Molecular Breeding (2005) 15: 409–422
DOI: 10.1007/s11032-005-0386-8

The tomato Orion locus comprises a unique class of *Hcr9* genes☆

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Received 1 July 2004; accepted in revised form 11 January 2005

Key words: Cladosporium fulvum, Contig, Fingerprinting, *Hcr9*, *Lycopersicon*, Resistance

Abstract

Resistance against the tomato fungal pathogen *Cladosporium fulvum* is often conferred by *Hcr9* genes (Homologues of the *C. fulvum* resistance gene *Cf-9*) that are located in the *Milky Way* cluster on the short arm of chromosome 1. These *Hcr9* genes mediate recognition of fungal avirulence gene products. In contrast, the resistance gene *Cf-Ecp2* mediates recognition of the virulence factor Ecp2 and is located in the *Orion* (*OR*) cluster on the short arm of chromosome 1. Here, we report the map- and homology-based cloning of the *OR Hcr9* cluster. A method was optimised to generate clone-specific fingerprint data that were subsequently used in the efficient calculation of genomic DNA contigs. Three *Hcr9s* were identified as candidate *Cf-Ecp2* genes. By PCR-based cloning using specific *OR* sequences, orthologous *Hcr9* genes were identified from different *Lycopersicon* species and haplotypes. The *OR Hcr9s* are very homologous. However, based on the relative low sequence homology to other *Hcr9s*, the *OR Hcr9s* are classified as a new subgroup.

Abbreviations: Hcr9 – homologue of the *Cladosporium fulvum* resistance gene *Cf-9*; *OR* – *Orion* locus; *R* gene – resistance gene; *Avr* gene – avirulence gene; HR – hypersensitive response; Ecp – extracellular protein; PR protein – pathogenesis-related protein; *MW* – *Milky Way* locus; *AU* – *Aurora* locus; cM – centiMorgan; LRRs – leucine-rich repeats; *NL* – *Northern lights* locus; *SC* – *Southern Cross* locus; CAPS – Cleaved Amplified Polymorphic Sequence; ORF – open reading frame; FPC – fingerprinted contigs; RGA – resistance gene analogue; *LoxC* gene – *LipoxygenaseC* gene; utr – untranscribed region; UPGMA – unweighted pair group method with arithmetic mean

Introduction

The fungal pathogen *Cladosporium fulvum* causes tomato leaf mould. In wild related species of tomato (*Lycopersicon esculentum*), many accessions have been identified that are resistant to *C. fulvum*. Plant breeders have introgressed the

☆Data deposition: The sequence of the *Cf-Ecp2 Hcr9* gene cluster and the orthologous *Hcr9* sequences have been deposited in the GenBank database (accession No. AY639600..AY639604)

C. fulvum resistance genes from these accessions (designated *Cf*-genes) into new cultivars, which provide effective protection of tomato against the fungus. The tomato – *C. fulvum* interaction has been extensively used as a model to study gene-for-gene interactions (Joosten and De Wit 1999). According to this model, resistance against the pathogen depends on the presence of at least two components: a resistance (*R*) gene in the plant and a matching avirulence (*Avr*) gene in the pathogen. An interaction between the resistance gene product and the Avr factor will invoke defence responses (accompanied a hypersensitive response; HR) eventually leading to complete inhibition of fungal growth.

Colonisation of tomato leaves by *C. fulvum* remains restricted to the apoplast and, consequently, the exchange of molecular signals between fungus and the plant occurs extracellularly. Detailed analyses of apoplastic fluids resulted in the identification, molecular isolation and characterisation of race specific Avr factors (reviewed by Joosten and De Wit 1999; Luderer et al. 2002; Westerink et al., submitted). In addition to the race-specific Avr factors, many other fungal low molecular weight peptides have been purified from apoplastic fluids from infected tomato leaves. Several of these corresponding Ecps (Extracellular Proteins, including Ecp1, Ecp2, Ecp3, Ecp4 and Ecp5) have been isolated and, except for Ecp3, the encoding genes have been isolated (Van der Ackerveken et al. 1993; Laugé et al. 2000). During pathogenesis, all strains of *C. fulvum* produce and secrete these Ecps abundantly. Inoculation of an Ecp2-deficient replacement mutant on susceptible tomato plants showed a reduced virulence and induced accumulation of pathogenesis-related (PR) proteins (Laugé et al. 1997). Consequently, Ecp2 was proposed to play a role in virulence of *C. fulvum* by suppression of host defence responses.

Various breeding lines and accessions of *L. pimpinellifolium* have been identified that recognise Ecps after injection of these proteins or by Potato Virus X-based expression of the corresponding cDNA in infected plant tissue (Laugé et al. 1998, 2000). The ability to recognise Ecp2 and to induce an HR is based on the single dominant gene *Cf-Ecp2* that confers resistance to *C. fulvum* strains producing the Ecp2 protein (Haanstra et al. 1999). As the *Cf-Ecp2* gene mediates recognition of the potential virulence factor Ecp2, it was hypothesised that *Cf-Ecp2* may confer durable resistance against *C. fulvum* (Laugé et al. 1998). Furthermore, Ecp2 is also specifically recognised by several *Nicotiana* species which are non-hosts to *C. fulvum* (Laugé et al. 2000; De Kock et al. 2004). This indicates that recognition of Ecp2 is widespread and can be found in both host and non-host plants.

A number of *Cf* resistance genes have been mapped at four different loci. *Cf-2* and *Cf-5* are closely linked and map on chromosome 6 (Dixon et al. 1996, 1998), *Cf-4, Cf-4E* and *Cf-9* have been mapped on the short arm of chromosome 1 at the *Milky Way* (*MW*) locus (Van der Beek et al. 1992; Balint-Kurti et al. 1994; Takken et al. 1998), *Cf-Ecp5* at the *Aurora* (*AU*) locus, 4 cM(centi-Morgan) proximal to the *MW* cluster (Haanstra et al. 2000) and *Cf-Ecp2* and *Cf-Ecp3* at the *Orion* (*OR*) locus, 12 cM proximal to the *MW* locus (Haanstra et al. 1999; Yuan et al. 2002), see also Figure 5. Several *Cf* genes, notably *Cf-9* (Jones et al. 1994), *Cf-2* (Dixon et al. 1996) *Cf-4* (Thomas et al. 1997), *Cf-4E* (Takken et al. 1998), *Cf-5* (Dixon et al. 1998) and *9DC* (Van der Hoorn et al. 2001a; M. Kruijt et al. 2004) have been cloned and sequenced. The *Cf*-genes encode membrane-anchored, extracytoplasmic glycoproteins with an extracellular domain mainly consisting of leucine-rich repeats (LRRs) which are predicted to mediate recognition of matching fungal elicitor proteins. The *Cf* genes cloned so far belong to two gene families, the *Cf* genes located in the *MW* locus (*Cf-4, Cf-4E*, *Cf-9 and 9DC*) are very homologous and are referred to as *Hcr9*s (Homologues of the *C. fulvum* resistance gene *Cf-9*) and similarly, the genes *Cf-2* and *Cf-5* are referred to as *Hcr*2s. Depending on the genotype, the *MW* locus can contain up to six *Hcr9*s (Parniske et al. 1997, 1999; Parniske and Jones 1999; M. Kruijt et al. 2004). The short arm of chromosome 1 harbours two additional clusters with *Hcr9*s, *Northern Lights (NL)* and *Southern Cross* (*SC*) (Parniske et al. 1999). The latter clusters do not contain *Cf* genes involved in resistance. RFLP analysis with a *Cf-9* probe demonstrated that *AU* and *OR* loci containing the *Cf-Ecp* genes also comprise *Hcr9*s (Haanstra et al. 1999, 2000; Yuan et al. 2002). So far, 19 *Hcr9*s have been sequenced. Sequence variation within Cf proteins is generally present in the first 16 LRRs that most probably

