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Abstract

While no R gene has been reported for resistance to *Phytophthora capsici* in pepper, all studied resistant accessions display a major effect QTL on chromosome P5. By clustering 14 QTLs detected on P5 from independent studies, we identified 3 linked metaQTLs. The QTL *Pc5.1* confers a broad-spectrum resistance to the 12 tested isolates, collected worldwide. A fine mapping of QTL *Pc5.1* delimited the locus to less than 0.4 cM. The physical map based on BAC libraries delivered a reference sequence covering ~1.1 Mb. Structural and functional gene annotation identified a huge proportion of transposable elements and a few ORFs. One ORF shows homology with a transcript differentially expressed in *P. capsici*-infected peppers, and exhibits SNPs between resistant and susceptible lines. By constructing a trait-specific core-collection and by sequencing strategic positions on the locus of interest, we are achieving an association study in order to identify the causal SNP(s). Identifying the responsible gene should facilitate marker-assisted selection, and the study of the molecular crosstalk between plant and Oomycete pathogen.

Keywords: *Capsicum* spp., *Phytophthora capsici*, quantitative disease resistance, QTL, candidate gene, germplasm collection, association analysis

Introduction

Plant diseases are major threats to crop production since pathogens and insects reduce yield potential by at least a quarter. Because governments strongly discourage pesticide use considering risks for human health and the environment, both identification of resistant genitors within genetic resources and breeding for broad spectrum resistance (BSR) to biotic stresses are continually major concerns for end-users. Many major-effect race-specific resistance genes (R genes), involved in gene-for-gene interactions and associated with the hypersensitive response (HR), have been introduced into various cultivars. When widely deployed in environments favourable to disease, however, improved cultivars may succumb. Conversely, it is often assumed that resistances under polygenic control are typically not race-specific, not involved in HR, nor overcome (Lindhout 2002; Hu et al. 2008; Ayliffe et al. 2008; Kou & Wang 2010). Quantitative trait loci (QTLs) conferring partial BSR are therefore considered valuable potential sources for improving durable resistance.

The fungal-like eukaryotic pathogens belonging to the Oomycete genus *Phytophthora* are resurgent and affect many economically important crops worldwide. *P. capsici*, first described by Leonian in 1922 on chili pepper (*Capsicum* spp.) in New Mexico (USA) and causing root rot, crown rot, fruit rot and foliar blight, is today responsible for one of the most destructive and widespread disease affecting pepper production worldwide. The control of *P. capsici* diseases in most developed countries relies extensively on fungicide applications despite their progressive banning and their limited efficacy. Additionally, sources of resistance to *P. capsici* are rare in *Capsicum* diversity, since very few resistant accessions were identified during previous screenings of genetic resources (Kimble & Grogan 1960, Candole et al. 2010, Cantet et al. submitted). The few well-known sources of resistance belong to *C. annuum*, although a small number of sources have also been reported in *C. baccatum* and *C. frutescens*. As no race-specific R genes have been reported, the pepper genitors exhibiting partial quantitative resistance are of particular agronomic interest. Breeding programs and mapping studies have therefore far concentrated on the few *C. annuum* genitors of resistance (Palloix et al. 1990; Thabuis et al. 2003). Mapping data indicate that resistances to *P. capsici* are under polygenic control and that a QTL region on pepper chromosome P5 has consistently been shown to play a major role on resistance (Mallard et al. 2013).

To investigate the major effect QTL region consistently detected on chromosome P5, our study had three strategic objectives: i) to determine whether this QTL region would correspond or not to the same locus in the different resistant genitors; ii) to assess its resistance spectrum; iii) to explore its genetic variability in a pepper core-collection in association with the quantitative evaluation of *P. capsici* resistance in the whole INRA germplasm collection of *Capsicum* spp.

Materials and Methods

Plant material

Three INRA pepper intraspecific *C. annuum* mapping progenies were considered: HV (H3 x Vania), PY (Perennial x Yolo Wonder (YW)), and F5YC (YW x Criollo de Morelos 334 (CM334)) described by Lefebvre et al. (2002) and Barchi et al. (2007). H3 and YW are susceptible to *P. capsici.* Vania, Perennial and CM334 are partially resistant. Vania was derived from the *P. capsici* resistant accession *C. annuum* PI201234 (PM217). Progenies and parental lines had previously been assessed for stem and root resistance to *P. capsici* isolates Pc101 or Pc197 for QTL detection according to the experimental design described in Lefebvre and Palloix (1996), Bonnet et al. (2007), and Thabuis et al. (2003).

