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Map- vs. homology-based cloning for the recessive gene *ol-2* conferring resistance to tomato powdery mildew

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Abstract The recessive gene *ol-2* confers papilla-associated and race-non-specific resistance to tomato powdery mildew caused by *Oidium neolycopersici*. In order to facilitate marker assisted selection (MAS) in practical breeding programmes, we identified two simple sequence repeat (SSR) markers and one cleaved amplified polymorphic sequence (CAPS) marker which are linked to the resistance locus and co-dominantly inherited. Aiming to provide a base for *ol-2* positional cloning, we used a large segregating F$_2$ population to merge these markers with all the *ol-2* linked amplified fragment length polymorphism (AFLP®) markers previously identified in an integrated genetic map. By screening a tomato bacterial artificial chromosome (BAC) library, we detected two BAC clones containing two expressed sequence tags (ESTs) homologous to the gene *mlo*, responsible for powdery mildew resistance in barley, as well as an *ol-2*-linked marker. Chromosomal mapping by Fluorescence in situ Hybridization (FISH) revealed major signals of the two BAC DNAs in the pericentromeric heterochromatin of the short arm of chromosome 4, in the same region where the *ol-2* gene was previously mapped. The genetic and cytogenetic co-localisation between *ol-2* and tomato *mlo*-homologue(s), in addition to the similarity of *ol-2* and *mlo* resistances for both genetic and phytopathological characteristics, suggests that *ol-2* is likely a *mlo*-homologue. Thus, a homology-based cloning approach could be more suitable than positional cloning for *ol-2* isolation.

Keywords Fluorescence in situ hybridization · Homology-based cloning · Integrated linkage map · Map-based cloning · *Oidium neolycopersici* · *Solanum lycopersicum*

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Introduction

Tomato powdery mildew caused by *Oidium neolycopersici* has recently been recognized as a world-wide emerging pathogen on tomato (Jones et al. 2001; Kiss et al. 2001). Several dominant genes have been characterized, which confer resistance to

O. neolycopersici and originate from wild tomato species (Bai et al. 2005; Kozik 1993; Lindhout et al. 1994). The exploitation of these genes in plant breeding is limited due to linkage drag phenomena, consisting in unfavourable wild alleles closely linked to the resistance loci (Zeven et al. 1983; Young and Tanksley 1989). In addition, resistance conferred by the dominant *Ol*-genes is based on race-specific hypersensitive response (HR), thus is likely not durable. In contrast, the recessive gene *ol-2*, located around the centromere of chromosome 4 (De Giovanni et al. 2004), derives from the germplasm of the cultivated tomato species, *Solanum lycopersicum* (Ciccarese et al. 1998), and confers race-non-specific resistance via papilla formation, a non-HR-based mechanism (Bai et al. 2005).

The introduction of *ol-2* resistance into tomato cultivars via traditional breeding methods is complicated by its recessive nature, as the selection of the *ol-2* allele needs to be tested in the selfing progeny of each backcross generation. Although marker assisted selection (MAS) with co-dominant molecular markers would allow the breeder to perform the selection of the *ol-2* allele in backcross generations, the low genetic variation within *S. lycopersicum* (Miller and Tanksley 1990) hindered the identification of polymorphic co-dominant markers linked to the resistance locus. So far, mainly amplified fragment length polymorphism (AFLP®) markers have been identified for the *ol-2* region (De Giovanni et al. 2004; Ricciardi et al. 2007). In practical breeding, AFLP® markers cannot offer efficient MAS as they are dominant and require advanced equipment. Moreover, AFLP® markers are patented and thus not utilizable by most breeding companies. Although several efforts were made, the conversion of *ol-2* linked AFLP® markers into PCR markers failed (Ricciardi et al. 2007).

Similarly to *ol-2*, the barley gene *mlo* (Büschges et al. 1997) is recessively inherited and confers durable resistance to barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) through the apposition of papillae at the cell wall (Piffanelli et al. 2002). The similarity of *ol-2* and *mlo* resistances for both genetic (recessive) and phytopathological (papilla-based mechanism) characteristics may indicate that *ol-2* confers *mlo*-based durable resistance.

In the present work, several attempts were carried out in order to obtain new simple PCR co-dominant markers linked to *ol-2*, allowing selection of the resis-

tance via MAS. Aiming at *ol-2* positional cloning, a segregating F_2 population of 316 individuals was analyzed to generate an integrated map of the *ol-2* region, including all the dominant AFLP® markers reported so far. Bacterial artificial chromosome (BAC) library screening and Fluorescence in situ Hybridization (FISH) were used to investigate the possibility that *ol-2* resistance is mediated by a *mlo*-based mechanism.

