989, 1991; Clulow et al. 1991; Wroth 1996; Nasir and Hoppe 1997; Kraft et al. 1998; Xue and Warkentin 2001; Khan et al. 2013). Most studies examining the genetics of the resistance to *D. pinodes* in pea have concluded that resistance is a polygenic trait controlled by several QTLs (Wroth 1999; Timmerman-Vaughan et al. 2002, 2004; Tar'an et al. 2003; Prioul et al. 2004; Zhang et al. 2006; Prioul-Gervais et al. 2007; Fondevilla et al. 2007, 2008).

The highest levels of resistance to *D. pinodes* have been identified in wild species of *Pisum* (Clulow et al. 1991; Wroth 1998; Fondevilla et al. 2005). Among these lines, *Pisum sativum* ssp. *syriacum* accession P665 has been shown to provide resistance to different isolates of *D. pinodes* (Fondevilla et al. 2005). A genetic map has been developed using a recombinant inbred line (RIL) population derived from the cross between P665 and the susceptible *P. sativum* ssp. *sativum* cv. Messire allowing the identification of seven QTL controlling resistance to *D. pinodes* (Fondevilla et al. 2007). However, further saturation of these genomics regions is needed to identify molecular markers more tightly linked to the resistance genes that could be efficiently used for marker-assisted selection (MAS).

The accuracy of a QTL analysis is highly influenced by precision in scoring the trait and the availability of high-density maps. Resistance to *D. pinodes* in pea has traditionally been evaluated by visually estimating disease symptoms with the help of several scales or indexes (Tivoli et al. 2006; Fondevilla et al. 2008, 2011; Khan et al. 2013). However, resistance to *D. pinodes* is a multi-component event where the observed symptoms are the result of a battery of resistance mechanisms acting at different phases of the infection process. Accordingly, previous histological studies (Carrillo et al. 2013) have shown that resistance in P665 is characterized by a reduced success of colony establishment and lesion size, associated with a high frequency of epidermal cell death and protein cross-linking. Dissecting specific cellular resistance responses is expected to allow a more accurate assessment of the trait. In addition, knowledge of the resistance mechanisms that control each QTL would facilitate the identification of the genes underlying the QTL.

Materials and methods

Plant material

The population used in the study consisted of 111 $F_{6:7}$ 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 *D. pinodes*, while cv. Messire is highly susceptible (Fondevilla et al. 2005).

Seeds were scarified and pre-germinated at 4 °C in darkness for 48 h. After that, they were maintained in darkness for another 48 h at room temperature (20 °C approximately). Germinated seeds were then sown, one seed per pot, in plastic pots containing 250 cm^3 of 1:1 sand–peat mixture. Plants were kept at 20 ± 2 °C in a growth chamber with 12 h light/12 h dark photoperiod during 3 weeks approximately (until the 4th leaf was completely developed). RILs were grown in a randomized complete block designed with three blocks, each block having four plants of each RIL family and the parental lines.

Single-nucleotide polymorphism markers (SNP) analysis

The DNA used to develop the previous P665 × Messire map was used for the SNP analysis (Fondevilla et al. 2008). SNP markers were analysed using the high-throughput genotyping method Illumina GoldenGate assay as described by Bordat et al. (2011). Primers were developed using the BeadXpress Primer Design (Illumina, San Diego, CA, USA) (Deulvot et al. 2010). Out of the 384 gene-based SNP markers surveyed, 333 SNP markers have been previously described (Razdan et al. 1992; Borisov et al. 2003; Aubert et al. 2006; Choi et al. 2006; DeMason and Weeden 2006; Edwards et al. 2007; Jing et al. 2007; Aubry et al. 2008; Deulvot et al. 2010; Krussell et al. 2011, Rameau pers. comm.) (Supplementary Information Table S1), whereas 51 are new SNP markers described for the first time in this work (Supplementary Information Table S2).

Map construction

Polymorphic SNP markers were included in the previous P665 × Messire RIL dataset (Fondevilla et al. 2012). The linkage groups were 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. Recombination fractions were converted to centiMorgans (cM) using the mapping function of Kosambi (1943).

Fungal material and inoculation

D. pinodes isolate CO-99 was used for the experiments. This monoconidial isolate was obtained from naturally infected pea material collected in commercial fields at Cordoba,

Spain in 1999 and selected for its high virulence. The isolate was multiplied in Petri dishes containing V8 medium and cloranphenicol (200 ml/l V8 vegetables extract juice + 40 g/l agar + 60 mg/l cloranphenicol) at 20 °C and subjected to 16 h light/8 h dark photoperiod during 13 days. The spore suspension was prepared by flooding the surface of cultures with sterile water, scraping the colony with a needle and filtering the suspension through two layers of sterile cheesecloth. The concentration of spores in the solution obtained was further determined with a haemocytometer and adjusted to 200,000 spores/ml. Then, Tween-20 (120 µl/100 ml of suspension) was then added as wetting agent.

Plants were inoculated at the 4th leaf stage in three independent replicates, each replicate having four plants per RIL family and parental lines. The spore suspension was applied on the leaflets with a paintbrush at a rate of 1 ml/plant. After inoculation, plants were maintained during the first 24 h in darkness and high humidity was ensured by ultrasonic humidifiers operating for 15 min every 2 h. Later on, plants were moved to a growth chamber (12 h light/12 h darkness photoperiod). High humidity was maintained by placing a perforated polyethylene plastic bag covering the trays containing the plants.