determine recognitional specificity (Thomas et al. 1997; Van der Hoorn et al. 2001b).

The resistance gene *Cf-Ecp2* has been accurately mapped on chromosome 1 at <0.3 cM distance from the Cleaved Amplified Polymorphic Sequence (CAPS) marker CT116 (Haanstra et al. 1999). Bonnema et al. (1997) showed by using a cross between *L. esculentum* and *L. peruvianum* LA2157, that near the CT116 locus a genetic distance of 1 cM corresponds to a physical distance of maximally 52 kb. The tight linkage of *Cf-Ecp2* with CT116 and the cosegregation with *Hcr9*s prompted us to use a combination of a homology-based- and map-based cloning approach to characterise the *OR Hcr9* gene cluster and to identify candidate *Cf-Ecp2* genes.

In the present study, the cloning and sequence analysis of the *Hcr9*s at the *OR* cluster is reported. Additionally, the presence of orthologous *OR* clusters was investigated. We isolated *OR Hcr9*s from other tomato haplotypes and discuss unique features of these *Hcr9*s and corresponding clusters.

Results

Library construction and screening

The *Cf-Ecp2* gene has previously been mapped within approximately 20 kb from the genetic marker CT116 (Haanstra et al. 1999). A five genome equivalent library was constructed in the pCLD04541 binary cosmid vector (Bent et al. 1994) with an average insert size of approximately 20 kb. Isolation of library clones containing *Hcr9*s and/or CT116 would enable us to isolate an overlapping series of clones (contig) covering the *OR* resistance cluster.

Due to a high background signal, hybridisation screening of pooled cosmid clones with *Cf-* and CT116 probes was not successful. Therefore, a PCR-based screening using *Hcr9-* and CT116 specific primer sets was performed. In total, 23 cosmids harbouring *Hcr9* sequences and one cosmid containing the CT116 marker were identified. Detailed characterisation of these cosmids by restriction mapping, DNA hybridisation and sequence analysis of *Hcr9*-derived PCR amplification products indicated that coverage of clones varied over the genome (data not shown). Many cosmids showed similar sequences to known *Hcr9* genes located in *NL, MW* or *SC* cluster. Two cosmids were identified that contained *Hcr9*s of unknown origin. Unfortunately, physical overlap of these clones with the clone containing CT116 was not present.

We used the binary cosmid vector since it allows a direct *Agrobacterium*-mediated transfer of cloned plant DNA into plant cells for complementation experiments. Although a contig covering the complete *OR* locus could not be constructed, we continued with the functional analysis. Cosmids carrying candidate *Cf-Ecp2* genes were transformed into *Agrobacterium tumefaciens* strain GV3101. Restriction analysis of several transformed cosmids (including 3.8G and 4.8G, located in the *OR* contig) showed deletions of insert-DNA (results not shown). This artefact is probably caused by the recombination of duplicated homologous sequences on the insert and hampered a straightforward functional analysis of candidate *Cf-Ecp2* genes.

A second genomic library was made in the λ-BlueStar vector (Novagen) which enables the screening of phages by DNA hybridisation and subsequent conversion of the isolated phages into high-copy plasmids (pBlueStar). A 16-genome equivalent library was constructed aiming to cover the complete *OR* cluster despite even in case of biased genome coverage. Hybridisation screening of this library resulted in 49 individual *Hcr9* clones and six CT116 clones, of which four clones contained both the CT116 CAPS marker and an *Hcr9* sequence confirming the tight linkage of CT116 with at least one *Hcr9*.

Contig construction and mapping

We expected that isolated library clones would align in different contigs corresponding to the different *Hcr9* clusters. Contig construction of the clones by (low-resolution) restriction mapping was very laborious and therefore a restriction-mediated PCR fingerprinting method was developed to obtain clone-specific, high-resolution fingerprints that facilitated a reliable contig establishment. Indeed, contiguous clones with similar fingerprint-patterns were clustered into a distance tree comprising distinct branches (Figure 1).

This restriction-mediated PCR fingerprinting method was initially optimised for the *Eco*RI/*Mse*I restriction enzyme combination. Additional