Materials and methods

Plant material

A F_2 mapping population of 316 individuals was used, which was derived from a cross between the resistant F_3 line R28 and the susceptible *S. lycopersicum* cv. Moneymaker (MM). The F_3 line R28 (homozygous for the *ol-2* allele) was developed from a cross between the resistant line LC-95 of *S. lycopersicum* var. *cerasiforme* and the susceptible *S. lycopersicum* cv. Super Marmande (SM) (Ciccarese et al. 1998).

Fungal material and disease test

The *O. neolycopersici* used in this study was collected from infected tomato plants in The Netherlands. One-month old plants were inoculated with a suspension of conidia as described by Bai et al. (2003). The inoculated plants were grown in a greenhouse compartment at $20 \pm 2°C$ with $70 \pm 15\%$ relative humidity (RH) and day-length of 16 h. Disease evaluation was carried out twice, 12 and 15 days post-inoculation (dpi), on the basis of presence or absence of fungal sporulation.

Marker analysis

Total DNA was extracted from plant leaves 2 days before inoculation by using a rapid cetyl trimethyl ammonium bromide (CTAB) isolation method (Brugmans et al. 2003).

Seven simple sequence repeat (SSR) markers on tomato chromosome 4 were chosen among the ones published by Suliman-Pollatschek et al. (2002) (Table 1). PCR reactions (10 µl) contained 50 ng of genomic DNA, $1 \times$ PCR buffer, 0.5 pmol of each forward and reverse primer (one of which was labelled

with IRD700 or IRD800), 0.2 mM dNTPs and 0.5 units of Taq-polymerase in demi water. PCR conditions were: 1 cycle at 94°C for 3 min followed by 30 cycles of 30 s at 94°C, 45 s at the optimal annealing temperature, 45 s at 72°C, and a final extension of 7 min at 72°C. Amplified products were scored on 6% polyacrylamide gel with a LICOR 4200 DNA sequencer and on 2.5% agarose gel stained with ethidium bromide.

The cleaved amplified polymorphic sequence (CAPS) marker U3-2*Hha*I (Table 1) was obtained by designing primers on the SCAU3$_{1500}$ sequence characterized amplified region (SCAR) sequence reported by De Giovanni et al. (2004) with the software Primer3 (Rozen and Skaletsky 2000) and digesting the amplification products with different restriction enzymes. PCR and digestion were carried out according to the conditions reported by Bai et al. (2003).

Nine markers with known sequence mapped on tomato chromosome 4 and two tomato expressed sequence tag (EST) sequences (cLEC-80-N18 and cTOC-20-K10) homologous to the barley *mlo* gene were selected from the Sol Genomics Database (http://www.sgn.cornell.edu, Table 1). Locus specific primers were designed to amplify genomic DNA as described by Bai et al. (2003). All the amplification products were subsequently digested with several restriction enzymes as described by Bai et al. (2004). AFLP® fingerprints of the *ol-2* linked markers identified by De Giovanni et al. (2004) and Ricciardi et al. (2007) were generated on a LICOR 4200 DNA sequencer as described by Bai et al. (2003).

Linkage analysis

The software JoinMap 3.0 (Van Ooijen and Voorrips 2001) was used to generate an integrated linkage map of the *ol-2* chromosomal region. A logarithm of odds (LOD) score of 4.0 was used as linkage threshold and the Kosambi mapping function was used to convert recombination frequency into genetic map distance.

BAC screening and fluorescence in situ hybridization

The *Hind*III BAC library of *S. lycopersicum* cv. Heinz 1706 (Budiman et al. 2000) was screened for the presence of the tomato *mlo*-homologous EST sequences cLEC-80-N18 and cTOC-20-K10 and the *ol-2* linked SCAR sequence U3-2. The screening was

performed by PCR with corresponding primers and conditions presented in Table 1.