Scoring specific cellular resistance responses to *D. pinodes* in the RIL population

Specific cellular resistance responses to *D. pinodes* in pea (Carrillo et al. 2013) were analysed in the RIL population P665 × Messire. The responses assessed were: colony establishment, lesion size, host epidermal cell death and host protein cross-linking. For the histological studies the true third-formed leaflets inoculated were excised at 48 h after inoculation and subjected to two different stains (Carrillo et al. 2013) depending on the trait to assess:

Percentage of germinated spores that established a colony (EstC), lesion size (LesS) and percentage of germinated spores causing host epidermal cell death (DeadC)

Cut leaflets were laid, adaxial surface up, on filter paper moistened with a 1:1 (v/v) mixture of glacial acetic acid: absolute ethanol for fixation. Leaflets were later stained by boiling in 0.05 % trypan blue in lactophenol–ethanol (1:2, v:v) for 10 min. Then, they were moved into a solution of chloral hydrate (5:2, p:v) to clarify the tissues. For histological assessments, samples were mounted on lactoglycerol and observed with a light microscope.

For scoring the percentage of germinated spores that established a colony (*EstC*), 100 germinated spores per accession and replicate were examined. A colony was considered established when a necrotic lesion was observed in mesophyll. For measuring the lesion size (*LesS*), 20 lesions formed in the mesophyll per replicate were assessed. Lesions were considered ellipses and their longer and shorter diameters were measured. The area of the lesions was calculated as area $= \pi * r_1 * r_2$, where r_1 and r_2 are the long and short radio of the ellipse, respectively.

For scoring the percentage of germinated spores causing host epidermal cell death (*DeadC*), 100 germinated spores per accession and replicate were observed for the presence of host epidermal cell death at the penetration site. Cell death was observed by epifluorescence under excitation with a 450–490 nm yellow filter and differential interference contrast (DIC) microscopy. Dead cells showed fluorescence at 450–490 nm, but at early stages of cell death this fluorescence was not strong enough to be detected easily. Because of that, we combined epifluorescence with DIC microscopy. By bright field microscopy, the walls and contents of dead cells were discoloured yellow or brown, and by DIC, the cell contents appeared granular and disorganized.

Percentage of germinated spores causing host protein cross-linking (ProtC)

Cut leaflets were stained according to Mellersh et al. (2002). Fresh samples were submerged in 1 % sodium dodecyl sulphate (SDS) for 24 h at 80 °C to remove soluble proteins. After that, samples were boiled for 8 min in a 0.1 % Coomassie solution in ethanol–acetic (4:1, v:v). Finally, samples were rinsed in ethanol–acetic 4:1 and were mounted on lactoglycerol. Presence/absence of protein cross-linking was studied in 100 germinated spores per accession and replicate, being scored as presence/absence of dark blue colour on the walls of the epidermal cells surrounding the penetration site.

Samples were observed using a light microscope (Leica DMLB; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

Resistance traits previously scored in the RIL population

The following quantitative traits have been previously scored in the RIL population (Fondevilla et al. 2008) and are included in the present QTL analysis:

1. Resistance to *D. pinodes* under controlled conditions (*DRseedl*): Disease under controlled environmental conditions was assessed at the seedling stage, 7 days after inoculation using a 0–5 scale defined by Roger and Tivoli (1996). For each plant, the average disease rating was calculated as the mean disease score over the first, second and third leaves.
2. Resistance to *D. pinodes* under field conditions: The RIL population and the parental lines were screened

for resistance to *D. pinodes* during two seasons, 2004–2005 (2005) and 2005–2006 (2006), in experimental plots located at Cordoba, Spain. Evaluation was performed in April, at the end of the crop cycle. Disease rating on leaves (leaflets and stipules; *DRl*) and stems (*DRst*) was scored separately using the 0–5 scale described by Roger and Tivoli (1996). The average disease rating for each organ was visually estimated in the first ten nodes of five plants situated in the middle of each row. Disease severity (*DS*) was also assessed in the same plants as the percentage of the whole plant area covered by symptoms.

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).

Quantitative trait analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc. 2004). Standard analysis of variance (ANOVA) was performed using PROC GLM to determine variation in the analysed traits. Variance components were estimated using PROC VARCOMP. Broad-sense heritability (h^2), that represents the part of genetic variance in the total phenotypic variance, was calculated as $h^2 = \delta_g^2 / \left(\delta_g^2 + \delta^2/r\right)$, where δ_g^2 is the genotypic variance, δ^2 is the error variance and r is the number of replications. Normality of residual distribution was checked using the Kolmogorov–Smirnov test. To improve the normality of data, variables *EstC, DeadC* and *ProtC* were transformed using angular transformation (y = arcsine $\sqrt{x/100}$), while square root transformation was performed for *LesS.*

QTL analysis was conducted using composite interval mapping (CIM) and multiple interval mapping (MIM) in Windows QTL Cartographer V2.5 (Wang et al. 2007). Markers to be used as cofactors for CIM were selected by forward–backward stepwise regression. The number of markers controlling the genetic background in CIM was set to five. The thresholds for the detection of QTLs were estimated by permutations analysis (Churchill and Doerge 1994) using 1.000 permutations. One- and two-LOD support intervals for the position of each QTL were calculated as described by Darvasi and Soller (1997).