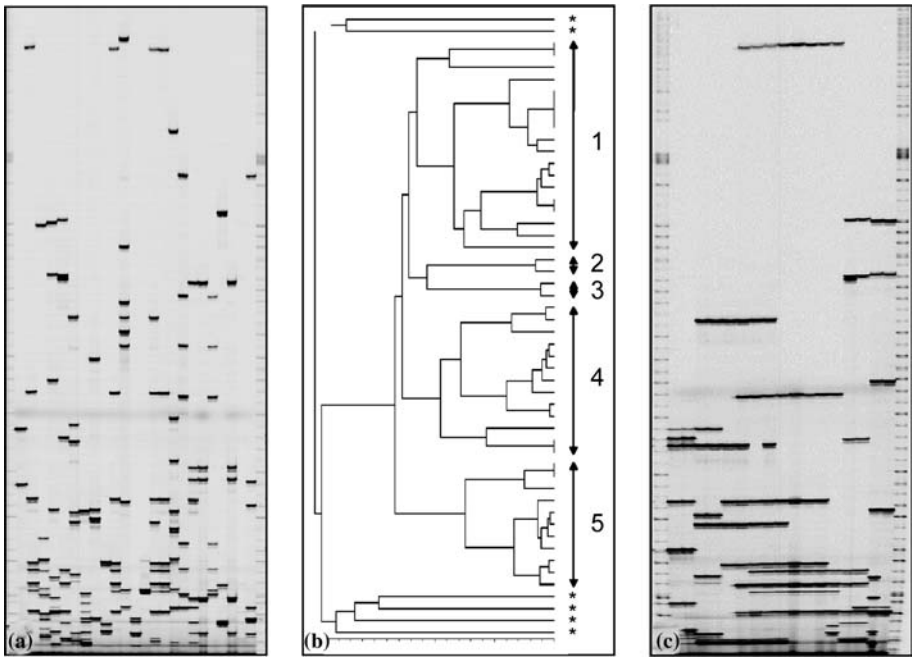

Figure 1. Ordering of library clones in contigs by PCR-fingerprinting and distance trees. (a) PCR fingerprint pattern of randomly ordered *Eco*RI/ *Mse*I-mediated pBlueStar library clones containing *Hcr9*s and/or CT116 (subset of clones). The fingerprint pattern of each clone was converted to a binary data set (presence or absence of a band), which, together with additional experimental data (not shown), enabled the calculation of a distance tree using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (b) Distance tree in which overlapping library clones were clustered. Arrows indicate five clades with contiguous clones. Clade 1 represents the *Northern Lights* cluster; clade 2 the *Milky Way* cluster, clade 3 is a cluster of unknown origin; clade 4 the *Orion* cluster; clade 5 the *Southern Cross* cluster. Clones, which could not be assigned to a contig, are indicated with an asterisk; (c) Fingerprint pattern of contiguous PCR fingerprinted library clones.

fingerprint data were obtained by using the five/ four-cutter restriction enzyme combination *Apo*I/ *Mse*I which allowed the unambiguous location of clones into one contig. Some of the clones appeared in separate clades with unrelated fingerprints and could not be assigned to *Hcr9* contigs. Additional hybridisation and PCR analysis showed that these clones did not contain *Hcr9* sequences and were false-positives from the library screening. Positioning fingerprinted clones according to the position in this tree enabled us to visually confirm the order of clones in a contig (Figure 1). With this method we could efficiently construct five contigs.

Positioning of contigs on the tomato genetic map

Based on the perfect sequence homology of *Hcr9*-derived PCR products with published *Hcr9* sequences, three contigs were assigned to the *NL, MW*, or *SC Hcr9* cluster. The fourth contig, spanning a 41 kb genomic DNA region, comprised the CT116 marker and therefore covers the *OR* locus (Figure 2a). The fifth contig with two overlapping clones contained one *Hcr9* of unknown origin. Physical overlap with one of the other contigs was absent. This contig may represent the *AU* locus of the *Cf-Ecp2* haplotype.

The Cf-Ecp2 Orion locus

The *Cf-Ecp2 OR* cluster represented by pBlueStar clones 19, 49 and J was completely sequenced. In the initial physical alignment, clone 19 was overlapping with clone 49 by three shared *Apo*I/ *Mse*I markers. Remarkably, the consensus DNA sequence of clone 19 and 49 could not be aligned. Analysis of both sequences showed that this was due to a perfect DNA repeat encompassing both clones. Therefore, additional selected cosmid

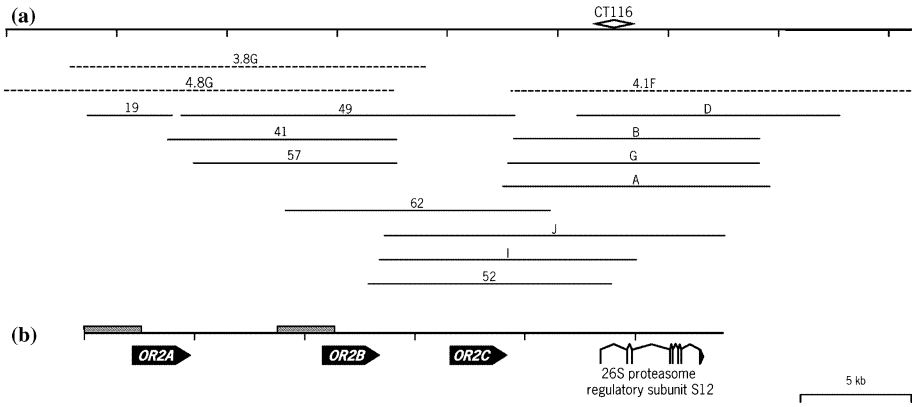

Figure 2. Physical map of the *Cf-Ecp2* locus. (a) A 41 kb contig spanning the *Orion Cf-Ecp2* resistance cluster was constructed with pBlueStar clones (solid lines) and pCLD04541 cosmid clones (dashed lines). The location of the CT116 CAPS marker is indicated by a diamond (◇). (b) A 29 kb segment was sequenced. The position and orientation of three intronless *Hcr9* ORFs is indicated by solid arrowed boxes. The position of the 26S proteasome regulatory subunit S12 exons are shown by connected boxes and triangle. The perfect tandem repeats are indicated by horizontal grey boxes.

clones were fingerprinted to assign them to *Hcr9* contigs. After additional restriction mapping, DNA hybridisation and sequencing of *Hcr9*-derived PCR products, three binary cosmid clones were identified which fitted in the *OR* contig. As a result, clone 19 and 49 showed to be separated by a 1 kb gap. A part of clone 4.8G was PCR-amplified and sequenced to close this sequence gap. Finally, a complete 29 kb sequence was obtained for the complete *Cf-Ecp2 OR* cluster.

Four putative open reading frames (ORFs) present in the *Cf-Ecp2 OR* cluster are shown in Figure 2b. The CT116 CAPS marker is located in the first intron of a gene encoding the 26S proteasome regulatory subunit S12 (*E*-value 5e − 21) and does not cover the open reading frame. The 26S proteasome is a 2 MDa proteolytic complex that degrades ubiquitinylated protein conjugates (Voges et al. 1999; Smalle et al. 2004). The proteasome pathway degrades proteins that arise from synthetic errors, spontaneous denaturation, free-radical-induced damage, improper processing or diseases (Hershko and Ciechanover 1998). There is no evidence that this gene, of which the open-reading frame is very conserved in the plant kingdom, is involved in the recognition of the *C. fulvum* Ecp2 elicitor.

The major part of the *OR* sequence contains three genes homologous to *Hcr9*s. These encode membrane-anchored receptor-like proteins with 27 extracellular LRRs and a short cytoplasmic tail (Figure 3). Following the nomenclature used by Parniske and Jones (1999), these genes were designated *OR2A, OR2B* and *OR2C*, after their genetic location *Orion*, their putative involvement in Ecp2 recognition and the alphabetic order of the gene in the *OR* cluster. The *OR* cluster contains a 2625 bp perfect tandem repeat including the promoter region and the first part of the coding region (432 bp) of *OR2A* and *OR2B*.