FISH of the BACs 184L18 and 205G16 on pachytene complements was performed as described by Zhong et al. (1999) and Chang (2004). In brief: flower buds of tomato cv. Tiny Tim containing anthers at meiotic prophase I were fixed in ethanol–acetic acid (1:3). After digesting the cell walls with a pectolytic enzyme mix, cells were spread in acetic acid 45% and post-fixed in ethanol–acetic acid (3:1). BAC clones 184L18 and 205G16 were directly labelled with FITC-dUTP and Cy3.5-dCTP, respectively. The probes were hybridized on the cell complements together with excess of Cot100 genomic DNA for blocking repetitive DNA sequences. The slides were counterstained with DAPI and studied under a fluorescence microscope with a high-sensitive CCD camera. Image capturing and analysis followed the procedures as described by Chang (2004).

Results

Disease test

A disease test was performed on the 316 individuals of the F_2 population of MM × R28. Ten MM and ten R28 plants were used as susceptible and resistant controls, respectively. Ten days post-inoculation all the MM plants showed powdery mildew sporulation on the upper side of the leaves, whereas no symptoms could be detected on R28 individuals. The F_2 population was rated for resistance at 12 and 15 dpi. In total, there were 240 susceptible and 76 resistant F_2 individuals, which fit into the expected 3:1 ratio for a single recessive gene model ($\chi^2 = 0.152$).

An integrated map for the chromosomal region harbouring *ol-2*

Aiming to fine-map the *ol-2* gene, we designed primers for nine chromosome 4 specific sequences located in the region bearing *ol-2* (Table 1). All the primer pairs amplified monomorphic fragments between MM and R28, which were then digested with different restriction enzymes. Unfortunately, no CAPS polymorphism was detected.

SSR markers have been reported to be powerful in detecting polymorphisms within *S. lycopersicum*.

Table 1 Markers tested to reveal polymorphisms between the parental lines *Solanum lycopersicum* cv. MM and R28

Marker name	Type	Source	Primers sequence (5′→3)	Size (bp)	Tm (°C)
Tom316	SSR	Suliman-Pollatschek et al. 2002	GAGTTGTTCTTTGGTTGTTT TAGATTTTTCGTGTAGATGT	134 + 138	50
Tom332	SSR	Suliman-Pollatschek et al. 2002	GATACCATTAAAGCTCATTC GGTTTCCGTCATTATGTCAG	115 + 120	50
Tom95-96	SSR	Suliman-Pollatschek et al. 2002	GTGGATGGATATGTGTGA GCACGGTAGGTCGCAGGCA	–	46
Tom268-269	SSR	Suliman-Pollatschek et al. 2002	AGGGTATGAGATGAGACAAT TTTTACCTTCTTTACTTGGA	–	48
Tom292-293	SSR	Suliman-Pollatschek et al. 2002	ATGTACTCTAATGGATGTTT ATTCATAGCATTGTCATAGG	–	47
Tom160-161	SSR	Suliman-Pollatschek et al. 2002	TGCTGAAGAATACAATGTTACC ATTGTTGGATGCTCAGTTTG	–	48
Tom61-62	SSR	Suliman-Pollatschek et al. 2002	GGCAAAGAAGGACCCAGAGC GGTGCCTAAAAAAGTTAAAT	–	48
U3-2	SCAR	Present work	AGTGGTTGGCGGATAGGTG TTGGCAAGGTGGGAAAACT	1200	55
T0707	COS	http://www.sgn.cornell.edu	TCGTGGATTATGGGCTTTCTT GGTAAGGCTGCAACACATCA	458	50
TG339	RFLP	http://www.sgn.cornell.edu	GAAACCTTACCCCTCTA CGCTGTTTCTTGCCATTT	436	46
TG652	RFLP	http://www.sgn.cornell.edu	CGGGGATCTGATAAGGAAGG ATAAGGGATCGTCAAGGAGTAACA	542	59
T1405	COS	http://www.sgn.cornell.edu	CACCAACAACTAGCCCTTGA AAGCAATTCCTCCAGCTTCA	535	55
cLEC7B23	EST	http://www.sgn.cornell.edu	GGAGAACACGGCTACCTCAG GGAGAACACGGCTACCTCAG	600	55
CT173	RFLP	http://www.sgn.cornell.edu	TGAGCACGGTATTACGCAG TTAGTAGTATGTGTGGCAGTCAC	500	55
TG2FS/R	RFLP	http://www.sgn.cornell.edu	TGTTGAAGAAGGCTCAGCAA TGCCCCTGTATATCCCTCAG	241	55
TG2FL/R	RFLP	http://www.sgn.cornell.edu	CCTCAATCCCAGGAATTTGT TGCCCCTGTATATCCCTCAG	403	57
TG516	RFLP	http://www.sgn.cornell.edu	GGCACTTCGCAAAACTGAAT GGTACGGCGGGTTTAAGAC0T	349	55
cTOC-20-K10	EST	http://www.sgn.cornell.edu	TGTGGTTTGCTTCATCTTGC ACCAACACTCTTGGGGACAC	360	56
cLEC-80-N18	EST	http://www.sgn.cornell.edu	CGTATCTTTGGGTGCCATTT CAGGGCGATTAAACCAGAAA	260	56