To obtain more precise information of QTL effects and positions and to evaluate the presence of digenic epistatic interactions across the QTL pairwise combinations, multiple interval mapping (MIM) (Kao et al. 1999; Zeng et al. 1999), as implemented in WinQTL Cartographer, was used by considering the CIM results obtained for the trait as initial QTLs. The initial CIM-derived QTL model was subjected to a search for significant epistatic interactions among QTLs. Both main additive effects and their epistatic interactions were tested for significance using the Bayesian information criterion (BIC) with the penalty function: c (n) = log (n), with n (sample size) = 111 (Zeng et al. 1999). The final main additive and epistatic QTLs effects and the R^2 values of the model were then estimated.

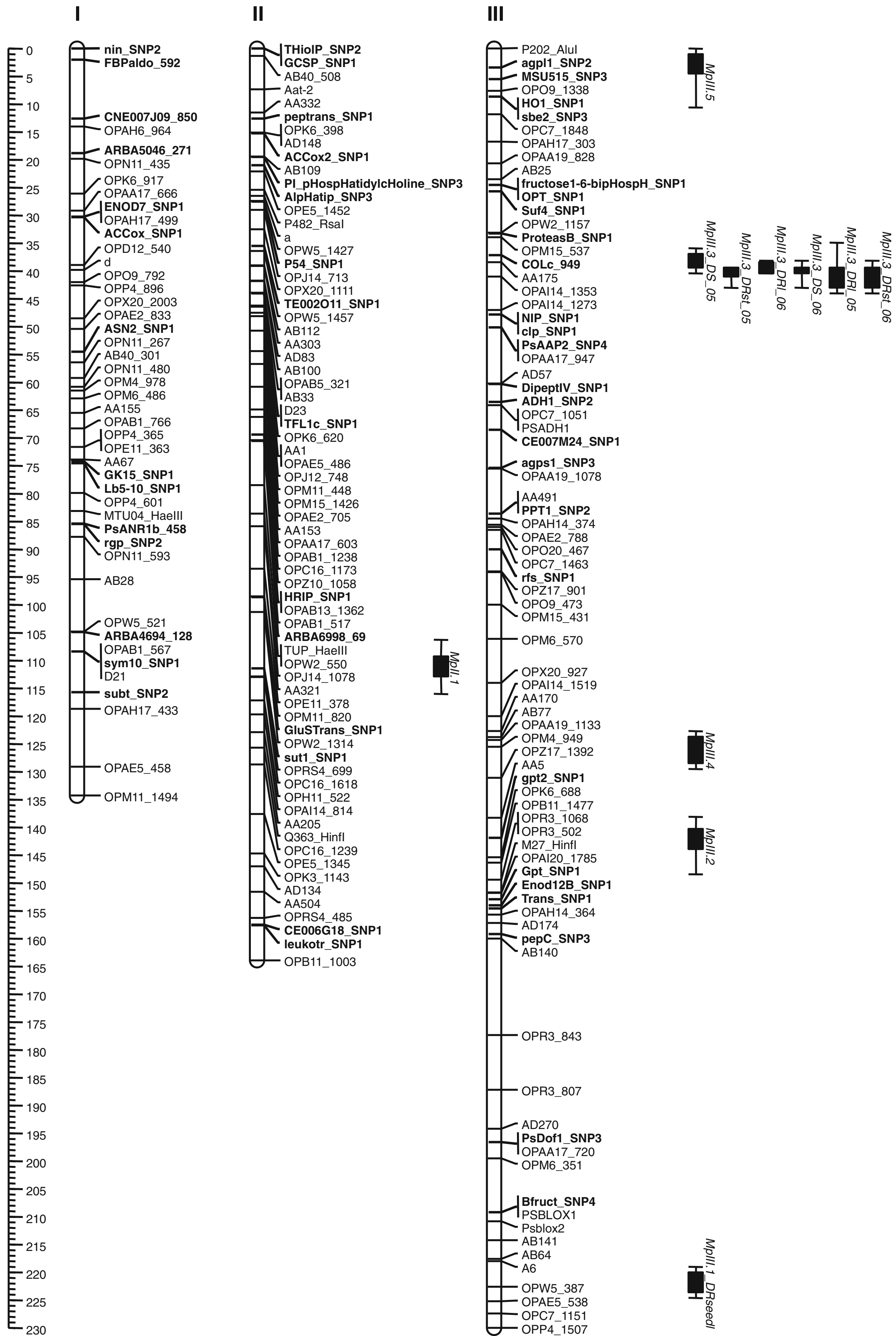
Fig. 1 Pea genetic linkage map constructed from a population ▶ formed by 111 $F_{6:7}$ recombinant inbred lines (RILs) derived from the cross between *Pisum sativum* subsp. *syriacum* accession P665 and *P. sativum* subsp. sativum cv. Messire. *Bar* positions indicate the locations of quantitative trait loci (QTLs): outer and inner intervals correspond to 1-LOD and 2-LOD support interval, respectively, and are indicated as a *full box* and a *single line*, respectively. SNP markers analysed in this study are shown in *bold*