Typical for Hcr9 proteins, the majority of the amino acid variation between members of this family is present in the B-domain and the first 17 LRRs of the C-domain. The alignment of the three newly identified Hcr9s shows that the amino-acid variation is spread throughout the protein (Figure 3). The signal sequence has only ±50% amino-acid homology with Cf-9 but still resembles a putative signal peptide for extracellular targeting. The cysteine-rich B-domain of OR2A/OR2B has low sequence homology with Cf-9 but the cysteine residues are conserved. In OR2C, a stretch of 17 amino acids is absent, including one cysteine residue. In the majority of the first 17 LRRs, amino-acid substitutions occur at the putative solvent-exposed domains (xxLxLxx) of LRRs. The amino acid variation in the OR2 proteins continues in the C-terminal part, including LRRs 18–24, the loop-out and the acidic E-domain.

Orthologous OR loci

To study the complexity of *OR Hcr9* clusters, an *Hcr9*-specific fingerprint method was developed

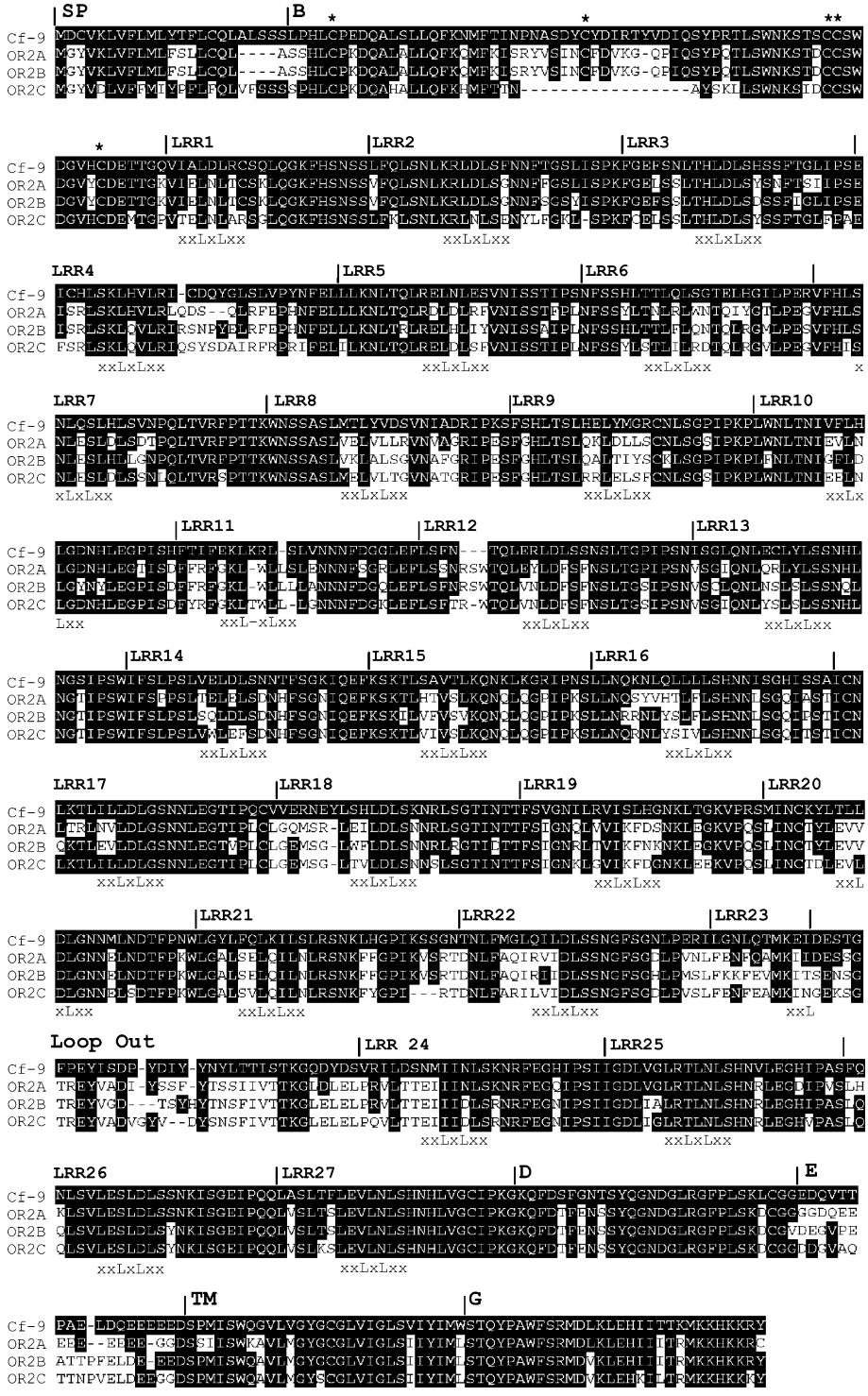

Figure 3. Alignment of the Cf-9, OR2A, OR2B and OR2C proteins. Amino acid residues identical to Cf-9 are indicated with solid black background. Sequence gaps inserted to maintain the alignment are indicated by dashes. Domains are indicated above the sequence as follows: SP – signal peptide (A-domain); B – cysteine-rich domain, cysteine residues are indicated with an asterisk; LRR 1 to 27 (C-domain), various β-sheets are indicated (consensus xxLxLxx) each of which contains five solvent-exposed amino acid residues (x); D – domain without obvious features; E – acidic domain; TM – putative transmembrane domain; G – basic domain, representing the short, putative, cytoplasmic tail.

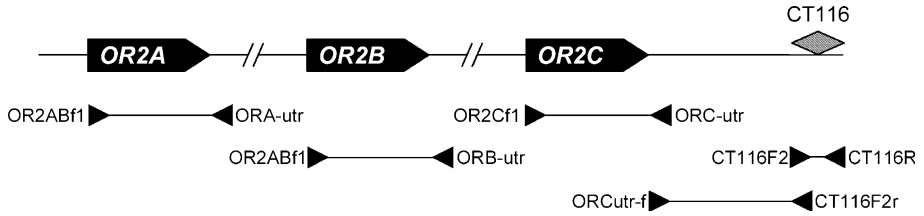

Figure 4. PCR-amplification strategy for the identification of *Orion* (*OR*) orthologous *Hcr9* genes and for the confirmation of physical linkage of *OR2C* orthologous genes with CT116. In the schematic representation of the *Cf-Ecp2 OR* cluster, the position and the orientation of the three *Hcr9*s are indicated by arrowed boxes, the CT116 CAPS marker is indicated by a diamond (◇). Triangles indicate annealing position and direction of primers. For further details see Experimental Procedures.