Therefore, we selected seven SSRs mapped on chromosome 4 and two of them, Tom316 and Tom332, revealed length polymorphisms between MM and R28 on 6% polyacrylamide gel. Polymorphism of the SSR marker Tom316 was also detectable on 2.5% agarose gel (Fig. 1).
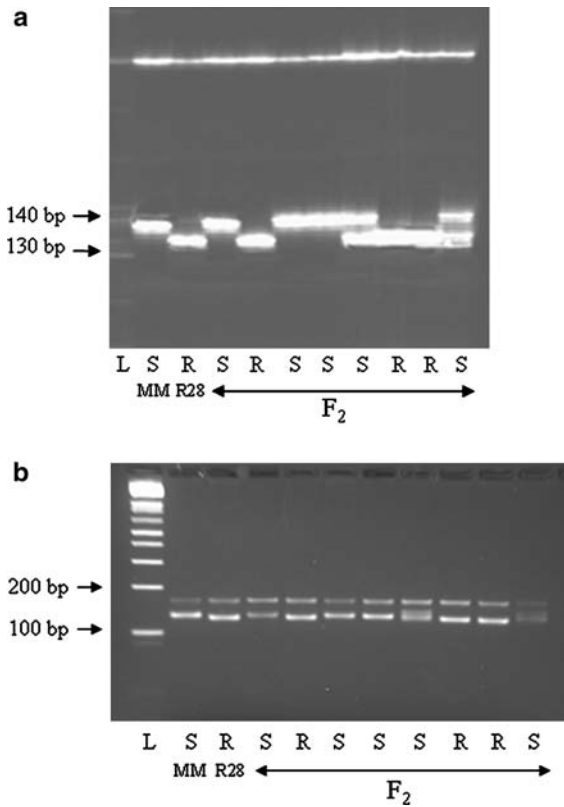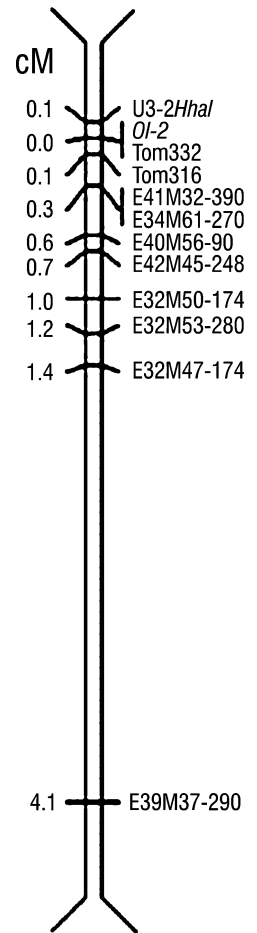
Fig. 1 SSR marker Tom316 tested on the susceptible (S) parent Moneymaker (MM), the resistant (R) parent R28 and eight F$_2$ individuals. Co-dominant length polymorphism is revealed on both 6% polyacrylamide gel (**a**) and 2.5% agarose gels (**b**). Lane L (ladder) points the molecular weight (bp)

Fig. 2 Integrated linkage map of the *Ol-2* locus region on tomato chromosome 4. Genetic distances were estimated by using Kosambi's mapping function

All the *ol-2* linked markers previously identified were used to search for polymorphisms between MM and R28. We failed to amplify the SCAU3$_{1500}$ SCAR sequence with the primers published by De Giovanni et al. (2004), thus new primers were designed. The amplification product, named U3-2, was subsequently digested and converted into a co-dominant CAPS marker, U3-2*Hha*I. Three out of the eight *ol-2* linked AFLP® markers so far reported, E34M61-270, E40M56-90 and E41M32-390, showed polymorphism between MM and R28.