Results

Linkage map

Out of the 384 SNP markers analysed, 119 showed polymorphisms between the parental lines. Of them, 117 SNP markers were successfully mapped resulting in a more saturated map containing 414 markers distributed in eight linkage groups (Fig. 1). Four RAPD markers previously mapped on a distal part of LGI (OPAI14_854, OPAI14_877 and OPR3_588) and LGII (OPM6_884) were discarded, as their position could not be determined unambiguously after the inclusion of the additional SNP markers. The new map covered 1,119.46 cM with an average inter-marker distance of 2.87 cM. All the SNP markers mapped in the expected LG according to Deulvot et al. (2010) and Bordat et al. (2011), except for *Gpt2* (LGIII) previously linked in LGIV. In addition, some genomic regions displayed an inverted order compared to Bordat et al. (2011). These regions were: in LGII the region containing AA332 and *Peptrans* and the region between AB100 and AA1; in LGIII the region between M27 and *Gpt*; in LGV the regions between AB23 and *SS*, and between *sbe* and AD68. In LGVI, the region containing AD68, *PCT* and *Sus3* was also inverted.

Twelve of the mapped SNP markers had not been previously mapped in any other pea genetic map. One of them (*COLc_949*) was located into a gene whose position in the pea genetic map is already known (Weller et al. 2012); but the remaining 11 corresponded to loci whose position was hitherto unknown. These 11 SNP markers were: *CNE007J09_850*, *ARBA5046_271*, *PsANR1b_458* and *ARBA4694_128* located in LGI; *ARBA6998_69* located in LGII; *ARBA10806_623* located in LGIVa; *Pea4_1_499* located in LGV; *PsDHN1_320*, *PsDHN2_485*, *ARBA3199_340* and *ARBB22348_378* located in LGVI (Supplementary Information Table S2) (Fig. 1).



I
II
III
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220
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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
AB109
PI_pHospHatidylcHoline_SNP3
AlpHatip_SNP3
OPE5_1452
P482_RsaI
a
OPW5_1427
P54_SNP1
OPJ14_713
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
sut1_SNP1
OPRS4_699
OPC16_1618
OPH11_522
OPAI14_814
AA205
Q363_HinfI
OPC16_1239
OPE5_1345
OPK3_1143
AD134
AA504
OPRS4_485
CE006G18_SNP1
leukotr_SNP1
OPB11_1003
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agpl1_SNP2
MSU515_SNP3
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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
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
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_DRst_05
MpIII.3_DRl_06
MpIII.3_DS_06
MpIII.3_DRl_05
MpIII.3_DRst_06
MpIII.4
MpIII.2
MpIII.1_DRseedl

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230

IVa

OPO20_295
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OPK6_450
non-pHospHorylating-GAPDH_SNP1
OPC16_354
AA219
HATPS_SNP2
OPAC1_432
OPAE2_479
OPB11_522
OPJ12_997
OPD12_1057
SPS_SNP2
OPAA17_737
OPD12_595
Xyft_SNP1
OPM4_513
OPZ12_898
OPJ14_680
OPZ12_845
OPH11_777
ARBA10806_623
OPM15_500
OPM15_569
NTH1_SNP1
OPK3_396

IVb

OPW2_1086
AA122
OPAI20_1050
OPP4_1466
OPAA11_694
OPO6_615
OPAA11_683
OPK3_643
P393
OPAE2_612
OPM11_270
OPZ12_590
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OPX20_719
OPR3_400
OPAH6_1302
OPC16_1837
OPR3_1233
CNGC4_SNP1
OPAB1_1522
OPB11_582
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OPRS4_782
cwi2_SNP2
AA315
OEE3_SNP1
OPC16_740
Vsr_SNP2
OPO9_531

MpIV.1_DS_06

V

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SS_SNP3
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OPE11_477
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CNE007I23_SNP2
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OPK6_887
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OPAE2_1551
OPB11_941
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RNAH_AluI
ArfB3_SNP1
AA399
OPK6_818
OPC7_1390
OPE5_512
GNS2_SNP2
OPE11_392
At2g44950_SNP1
OPR3_465
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OPM11_771
Pea4_1_499
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AA241
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OPAE2_1173
rbcs_SNP1

MpV.3
MpV.2
MpV.1_DRseedl

VI

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PIN1_SNP1
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OPW5_860
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OPAB1_1158
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Dof6_SNP1
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RBBP_HaeIII
FVE_SNP6
RBBP_SNP1
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sus3_SNP1
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PCT_SNP2
OPW5_565
Kao2_SNP2
OPN11_750
PFK_SNP1
OPAC1_305
AA335
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OPD12_441
PI
Dof3_SNP3

MpVI.1

VII

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OPM4_831
Q500_HaeIII
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OPZ17_513
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Htrans_SNP2
OPAI14_665
OPAI14_734
AA160
Dof4_SNP3
Ptrans_SNP1
PlcSyn_SNP1
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Selbin_SNP2
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AB114
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OPM11_503
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AA446
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clpser_SNP1
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DNAPB
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pip2_SNP1
Arf1350_SNP1
OPK3_726
OPE5_847
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OPAA17_997
OPW5_638
OPW2_421