(De Kock 2004). In a mapping population segregating for *Cf-Ecp2* resistance, certain *Hcr9* markers cosegregated with *Cf-Ecp2* resistance, while one *Hcr9* marker was in repulsion phase with *Cf-Ecp2* resistance. Probably, this *Hcr9* marker corresponds to an orthologous gene in the *OR* locus of the susceptible parent MM-Cf0. The *OR* clusters in other haplotypes were subsequently investigated. The low sequence homology at primer-annealing site of the *OR Hcr9*s compared to the consensus sequence of *Hcr9*s enabled us to design *OR* specific primers that were used to identify orthologous *OR* genes in other *Lycopersicon* accessions. By a PCR-based cloning strategy using primer sets specific for each of the three *OR2s* (Figure 4), orthologous *OR Hcr9*s from Cf0-, Cf-Ecp3 and Cf-Ecp5 haplotypes were identified. Following the above nomenclature, these genes were designated after their genetic location *Orion*, the Cf-haplotype (Cf0, Cf-Ecp3 and Cf-Ecp5) and the alphabetic order of the gene in the *OR* cluster. The prefix Ψ indicates a pseudogene. The organisation of all the 32 presently known *Hcr9*s is depicted in Figure 5.

The *OR* orthologue in Cf-0, designated Ψ*OR0A*, is homologous to *OR2C* (99.7% sequence homology) but has a 10 bp deletion resulting in a frame shift. The MM-Cf-Ecp3 and MM-Cf-Ecp5 orthologues of *OR2C* (designated Ψ *OR3B* and Ψ *OR5A*, respectively) showed 99.5% and 99.1% sequence homology to *OR2C*. Both genes encode a truncated *Hcr9* caused by a point mutation resulting in a stop-codon (Cf-Ecp3 haplotype) or a nucleotide insertion (Cf-Ecp5 haplotype). By using primer sets specific for *OR2A*, no orthologous genes in other haplotypes were identified, while one orthologous gene in the *Cf-Ecp3 OR* cluster was identified by using a primer set specific for *OR2B* designated *OR3A*. The encoded protein showed 92% sequence homology to *OR2B*. The Cf-Ecp3 and Cf-Ecp5 haplotypes were not studied by RGA-fingerprint analysis. Therefore, the presence of additional *Hcr9*s at these loci cannot be excluded.

Physical linkage of *OR3A* and Ψ*OR3B* to the CT116 marker was proven by the analysis of *Cf-Ecp3* genomic library clones harbouring the *Cf-Ecp3 OR* locus (Y. Yinan, personal communication). Finally, physical linkage of Ψ*OR0A*, Ψ*OR3B* and Ψ*OR5A* to CT116 was investigated by PCR analyses. The 4.2 kb DNA fragment that spans the distance between these *OR2C* orthologous genes and the CT116 locus could be PCR-amplified from all tested haplotypes. With these results we show the existence of orthologous gene clusters at the *OR* locus as was previously described for the *MW* cluster.

Discussion

Isolation and characterisation of binary cosmid library clones

In a genomic library of more than five genome equivalents, statistically more than 99% of the genome should be covered by cosmid library clones. In our study, the constructed binary cosmid library was incomplete at the *OR* locus. Therefore, it seemed not possible to isolate contiguous binary cosmid clones covering the complete *OR* cluster. Two additional reasons that prompted us to decide to construct a new genomic library in another vector were (*i*) the risk that *Cf-Ecp2* candidates that do not perfectly match to the degenerate *Hcr9* primers could have been

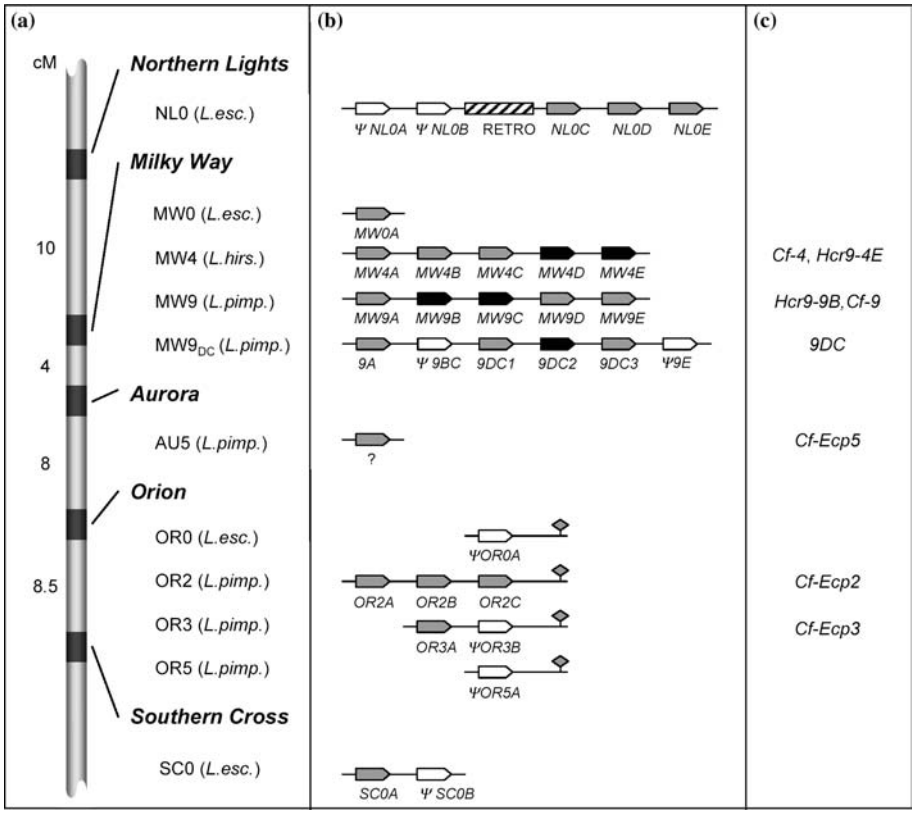

Figure 5. Map position, genetic distance (cM) and physical structure of the *Northern Lights, Milky Way, Aurora, Orion* and *Southern Cross* loci harbouring clusters of *Hcr9*s on the short arm of chromosome 1. (a) A genetic map of the various clusters showing the position of five *Hcr9* loci relative to each other. (b) The physical organisation of each *Hcr9* cluster is shown. (c) The *Cf* resistance genes present in the cluster are indicated. Arrowed boxes indicate the relative position and orientation of *Hcr9*s; white arrowed box: *Hcr9* pseudogene; grey arrowed box: *Hcr9* with unknown function; black arrowed box: *Hcr9* resistance gene. *Hcr9* clusters are derived from different haplotypes: NL0: *L. esculentum* Cf0; MW0: *L. esculentum* Cf0; MW4: *L. hirsutum* Cf4; MW9: *L. pimpinellifolium* Cf9; $MW9_{DC}$: *L. pimpinellifolium* 9DC (Kruijt et al. 2004). The organisation of the *OR* cluster was determined in this study: OR0: *L. esculentum* Cf0; OR2: *L. pimpinellifolium* Cf-Ecp2; OR3: *L. pimpinellifolium* Cf-Ecp3; OR5: *L. pimpinellifolium* Cf-Ecp5. SC0: *L. esculentum* Cf0. The organisation of the *Aurora* cluster is unknown. The CT116 CAPS marker at the Orion locus is indicated by a diamond (◇); RETRO denotes a retrotransposon insertion in the *NL* cluster.

missed in the PCR-selection of clones; (*ii*) DNA-instability of the selected cosmid inserts in *A. tumefaciens* hampered functional analysis of candidate *Cf-Ecp2* genes. Instability of insert-DNA in the binary cosmid vector pCLD04541 upon transfer to Agrobacterium has previously also been reported by Dixon et al. (1996). Although the vector pCLD04541 has frequently been used for the cloning of resistance genes, e.g. *Cf-2, Cf-4*, *Cf-5, Hero* (Dixon et al. 1996; Thomas et al. 1997; Dixon et al. 1998; Ernst et al. 2002), this vector was not suitable to clone *Cf-Ecp2*.