By marker-based haplotyping analysis, we chose 11 lines from the 3 mapping progenies that carry the allele associated with resistance at markers located within the major effect QTL *Pc5.1*, and 8 lines with the allele associated with susceptibility at the same markers. These 19 lines varied for alleles at the other *P. capsici* resistance QTLs detected by Thabuis et al. (2003) and Bonnet et al. (2007).

A core-collection of 60 accessions of *Capsicum* spp. was constituted from the INRA pepper collection to describe the polymorphism at *Pc5.1* candidate genes (see Sage-Palloix et al. 2013, in this issue). The core-collection is structured into two subsets: the "ingroup" subset dedicated to intra-species analyses and the "outgroup" subset that will be used to perform inter-species analyses. Attention was paid to balance phenotypes of high resistance, moderate resistance and susceptibly to *P. capsici* (Cantet et al, in prep).

Pathogen material, resistance assessment and experimental design

The artificial "stem inoculation test" was performed with 4 *P. capsici* isolates (Pc101, Pc107, Pc197 and Pc204) as described by Lefebvre & Palloix (1996). Plants were grown in a nursery greenhouse until the six-leaf stage, and a 4-mm mycelium plug of *P. capsici* was deposited on a

decapitated stem. Inoculated plants were kept in growth chambers with a 12-h-photoperiod at 22°C night/24°C day. From the day of inoculation onwards, the pathogen progressively grew to the bottom of the stems, causing stem necrosis. The length of necrosis was measured at 3, 7, 10, 14, 17, and 21 days post-inoculation (dpi). Then we calculated the speed of the necrosis spread for each scoring date (S3, S7, S10, S14, S17 and S21).

The 5 parental lines and 19 selected lines were tested against the 4 *P. capsici* isolates. For each pathogen isolate, six plants of each line were inoculated.

Sequencing of Pc5.1 candidate genes

Candidate genes were amplified either by standard PCR or long-range PCR (Cantet et al. in prep), and libraries for the 60 accessions were constructed to sequence on Illumina HiSeq 2000. The nucleotide diversity was calculated from the consensus sequence of the 60 accessions, using DnaSP v5.10, and haplotype networks were constructed by TCS software v1.21.

Data analysis

Statistical analysis was performed using the statistical software R version 2.14.1 (R Development Core Team, 2011). Analyses of variance (ANOVA) were performed for the resistance measurements on the 19 selected lines to determine the effects of the variables 'Plant genotype', 'Locus *Pc5.1*' and 'Isolate' along with the interaction 'Locus *Pc5.1* x Isolate'. Comparisons of all pairs of adjusted means were performed using Tukey's test with a probability level of $P < 0.05$.

Segregation data for each marker were analysed using MAPMAKER/EXP software, version 3.0. Markers were mapped onto the previously constructed P5 linkage groups (Bonnet et al. 2007; Thabuis et al. 2003). The three chromosome P5 maps were constructed independently and aligned using anchor markers.

QTLs contributing to *P. capsici* resistance were independently detected de novo on the improved maps of the three INRA pepper progenies. Phenotypic datasets previously analysed by Thabuis et al. (2003) and Bonnet et al. (2007) were used along with QTL Cartographer software for this analysis. To detect significant QTLs, a permutation test was performed to estimate the appropriate critical LOD threshold for each trait using the composite interval mapping (CIM) model. LOD thresholds were determined after 1000 permutations, corresponding to a genome-wise significance level of alpha = 0.05.

To determine the most likely number of "real" QTLs on chromosome P5 from the QTLs detected in independent studies (published and from this paper), individual genetic maps were compiled, and a meta-analysis was performed using BioMercator, version 2.0 (http://www.genoplante.com/) (Arcade et al. 2004). The meta-analysis clustered projected individual QTLs. The best clustering model having the lowest Akaike criteria value indicated the optimal number of meta-QTLs that explains the observed QTL distribution on chromosome P5.