Fig. 1 continued

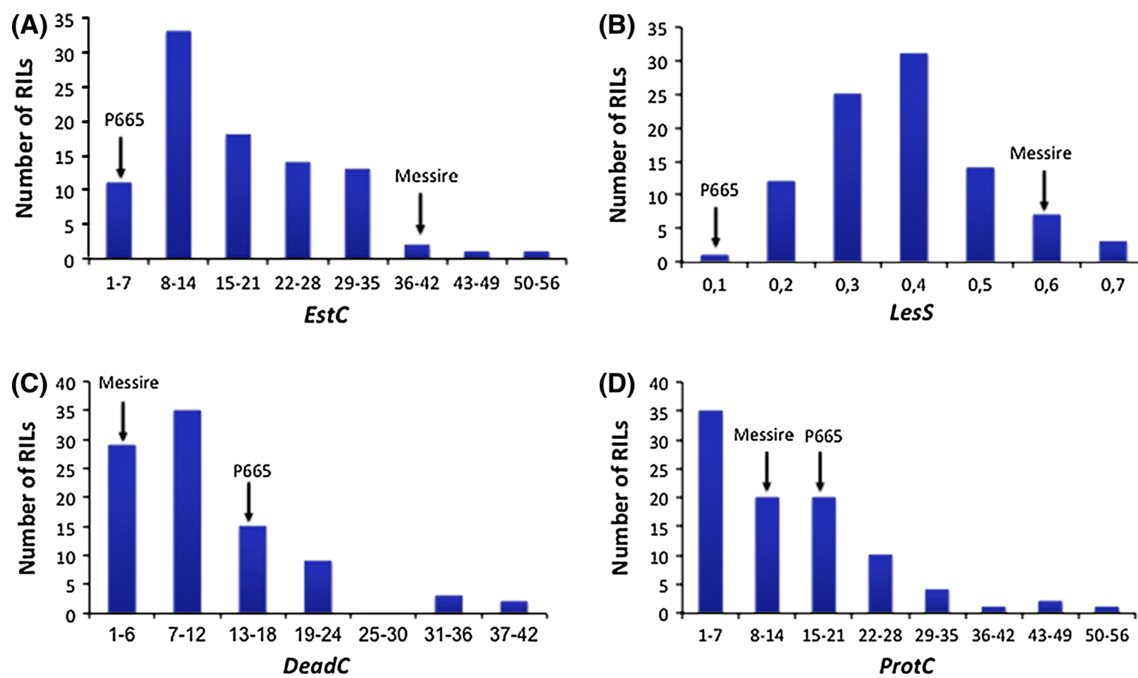

Fig. 2 Frequency distributions of the histological traits quantified: **a** *EstC* percentage of germinated spores that established a colony; **b** *LesS* lesion size (mm^2); **c** *DeadC* percentage of germinated spores causing host epidermal cell death; **d** *ProtC* percentage germinated spores causing protein cross-linking in epidermal cells. *Arrows* indicate the means of the resistant (P665) and the susceptible (cv. Messire) parental line

Assessing specific cellular responses to *D. pinodes* in the RIL population

P665 accession was significantly more resistant to *D. pinodes* than Messire for all the histological resistant traits scored (ANOVA $p < 0.05$). Thus, P665 displayed an average percentage of established colonies (*EstC*) of 3.7 % against the 42.3 % observed in Messire. Lesions (*LesS*) were also significantly smaller in P665 (0.1 mm^2 as average) than in Messire (0.6 mm^2 as average). Furthermore, P665 also showed a higher percentage of germinating spores causing host epidermal cell death (*DeadC*) and protein cross-linking (*ProtC*) compared to Messire (14.7 vs 4 % and 16.7 vs 8.7 %, respectively).

The ANOVA also revealed highly significant differences between the RIL families for all specific cellular responses of resistance studied ($p < 0.001$). Histological traits followed continuous distribution with several transgressive RIL families showing higher susceptibility or resistance comparing to the parental lines (Fig. 2). Broad sense heritability value for the resistance traits scored was high, ranging from 0.95 to 0.99 (Table 1).

QTL analysis

The QTL analysis was performed on the specific cellular responses of resistance scored in this study and also on those resistant traits scored before by Fondevilla et al. (2008) to refine the position of the *D. pinodes* resistance QTLs previously identified. The analysis confirmed the involvement in resistance of the genomic regions reported in Fondevilla et al. (2011) (Table 2). As an exception, in the region of LGII where the QTL *MpII.1_DRseedl* was previously reported (Fondevilla et al. 2011) no QTL reached the required LOD threshold (there was a peak with a LOD = 2.05 in this region while the threshold was 3.09). In some cases, QTLs located in these regions corresponded to different resistance traits. Thus, the previous *MpIII.2_DRl_05* associated with resistance to *D. pinodes* in leaves was not detected, but a new QTL associated with resistance in stems (*MpIII.2*) was identified in the same region. Similarly, *MpIII.2_DRseedl* was not identified in the new analysis but a QTL involved in epidermal cell death (*MpIII.4*) was found in the same region. Also in LGIII, the previous QTL *MpIII.1_DRst_05* was not identified; however, *MpIII.1_DRseedl* located in the same region, was maintained after the new QTL analysis. In LGVI, the previous QTL *MpVI_DS_05* was substituted by *MpVI.1*.