Isolation and contig construction of pBlueStar clones

To avoid the problems described above, a larger, 16-genome equivalent library in λBlueStar was made and phages were screened by DNA hybridisation. After restriction-mediated PCR fingerprinting and contig calculation, selected clones were positioned into five different *Hcr9*-containing contigs representing the known *NL, MW* and *SC Hcr9* clusters and the *OR Hcr9* cluster. It is possible that the fifth contig belongs to the *AU* locus. The restriction-mediated PCR fingerprinting and

subsequent calculation of contigs appeared to be a very efficient and reliable method for contig construction. Our method follows the same strategy of FPC (fingerprinted contigs) described by Soderlund et al. (1997), but a different type of fingerprint data is used and contig calculation is less complex. Selection of frequent or rare cutting restriction enzymes for fingerprinting is based on the required resolution and sizes of template DNA. After all, integration of two genomic libraries representing in total 21 genome equivalents was necessary for the contig construction and to sequence the *OR Hcr9* cluster.

The Cf-Ecp2 Orion cluster

The contig is at one side flanked by the CT116 CAPS marker, but the physical end on the other site of the contig remains obscure and thereby the number of *Hcr9*s was not known. To confirm the number of *Hcr9*s in the *OR* cluster, an *Hcr9* resistance gene analogue (RGA) fingerprint method was developed (De Kock 2004). With this method *Hcr9* gene-specific markers are generated. All *Hcr9* markers which cosegregated with *Cf-Ecp2* resistance corresponded with the three *Hcr9*s of the *OR* contig. Therefore, we concluded that the *Cf-Ecp2 OR* cluster contains three *Hcr9*s.

The *Cf-Ecp2 OR* cluster harbours a large duplication of 2.6 kb, which is probably a result of a recent intergenic unequal crossing over. Interestingly, the first 576 bp of this duplicated region shows high sequence homology (92%) with the promoter region of *Hero*, an NBS-LRR resistance gene located on chromosome 4 conferring broad spectrum resistance against potato cyst nematodes (Ernst et al. 2002). For *Hero, OR2A* and *OR2B* the conserved 576 bp region is located approximately 1.5 kb upstream of the ATG start codon and may therefore act as cis-acting binding domain that regulates the transcriptional activity of the upstream gene.

The *OR* Hcr9 proteins show the characteristic domains of plasmamembrane-anchored glycoproteins of which the extracytoplasmic domain mainly consists of LRRs. The LRR domains of R proteins were suggested to be involved in the recognition of the corresponding elicitor or co-acting proteins (Jones and Jones 1996). Consistent with this theory, it was found that Hcr9 proteins with specificity for different Avr factors predominantly differ in amino acid residues located at putative solvent-exposed positions in the N-terminal LRRs (Parniske et al. 1997, Van der Hoorn et al. 2001b; Wulff et al. 2001). However, in contrast to the previously known Hcr9 proteins, the variation in the *OR* Hcr9 proteins continues in the C-terminal LRRs. Additionally, the loop-out and the acidic E-domain vary, whereas these domains are rather conserved in the *NL, MW, SC* Hcr9 proteins (Figure 6). The loop-out of the BRI1 receptor protein, a receptor-like kinase located on the cell surface that is involved in brassinosteroid signalling (Li and Chory 1997; Wang and He 2004), was proven to facilitate brassinosteroid binding. Therefore, the abundant variation in the loop-out domain and the other C-terminal domains of the *OR* Hcr9s may indicate that elicitor perception and signal transduction mediated by the *OR* Hcr9s is different compared to the other Hcr9s.

Orthologous Orion Hcr9 clusters

The *OR Hcr9*s are highly homologous. *OR0A* is present in the Cf-0 *L. esculentum* haplotype, whereas the other genes are from different *L. pimpinellifolium* introgressions. Apparently, these sequences are very conserved in two *Lycopersicon* species although only *OR2C* encodes a full-length protein. Polymorphic sites can distinguish the individual members and allow speculation about the origin and relation to other members of this gene family. Based on the shared polymorphic sites, the *OR Hcr9*s are subsequently most related to Ψ*NL0A*, Ψ*NL0B, NL0D* and *NL0E*. The *Hcr9s* in the *OR* cluster represent a distinct subgroup of *Hcr9*s when the sequences are aligned with *NL, MW, SC Hcr9s* and *Hcr2*s (Figure 7). In contrast, the intergenic regions are very unique for the *OR* locus. The *MW* and *SC* clusters harbour several *LipoxygenaseC* (*LoxC*) exons that are thought to have coduplicated with *Hcr9*s (Parniske et al. 1997). These *LoxC* sequences are absent in the *OR* cluster as they are also absent in the *NL* cluster. Parniske and Jones (1999) suggested that the divergence of the *NL Hcr9*s was probably a consequence of its genetic isolation. This suggestion is now contradicted by our finding of the relative