Results and discussion

Anchor markers and de novo *QTL detection in INRA pepper chromosome P5 maps*

Three markers permitted the alignment of the 3 INRA P5 maps within the *Pc5.1* confidence interval (AFLP E38M61-139, RFLP GC015_1, CAPS Mfvt_M22). In addition, we mapped 7 new CAPS markers derived from tomato sequences of chromosome T4 available on the SGN website and 11 SSR, COSII and CAPS markers from published pepper chromosome P5 maps (Table 1).

Three resistance QTLs, *Pc5.1*, *Pc5.2* and *Pc5.3*, were detected on the P5 chromosome of HV and PY maps (Fig.1). A single QTL, corresponding to *Pc5.1*, was detected on the P5 chromosome of F5YC map. Individually, QTLs explained between 6.71 and 52.72% of the phenotypic variation (R^2). The QTL *Pc5.1* affected several resistance components in the three progenies, whereas *Pc5.2*

and *Pc5.3* only affected a few resistance components. *Pc5.1* had a larger R^2 value and additive effect than the other two QTLs.

Table 1 New CAPS markers mapped onto the INRA pepper chromosome P5 maps

Unigene / EST / Marker added on INRA P5 pepper maps	Corresponding tomato BAC containing a sequence homologous to the unigene / EST		Corresponding tomato marker homologous to the unigene	Primers for *Capsicum annuum* amplification (5'-3')		Enzyme used for CAPS	INRA map in which the marker has been mapped
	BAC name	Tomato markers contained in the BAC sequence		Forward primer (5' - 3')	Reverse primer (5' - 3')		
Tomato chromosome T4-derived CAPS markers							
SGN-U196349	C04HBa0070F01	T1068	-	CCTGGGAGAGGAGTCCTACA	GCAAGAAACAGCGCCTTTAG	*Nla*IV	HV
SGN-U204895	C04HBa0070F01	T1068	-	TGTCGATGTTACAAGGCCATA	CATGCGGTGACAATACCAAG	*Hpy*CH4IV	F5YC
SGN-U202638	C04HBa0049A17	TG370, TG437	-	GCTTTGAAGATGAGGCAAG	GGTGTACACATCGCCAGAT	*Hph*I	F5YC
SGN-U198114	C04SLm0040B16	TG123	-	TTGGGCTCAATTAAACCATACA	GCACCCCTTGATTGAGAGAA	*Alw*NI	HV
CK901616	C04HBa0008H22	T1792, C2At1g8620, C2At1g8630, C2At1g8640	-	CTCCAAATCGTGTCTGGTCA	ATCACGCTTCCTTCACATCC	*Hph*I	F5YC
SGN-U197890	-	-	TG437	CCATATGGTCCTCCTCCAGA	CTTTCAACCACTTCGGCAAT	*Hph*I	PY
SGN-U196183	-	-	T1261	CACACGTTTCTGGGAGATGA	TCAGCAGCCTTGATGATGTC	*Hha*I	HV
Pepper chromosome P5-derived CAPS markers							
Sn-2 (a)	-	-	-	TTCGATCCACACCATCATCT	TCCTTCAATGGCTTTCCATC	*Eco*RI	HV
P5-SNAP-CM (b)	-	-	-	TCATGAGGTTGCTATTAAG ATTGGTCCTGTTATATA	CATAGAAAGGGATATCATCT GGTACATGCAGAAA	*Hpy*188I	F5YC

(a) Primers for the Sn-2 CAPS marker were designed using the Genbank sequence X79231.1 (Pozueta-Romero et al. 1995).
(b) Primer sequences for the CAPS P5-SNAP-CM marker were supplied by Kim et al. (2008).

Comparison of QTL locations between pepper chromosome P5 maps

The addition of markers on the INRA chromosome P5 maps reinforced their connection with published maps in which *P. capsici* resistance QTLs have been reported (Kim et al. 2008; Minamiyama et al. 2007; Ogundiwin et al. 2005; Sugita et al. 2006; Truong et al. 2012). The integration of fourteen individual maps from independent studies (published and from this paper) produced a chromosome P5 consensus map containing 199 markers over 175 cM. Clustering of 14 individual resistance QTLs against *P. capsici* resulted in 3 meta-QTLs, namely MetaPc5.1, MetaPc5.2 and MetaPc5.3 (Fig. 1). Their confidence intervals ranged between 2.21 and 4.61 cM. The projection of the QTL detected on the top of P5 by Kim et al. (2008) positioned it above MetaPc5.3. The QTL Phyto-P detected by Ogundiwin et al. (2005) was not included in the meta-analysis due to the lack of anchor markers with other published maps. The meta-analysis reduced the number of resistance QTLs by 4.6-fold and the QTL confidence interval mean by 3.7-fold (mean CI for individual QTLs = 12.29 cM, mean CI for meta-QTLs = 3.30 cM).