The scoring of the cellular responses of resistance at histological level and the inclusion of new SNP markers allowed the identification of new genomic regions involved in resistance to *D. pinodes*. Thus, a new region (*MpII.1*) in LGII, other in LGIII (*MpIII.5*) and another two in LGV (*MpV.3*; *MpV.2*) were *de novo* identified. Furthermore, this

Table 1 Broad sense heritabilities (h^2) of the studied traits

Histological traits[a]				Traits visually scored in whole plants[b]						
EstC	*Dead*	*ProtC*	*LesS*	*Drseedl*	*DRl2005*	*DRst2005*	*DS2005*	*DRl2006*	*DRst2006*	*DS2006*
0.99	0.98	0.96	0.94	0.83	0.75	0.69	0.81	0.71	0.67	0.88

[a] Histological traits scored in leaflets: *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)

[b] Traits visually scored in whole plants: *DRseedl* resistance to *D. 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

Table 2 Quantitative trait loci (QTL) for resistance to *Didymella 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	Flanking markers	Peak[e]	LOD[f]	TLOD[g]	Add[h]	R^{2i}
EstC		V	*MpV.2*[c]	OPM4_490/OPK6_887	79.95	3.78	3.02	−0.06	15.05
	Total								*15.05*
DeadC		III	*MpIII.4*[c]	OPAA19_1133/OPM4_949	124.36	3.55	3.32	−0.04	9.67
DeadC		III	*MpIII.5*[c]	*agpl1_SNP2/MSU515_SNP3*	3.45	3.30		0.04	8.40
	Total								*18.07*
DRseedl		III	*MpIII.1_Drseedl*[d]	OPW5_387/OPAE5_538	222.59	5.59	3.09	−0.29	13.99
DRseedl		V	*MpV.1_Drseedl*[d]	OPK6_818/OPC7_1390	96.83	4.74		−0.27	14.66
DRseedl		V	*MpV.3*[c]	OPZ10_576/*Sugtrans_SNP3*	61.22	3.86		−0.21	11.39
	Total								*40.04*
DRl2005		III	*MpIII.3_DRl_05*[d]	OPAI14_1353/OPAI14_1273	40.96	9.89	3.07	−0.31	31.16
	Total								*31.16*
DRst2005		III	*MpIII.3_DRst_05*[d]	AA175/OPAI14_1353	40.42	13.08	3.16	−0.41	38.88
DRst2005		III	*MpIII.2*[c]	*gpt2_SNP1*/OPK6_688	141.89	4.39		−0.22	7.12
DRst2005		VI	*MpVI.1*[c]	OPAB5_498/OPAB11_598	59.46	4.08		−0.22	7.43
DRst2005		II	*MpII.1*[c]	*sut1_SNP1*/OPRS4_699	112.43	3.28		0.13	1.76
	Total								*55.19*
DS2005		III	*MpIII.3_DS_05*[d]	AA175/OPAI14_1353	38.42	13.81	3.14	−4.89	40.04
	Total								*40.04*
DRl2006		III	*MpIII.3_DRl_06*[d]	AA175/OPAI14_1353	40.42	16.21	3.13	−0.37	45.58
	Total								*45.58*
DRst2006		III	*MpIII.3_DRst_06*[d]	OPAI14_1353/OPAI14_1273	41.96	12.60	3.61	−0.31	37.06
	Total								*37.06*
DS2006		III	*MpIII.3_DS_06*[d]	AA175/OPAI14_1353	40.42	13.65	3.15	−4.99	52.59
DS2006		IVB	*MpIV.1_DS_06*[d]	AA315/*OEE3_SNP1*	64.46	4.28		1.68	7.29
	Total								*59.88*

[a] *EstC* percentage of established colonies, *DeadC* percentage of germinates spores causing epidermal cell death, *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] New QTL identified in this study

[d] QTL identified in previous studies (Fondevilla et al. 2008 and 2011)

[e] *Peak* QTL position (cM)

[f] *LOD* the peak LOD score

[g] *TLOD* LOD threshold derived from 1,000 permutations at $p = 0.05$

[h] *Add* the additive effect

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

more accurate analysis revealed that the region previously assigned as *MpIII.2* (Fondevilla et al. 2011) was composed in fact by two independent QTLs named *MpIII.2* and *MpIII.3* in this study. The QTLs explained individually from 1.8 to 52 % of the phenotypic variation, depending on the trait scored, and together from 15 to 59.9 % (Table 2).

QTLs controlling specific cellular responses of resistance to *D. pinodes* scored at histological level are first reported in this study. Two QTLs were identified to be associated with an increased presence of host epidermal cell death (*EstC*) and one with a lower succeed in colony establishment (*DeadC*). For the traits percentage of germinated spores causing host protein cross-linking (*ProtC*) and lesion size (*LesS*) no QTL reached the thresholds estimated by permutations analysis. Nevertheless, for *ProtC* a peak with a LOD of 3.17 (LOD threshold for this trait being 5.17) was detected in LGIII between markers *MSU515_SNP3* and OPO_1338. This is the same genomic region where the QTL *MpIII.5* was detected. Additionally, a peak with a LOD value of 3.09 (threshold LOD 3.15) was associated with *LesS* in LGI, between markers *ARBA5046_271* and OPN11_435.