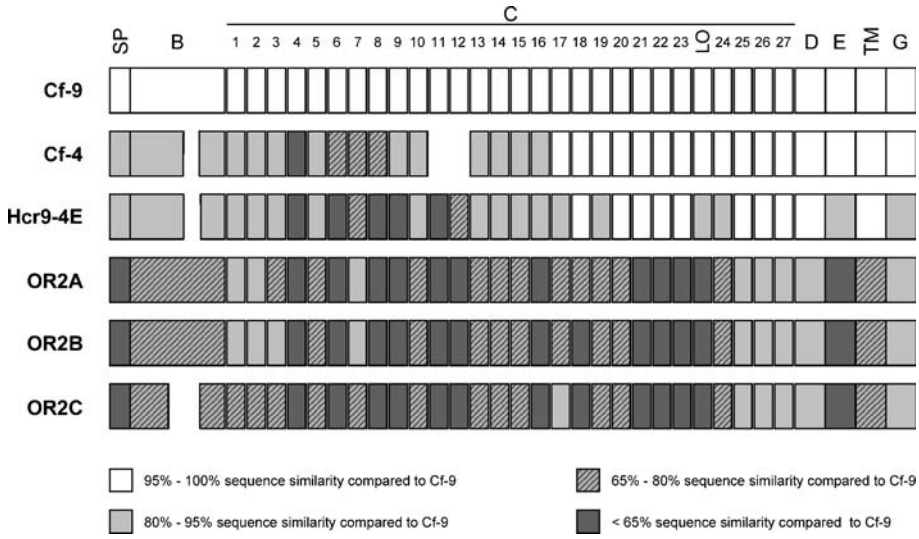

Figure 6. Schematic presentation of amino acid similarity of the *Cf* resistance proteins Cf-4, Hcr9-4E and the Orion Hcr9 protein OR2A, OR2B and OR2C compared to the Cf-9 resistance protein; structural protein domains: SP – signal peptide; B – cystein-rich domain; C – domain containing 27 leucine-rich-repeats (LRRs), LO – Loop Out; D – domain without conspicuous features; E – acidic domain; TM – putative transmembrane domain; G – basic cytoplasmic domain. Grey scale indicates the level of amino acid similarity compared to the Cf-9 protein.

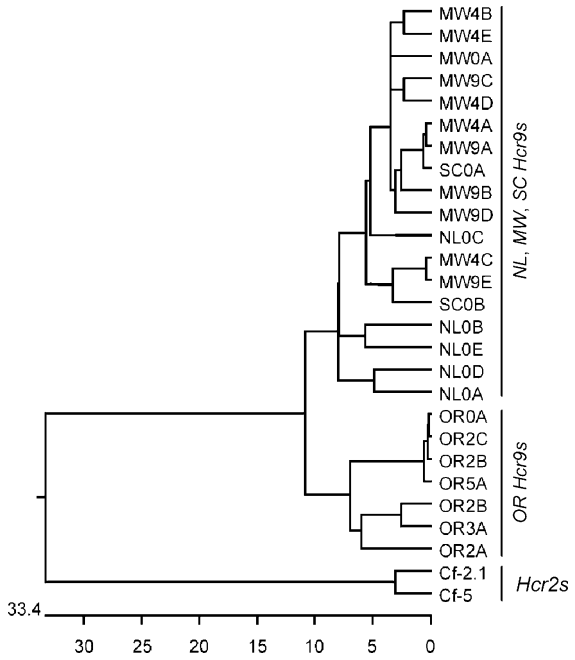

Figure 7. Phylogenetic relationships between *Hcr9*s and *Hcr2*s. The nucleotide sequences were aligned using the Clustal method (Higgins and Sharp 1989) and the neighbour-joining method was employed to construct a phylogenetic tree. Three different clades are constructed representing (*i*) the *NL, MW, SC Hcr9*s, (*ii*) the *OR Hcr9*s and (*iii*) the *Hcr2*s. The scale bar indicates the degree of nucleotide dissimilarity.

high homology of the *OR Hcr9*s to the *NL Hcr9s*, although the *OR* cluster is located between the *MW* and *SC* cluster.

Homology searches in databases using *Cf* gene and protein sequences showed that numerous sequences highly homologous to *Cf* genes (*E* value $< 1e-50$ at nucleotide level) are present in *Lycopersicon, Solanum* and *Capsicum* species. Interestingly, sequences most homologues to the *OR Hcr9*s are not found in *Lycopersicon* species, but are present in *Solanum tuberosum*. This suggests the existence of common ancestral *OR* genes before *Lycopersicon* and *Solanum* speciation and indicates that the unique features of the *OR Hcr9* genes remained conserved during evolution.

Depending on the haplotype, the identified *OR Hcr9*s are candidate genes for *Cf-Ecp2* and *Cf-Ecp3* function. Complementation analysis with these candidate *Cf-Ecp2* and *Cf-Ecp3* genes has to reveal which genes are involved in the perception of the *C. fulvum* elicitor Ecp2 and Ecp3, respectively and trigger HR-based resistance.

Experimental procedures

pCLD04541 binary cosmid library construction and screening

Genomic DNA was isolated according to Van der Beek et al. (1992) from four-week-old leaves of the breeding line Ontario 7518 (Cf18) showing *Cf-Ecp2*-mediated resistance (Laugé et al. 1998). DNA was partially digested with *Sau*3A I to an average size of 40 kb. Partially filled-in insert DNA (1 μg) was ligated at 4 °C for 16 h in a total volume of 10 μl with 500 ng of *Xho*I digested and partially filled-in binary cosmid vector pCLD04541 (Dixon et al. 1996). Ligated DNA was packaged using commercial extracts with size-selection (GigapackIII XL, Stratagene) according to manufacturer's instructions and transfected to XL1-Blue MRA *Escherichia coli* (Stratagene). Recombinant bacteria were plated onto agar at a density of 1000–2000 bacteria per plate. After growth at 37 °C, the bacteria of each plate were pooled into 5 ml of LB medium. Subsequently, 4 ml was used for cosmid DNA isolation, while the remainder was kept as glycerol stock. The entire library consisted of 2.7×10^5 clones in 180 pools representing 5.6 haploid genome equivalents based on an average insert size of 20 kb. Cosmid pools were screened by PCR with primers of the CT116 CAPS marker (Bonnema et al. 1997) and with degenerate primers which amplify LRR 1 to 17 of *Hcr9*s (HCR9C1F: 5′-catgggatggmrttsattgtgac-3′ and HCR9C1R: 5′-catwgtgggattgtyccctcc-3′). Pools that yielded a PCR product were selected. To isolate single clones, 7.5×10^3 bacteria of selected pools were screened for hybridisation with the two PCR products. Plasmid DNA of selected bacteria was isolated for further analysis.

λ-BlueStar library construction and screening

Genomic DNA from 4-week-old leaves of the breeding line Ontario 7518 (Cf18) showing *Cf-Ecp2*-mediated resistance (Laugé et al. 1998) was isolated according to the protocol described of Van der Beek et al. (1992). DNA was partially digested with *Sau*3A I to an average size of 40 kb and size-fractionated on a 10–40% sucrose gradient (Sambrook et al. 1989). Fractionated insert DNA with an average size of 20 kb was ligated at 4 °C for 16 h with 0.5 μg of *Bam*HI-digested, dephosphorylated λBlueStar arms (Novagen). Ligated DNA was packaged using commercial extracts with size-selecting features (GigapackIII XL, Stratagene) according to manufacturer's instructions. Phages were transfected to host strain ER1647 (Novagen). The entire library consisted of 1.1×10^6 phages representing 16 haploid genome equivalents based on an experimental average insert size of 15 kb. Phages were screened by hybridisation with the CT116 probe and a *Cf-9* probe covering the entire gene. Selected phages were automatically subcloned into plasmid by Cre-mediated excision from λBlueStar in host strain BM25.8 (Novagen). Plasmid DNA of selected bacteria was isolated for further analysis.