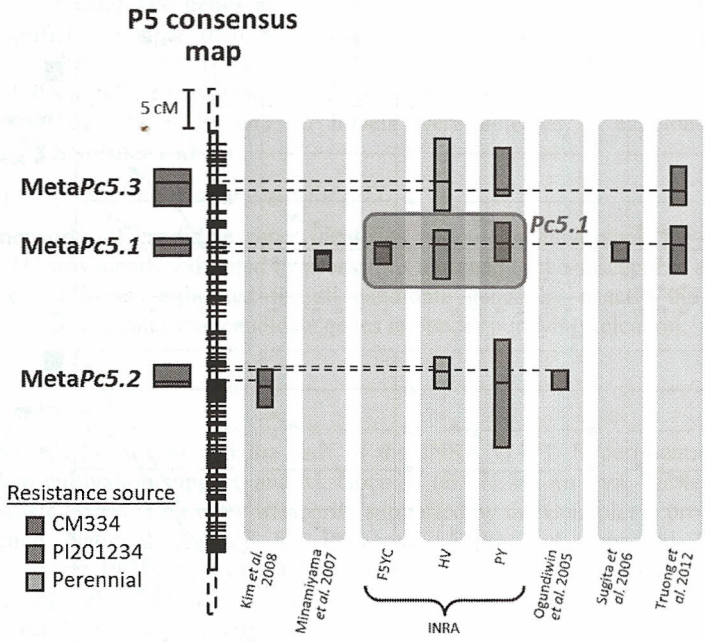

Figure 1: Consensus map of pepper chromosome P5 with projected individual resistance QTLs to P. capsici and computed meta-QTLs. Confidence interval and maximum LOD thresholds of each QTL are represented. For each map, corresponding references are indicated. The consensus map is only partially represented due to the high density of markers.

Effect of QTL Pc5.1 *on genetically distinct P. capsici isolates*

Tukey's test clearly separated susceptible and resistant genotypes. YW and H3 were susceptible to the 4 isolates we tested and displayed a comparable level of susceptibility. On the contrary, Vania, Perennial and CM334 were resistant. For isolates Pc107 and Pc197, CM334 were significantly more resistant than Vania and Perennial (Fig. 2).

Progeny lines with the resistant allele at *Pc5.1* were more resistant than those carrying the susceptible allele, regardless of the progeny considered. The QTL *Pc5.1* exhibited a significant effect on all resistance components assessed with the 4 isolates ($P < 0.0001$) and explained between 55 and 70% of the variation according to the component.

A significant 'isolate' effect was detected for all resistance components ($P < 0.0001$), and resistant parental lines actually behaved differently with tested isolates. Tukey's test classified the 4 *P. capsici* isolates into 3 levels of aggressiveness, with Pc107 and Pc197 being the most aggressive, Pc204 exhibiting an intermediate aggressiveness and Pc101 being the least aggressive.

Lastly, the interaction '*Pc5.1* x isolate' was not significant for REC ($P = 0.609$). While it was significant for the two other resistance components ($P < 0.0001$ for IND, $P = 0.003$ for STA), it explained less than 4% of the observed variation. We therefore concluded that no major host differential reaction occurred with the tested isolates.

Those data with the meta-analysis result demonstrate that *Pc5.1* is active in various genetic backgrounds and against the 12 tested isolates collected worldwide: France, Turkey, North America, Japan, Korea, and Taiwan. As a major BSR QTL against *P. capsici* with a robust effect in different backgrounds, *Pc5.1* should be the major target for breeders.