Resistant alleles derived from the resistant parent P665 except for *MpII.1*, *MpIII.4* and *MpIV.1_DS_06* where alleles conferring resistance originated from the susceptible parent Messire (Table 2). Epistatic interactions among QTLs were not significant according to MIM for any of the analysed traits.

Discussion

Ascochyta blight caused by *D. pinodes* is a major challenge to pea growers in temperate and Mediterranean regions. Complete resistance to this disease has not been identified so far. However, incomplete resistance has been reported in wild *Pisum* accessions, which could be used to develop pea cultivars with increased resistance (Khan et al. 2013; Rubiales and Fondevilla 2012). *P. sativum* ssp. *syriacum* accession P665 shows good levels of resistance at both seedling and adult plant stages and to different isolates of *D. pinodes* (Fondevilla et al. 2005), being a suitable source of resistance to this disease. The introgression of resistance to *D. pinodes* from P665 into elite cultivars will be facilitated by the absence of epistatic interactions among the genes controlling the resistance. However, the use of P665 as a source of resistance to ascochyta blight is hampered by the complex inheritance of the resistance. Despite considerable progress in identifying the genomic regions involved in incomplete resistance to *D. pinodes* in pea in recent years, there is still a need to identify the genes underlying the QTLs involved in resistance or at least molecular markers more tightly linked to them, which could be used for efficient MAS. Towards this objective, in this study we have dissected the resistance into its components to perform a better scoring of the trait and to improve the genetic map by adding SNPs markers located in genes.

Gene-based markers are highly reproducible markers suitable for MAS and for comparative mapping within and between species. In addition, they are a source of candidate genes through their co-localization with QTLs. Out of the 384 SNP markers analysed 117 have been mapped enriching the previous P665 × Messire genetic map by almost 30 %. The new map covers a genetic distance of 1,132.23 cM and contains 416 markers uniformly distributed throughout eight linkage groups (Fig. 1), being comparable with the latest consensus map (1,389 cM) (Bordat et al. 2011) and being connected to it by approximately 36 % of total markers. Out of the total 384 SNP markers surveyed, 51 SNP markers have been described for the first time in this work (Supplementary Information Table S2) and 11 loci have been successfully mapped for the first time in a pea genetic map (Fig. 1), thus increasing the number of markers available in pea.

All the SNP markers have been mapped in the expected LG according to Bordat et al. (2011) with the exception of *Gpt2*, located in LGIII instead of LGIV. This discrepancy might be explained due to differences between *P. sativum* ssp. *sativum* and *P. sativum* ssp. *syriacum*. The RIL populations used in Bordat et al. (2011) derived from a cross between two *P. sativum* ssp. *sativum* accessions, while our map is based on a cross between *Pisum sativum* ssp. *syriacum* and *P. sativum* ssp. *sativum*. In addition, some SNP markers displayed an inverted order compared to Bordat et al. (2011). These markers were closely linked and, therefore, it is difficult to determine accurately their relative order.

In addition to the saturation of the genetic map with gene-based SNP markers, we have performed a more detailed evaluation of the resistance than in our previous work (Fondevilla et al. 2008), thus increasing the accuracy of the QTL analysis. While resistance was previously scored by visual assessment of the final disease symptoms under controlled and field conditions, in the present study we have assessed the specific cellular resistance responses contributing to these final symptoms (Carrillo et al. 2013) on the RIL population. This approach has allowed the identification for the first time of genomic regions controlling host epidermal cell death and a lower success in colony establishment. For other traits, such as *ProtC* and *LesS,* no QTL reached the threshold LOD value estimated by permutations, but one region in LGI and other in LGIII were associated with these traits, respectively and reached a LOD > 3. Therefore, it is 1,000 times more plausible that these regions are associated with the traits that they are not. Thus, although we are unable to confirm that these regions are involved in the control of *ProtC* and *LesS*, this cannot

be excluded. Previous studies (Clulow et al. 1991; Fondevilla et al. 2005, 2008; Prioul et al. 2003, 2004; Wroth and Khan 1999) have shown good correlations between *D. pinodes* disease assessments in seedlings under controlled conditions and adult plants in the field, suggesting the existence of genetic factors controlling resistance effective in different developmental stages and environments (Fondevilla et al. 2008). Growth chamber experiments are more suitable than field tests to determine intrinsic resistance levels as the control of the environmental conditions allows a better detection of genetic resistance alleles; whereas field assessments are subjected to strong interactions with environmental conditions (Tivoli et al. 2006). In this study, by conducting the experiment under controlled environmental conditions, we have been able to detect such genetic resistance alleles controlling different host resistance mechanisms.

Furthermore, the combined approach of improving the genetic map and the evaluation of the resistance has allowed the identification of four new regions involved in resistance, one in LGII (*MpII.1*), one in LGIII (*MpIII.5*), and two in LGV (*MpV.2* and *MpV.3*). Of them, *MpIII.5* may correspond to the QTL *mpIII.2*, reported by Prioul et al. (2004). Both *mpIII-2* and *MpIII.5* are specific for seedling resistance under controlled conditions, explaining a similar percentage of the phenotypic variance of the trait (7–9 and 8.4 %, respectively) and sharing the anchor marker P202. In addition, the involvement in resistance of most of the regions reported in Fondevilla et al. (2011) has been also confirmed using the present improved map.