Restriction-mediated PCR fingerprinting and contig construction

To produce clone-specific markers, a restriction-mediated PCR fingerprinting method was optimised. Basically, the protocol consists of four

steps: (1) digestion of plasmid DNA with two restriction enzymes; (2) ligation of matching adapters to sticky ends (3) pre-amplification and (4) labelled amplification which allows size-separation on sequencing type gels. For both plasmid- and cosmid-library clones, 50 ng DNA was digested with *Eco*RI or *Apo*I and *Mse*I at 37 °C. Simultaneously, adapters compatible to the restriction site were ligated. The *Eco*RI-adapter, which is also compatible to the *Apo*I restriction site, originated from the AFLP-protocol (Vos et al. 1995), the adapter compatible to the *Mse*I site was adapted from the Universal Genome-Walker kit (Clontech, Palo Alto, CA). PCR amplification was essentially performed according to the standard AFLP protocol (Vos et al. 1995) on 10 times diluted restriction-ligation mixture. E-1_EX (5′-ctcgtagactgcgtaccaatt-3′) and the AP1 (5′- taatacactcactatagggc-3′) were used as primer set. A second, nested PCR using an fluorescently labelled, internal primer AP2 (5'−$_{IRD700}$actatagggcacgcgtgga-3') in combination with the E-1_EX-primer were performed on 25 times diluted amplification product of the first PCR. Samples were denatured and separated on a 5.5% polyacrylamide sequencing type gels using LI-COR Global IR^2 Systems. The presence or absence of polymorphic bands was scored by visual interpretation of outputs of the LI-COR system using the image interpretation software CROSSCHECKER (Buntjer 2000; http://www.dpw.wau.nl/pv/pub/CrossCheck/download.html). The resulting binary data set was subsequently used for clustering by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using the software package NTSYSpc2.0 (Applied Biostatistics Inc.). UPGMA is the simplest method of distance tree construction. The calculated phylogenetic tree should represent contiguous clones clustered in clades. To confirm the position of each clone in a clade, fingerprinted samples were re-loaded on a gel in the order as indicated in the phylogenetic tree. A combination of restriction mapping, PCR analysis and DNA hybridisation resulted in additional data used for accurate aligning of library clones into a single contig. The origin of *Hcr9*s located on a library clone was identified by *Hin*fI, *Ava*II or *Taq*I digestion of the PCR amplified region of *Hcr9*s containing LRR 1 to 17 (primers: HCR9C1F/-R) and subsequently size-separation on 1.5% agarose gel.

Sequencing and sequence analysis

Selected library clones were sequenced by shotgun sequencing or by transposon-based sequencing using the GPS-LS linker scanning system (New England Biolabs). Greenomics (Wageningen, The Netherlands) performed sequencing of pBlueStar library clones. Evaluation of sequencing data and construction of sequence contigs was performed with Lasergene (DNASTAR Inc., Madison, WI, USA) software packages. PCR products selected to be sequenced were subcloned into pGEM-T Easy (Promega) or PCR-Script (Stratagene). Sequencing of these constructs and insequencing of the insert was done by BaseClear (Leiden, The Netherlands). DNA sequence similarity analysis was performed using BLASTN and BLASTX (Altschul et al. 1997). The presence of retrotransposon sequences or repeated sequences of other nature was analysed by The TIGR Plant Repeat Database (http://www.tigr.org/tdb/e2k1/plant.repeats/index.shtml). Promoter regions and polyA signal sites were analysed with the GeneBuilder prediction program (http://l25.itba.mi.cnr.it/~webgene/genebuilder). Protein structure predictions were performed using publicly available programs (http://www.expasy.ch/tools), signal peptide motif was identified by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP).

Identification of orthologous Orion Hcr9s

Orthologous *Hcr9* genes were PCR-amplified using *OR Hcr9*-specific primers sets with *Pfu-Turbo* proofreading DNA polymerase (Stratagene) at $T_m = 50$ °C, 30 cycli, using genomic DNA of tomato haplotypes Cf0 (MoneyMaker), Cf-Ecp2 breeding line Ontario 7518 (Cf18), Cf-Ecp3 (*L. esculentum* G1.1153) and Cf-Ecp5 (*L. esculentum* G1.1161) and selected Cf-Ecp2, Cf-Ecp3 or Cf-Ecp5 genomic library clones as template and an extension time of 3.5 min at 72 °C. Tomato Cf-Ecp3 and Cf-Ecp5 genomic libraries were donated by Y.Yuan and F. Meijer-Dekens, respectively (Wageningen University, The Netherlands). Positions of the primer sets are depicted in Figure 5. The forward primers were located at the first 30 nucleotides of a specific *Hcr9*(OR2ABf1: 5′-atgggttacgtaaaacttgtttttttaatg-′3, OR2Cf: 5′-atgggctac-

gtagaccttgtatttttatg-3'), the reverse primer were located at the gene specific 3′ untranscribed region (utr) (OR2A-utr: 5′-ctaatgcttttattacttagggaaatg-cac-3′, OR2B-utr: 5′-atagagattaagttgaatacctggagg-3′, OR2C-utr: 5′-gaaaaatatcaagttgaatacctggag-3′). Partial *Cf-Ecp3 OR* sequences (Y. Yinan, unpublished data) were aligned to optimise primer sequences. PCR-amplification products were cloned into pGEM-T (Promega) and DNA sequencing was performed by BaseClear (Leiden, The Netherlands). Two independent clones per PCR-product were completely sequenced to avoid base pair changes introduced during PCR. To verify the physical linkage of OR2C orthologous genes with the CT116 CAPS marker, a PCR analysis was performed using the forward primer ORCutr-f, located at the 3′utr region an *OR2C* orthologue (ORCutr-f: 5′-aacctccaggtattcaacttg-3′) and reverse primer in the CT116 CAPS locus (CT116F2r 5′-ttaccttctcaatcggcctcg-3′). PCR-amplification was performed on 200 ng of genomic DNA or 20 ng of plasmid DNA in 25 μL reaction volume using *Supertaq* (HT Biotechnology) DNA polymerase at annealing temperatures of 48 °C and an extension time of five minutes at 72 °C. PCR-amplification products were checked for size on a 0.8% agarose gel.

Acknowledgements

We are grateful to Yuan Yinan, Marjan Bovers and Fien Meijer-Dekers for supplying the Cf-Ecp tomato genomic libraries. We acknowledge Matthieu Joosten for critically reading the manuscript. This project is sponsored by the Dutch Technology Foundation (STW, grant No. WGC5107) and Dutch breeding companies.

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