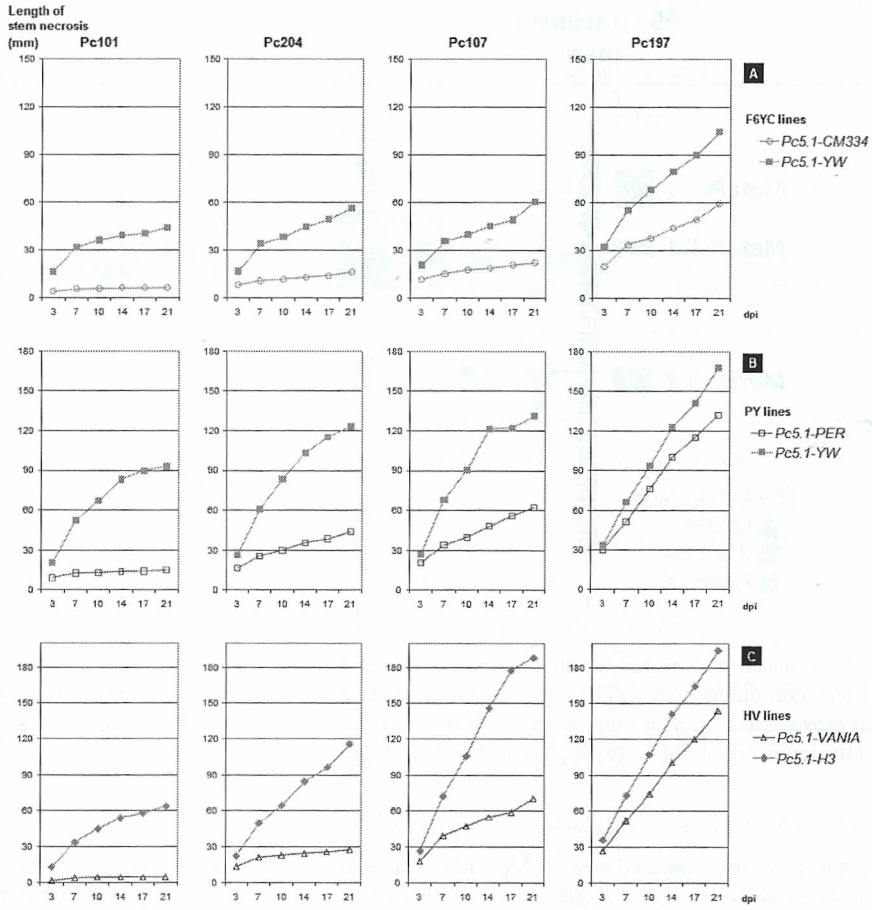

Figure 2: Mean length progression of stem necrosis over 21 days post inoculation (DPI). Stem necrosis was assessed in lines possessing the resistant or the susceptible allele at QTL Pc5.1 for F6YC, PY and HV progenies (A, B, and C, respectively) after inoculation with *P. capsici* isolates Pc101, Pc107, Pc197 and Pc204.

Fine mapping and physical map of QTL Pc5.1

The position of the locus *Pc5.1* was first downsized to less than 5 cM in the F5YC RIL progeny (Bonnet et al, 2007). Thanks to a positional cloning approach, it was yet downsized to less than 0.4 cM by screening a large selfing progeny derived from an introgression line heterozygous at *Pc5.1* in a fixed YW genetic background. Then, we constructed a physical map by anchoring BAC clones to the fine genetic map, and sequenced BAC clones constituting the minimum tilling path. Assembling of reads delivered a reference sequence of ~1.1 Mb. This sequence contained more than 90% of repeated elements corroborating the finding of Park et al (2011). Structural and functional annotation delivered less than 10 ORFs, with predicted functions for a few of them. One ORF shows homology with a transcript differentially expressed in *P. capsici*-infected peppers, and exhibits SNPs between resistant and susceptible lines.

Pattern of polymorphism at candidate genes for QTL Pc5.1

Six of the *Pc5.1* candidate genes and 14 loci distributed all over the pepper genome were successfully amplified for more than 55 accessions of the 60-accessions-core-collection. Their sequencing yielded between 4×10^5 to 202×10^5 of reads per accession. The entire trimming process removed 4% to 33% of sequenced reads depending on accessions. After applying filtering parameters, a total of 124 SNPs and no InDels were detected at candidate genes which corresponded to 9.8 SNPs per 1-kb.

Haplotype analysis of Pc5.1 *candidate genes for QTL*

Haplotype networks of candidate genes identified two major haplotypes, H1 and H2, for all analysed genes. H1 was mostly exhibited by accessions assigned to the susceptible and intermediate resistance clusters. H2 was exhibited for all candidate genes by exactly the same resistant accessions. This result indicates that candidate genes are under purifying selection.

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