The alleles conferring resistance derived from the resistant parental P665, except for *MpII.1, MpIII.4* and *MpIV.1_DS_06* QTLs, where alleles conferring resistance originated from the susceptible parental Messire. In agreement with that, transgressive lines more resistant than P665 have been identified for the traits *DRst_05* and *DeadC* suggesting that cv. Messire also holds desirable genes for these traits which in combination with P665, could improve the level of resistance provided by P665.

SNP markers are robust markers suitable for MAS. In this study, the inclusion of 117 new gene-based SNP markers in P665 × Messire map has saturated the genomics regions containing QTLs for resistance to *D. pinodes* and has provided a set of reproducible markers for MAS. Only QTLs not containing any SNP marker (*MpIII.4* and *MpIII.1_DRseedl*) contain SSR markers that are also robust markers suitable for MAS.

The mapping of gene-based markers is a source of candidate genes through their co-location with QTLs. Most genes located into the 2-LOD interval of the QTLs (*Agpl1*, *MSU515*, *H01*, *Sut1*, *Gpt2*, *Cwi2*; *Sugtrans*, *CNE007123*) were involved in carbohydrate metabolism or photosynthesis, probably due to the enrichment of the Illumina GoldenGate Assay used in genes involved in these processes. However, some genes co-localized with the QTLs may have an interesting role in defense and therefore, they could be candidate genes involved in resistance to *D. pinodes* in P665. *ArfB3*, located into *MpV.1_DRseedl*, encodes for auxin response factor B3 domain. Auxin response factors are transcription factors that bind to TGTCTC auxin response elements in promoters of early auxin response genes (Tiwari et al. 2003). Plant hormones regulate developmental processes and signalling networks as parts of plant responses to a wide range of stresses including biotic stresses (Bari and Jones 2009). Interestingly, in a recent transcriptomic study (Fondevilla et al. 2013) the auxin indole-3-acetic acid pathway was up-regulated after infection of P665 with *D. pinodes* suggesting that this hormone may have a role in the response against *D. pinodes*. In addition, this transcriptomic study identified members of the Glutathione-S transferase (GSTs) gene family up-regulated after *D. pinodes* infection in the resistant accession P665. Auxin activates the family of GSTs (Abel and Theologis 1996) that detoxify various dangerous compounds including microbial toxins (Marrs 1996) and could, therefore, be involved in the detoxification of the toxins produced by *D. pinodes*.

Another interesting candidate gene is *CE007J22*. This gene co-localizes with the QTL *MpVI.1* and is homologue to the *hypersensitive-induced reaction protein 4* (*HIR4*) gene from *A. thaliana* (Boisson et al. 2003; Ascencio-Ibáñez et al. 2008). A homologous of this gene in pepper was found to be capable of inducing cell death when ectopically expressed in tobacco and Arabidopsis and has been suggested to play a role in ETI (effector-triggered immunity) (Qi and Katagiri 2012). In previous histological studies (Carrillo et al. 2013), epidermal cell death has been shown to have a relevant role in the resistance to *D. pinodes* in P665.

Other authors have also suggested candidate genes for resistance to *D. pinodes*. Prioul-Gervais et al. (2007) suggested *PsDof1* gene (elicitor-responsive Dof protein, Seki et al. 2002) as a candidate gene for the QTL associated with resistance *mpIII.1*. This QTL seems to correspond to our QTL *MpIII.1_DRseedl*. However, in our study *PsDof1* was not located in the QTL *MpIII.1_DRseedl*. Other two candidate genes, pea defensin *DRR230-b* (Prioul-Gervais et al. 2007) and *RGA1.1* (Timmerman-Vaughan et al. 2002) have also been reported in LGIII. It would be interesting to map these genes in our population to check their possible co-localization with *MpIII.4* and *MpIII.2*. Similarly, it would be interesting also to map the *Chi2* gene. This gene, that codifies an endochitinase A2, is closely linked to the marker P202 (Prioul-Gervais et al. (2007) flanking *MpII.5*. Chitinases are important pathogenesis-related proteins that protect plants from fungal pathogens by hydrolysing

glycosidic bonds in the chitin of fungal cell walls (Chang et al. 1995; Renner and Specht 2012).

This study has also allowed the identification of candidate genes for the trait flowering time. The SNP *COLc_495*, mapped in our study, corresponds to a pea CONSTANS-like C gene. This SNP was located in the same genomic region of the QTL *dfIII.2,* a QTL associated with flowering time in Fondevilla et al. (2011) that explains 60 % of the trait. Studies in Arabidopsis have shown that the CONSTANs gene family has an important role in the photoperiod pathway, which is one of the four regulatory pathways controlling the timing of flowering (Griffiths et al. 2003). Interestingly, *COLc* maps in the vicinity of *Elf3*, another gene involved in pea photoperiod (Weller et al. 2012). Therefore, *COLc* and *Elf3* genes are potential candidate genes for *dfIII.2*.

The identification of candidate genes is the first step in identifying the genes controlling a trait. However, additional studies as functional analysis are needed to discern the real role of these genes in resistance to *D. pinodes* in pea. The suitability of these genes as candidates for resistance to *D. pinodes* would facilitate efficient MAS.

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

Ethical standards The experiments comply with the current laws of the country in which they were performed.

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