By screening the F$_2$ population of MM and R28 with the three polymorphic AFLP® markers, the CAPS marker U3-2*Hha*I and the two SSR Tom316 and Tom332, a map of the *ol-2* chromosomal region was constructed and integrated with the *ol-2* maps reported by De Giovanni et al. (2004) and Ricciardi et al. (2007) (Fig. 2). Remarkably, almost identical segregation patterns were observed for nearly all the

markers and the resistance, indicating that the *ol-2* gene is located in a chromosomal region characterized by recombination suppression.

Physical co-localisation of the *ol-2* gene and tomato *mlo*-homologue(s)

As both the genetic and phytopathological features of the *ol-2*-associated resistance mimic the well-characterized *mlo*-based resistance in barley, it is logical to argue that *ol-2* is likely a *mlo* homologue. By inspecting the tomato EST collection, two tomato ESTs (cLEC-80-N18 and cTOC-20-K10) were identified that are homologous to barley *mlo*. Unfortunately, genetic mapping of these two ESTs in the F$_2$ population was hampered by the lack of polymorphism between MM and R28. To visualize the relative chromosomal location of the *ol-2* gene and tomato *mlo*-homologue(s), we first screened the Heinz BAC

library with PCR primers amplifying the tomato *mlo*-homologous ESTs cLEC-80-N18 and cTOC-20-K10 (Table 1). Two BAC clones, 184L18 and 205G16, were identified, which were positive for both the ESTs. These two BAC clones were further inspected for the presence of the *ol-2* linked SCAR sequence U3-2. Remarkably, both BACs were positive for the U3-2 sequence, suggesting the co-localization of the *ol-2* gene with *mlo* homologue(s). In addition to the expected PCR fragment of 1200 bp for the U3-2 sequence, other amplification products were obtained, indicating that the two BACs contain repetitive sequences.

FISH of 184L18 and 205G16 BACs on pachytene chromosomes of tomato revealed in both cases major signals on chromosome 4, in the heterochromatin block of the short arm near the centromere (Fig. 3). In spite of blocking the BAC probes with Cot100 repetitive DNA, we also found small signals in heterochromatic regions of other chromosomes, which indicate that the BACs are rich in pericentromeric heterochromatic specific dispersed repeats. Repeat signals were more abundant for 184L18 (Fig. 3). As the *ol-2* gene is located on chromosome 4 around the centromere (De Giovanni et al. 2004), the presence of tomato *mlo*-homologue(s) in the pericentromeric heterochromatin of the same chromosome further suggests that *ol-2* is a *mlo*-homologue.

Discussion

Although several powdery mildew resistance genes have been reported, most of the current commercial tomato cultivars are susceptible to powdery mildew. The *ol-2* source of resistance, which originates from the cultivated tomato species *S. lycopersicum*, might allow circumvention of linkage drag problems which are encountered during the transfer of wild tomato resistance genes into cultivated backgrounds. On the other hand, the genetic relatedness between the donor parent and elite genotypes complicates the identification of polymorphic markers to be used for MAS breeding programmes. This was demonstrated by the failure in trying to convert several sequences located on the *ol-2* chromosomal region into PCR markers.

Suliman-Pollatschek et al. (2002) reported percentages of polymorphism within *S. lycopersicum* were 52% for SSR, 10.8% for AFLP® and 5% for RFLP
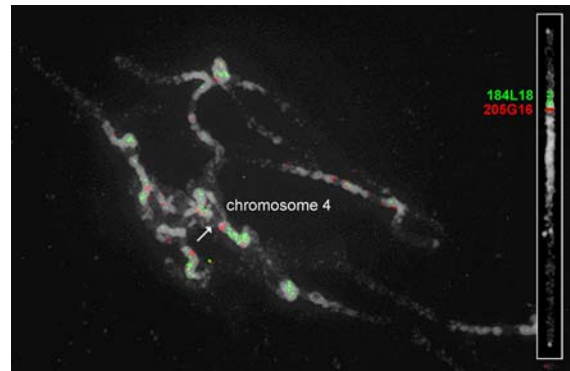

Fig. 3 FISH signals of the BAC clones 184L18 (labelled with FITC-dUTP) and 205G16 (labelled with Cy3.5-dCTP). The BACs display small and variable signals, close together, in the heterochromatic area near the centromere of chromosome 4. 205G16 gives a major focus next to the centromere and few minor signals more distally, whereas 184L18 produces a variable number of smaller foci in the whole pericentromeric heterochromatic region. Some of the green and red signals overlap giving yellow fluorescence. Both BACs also give some background signals in heterochromatin areas of other chromosomes, due to cross hybridization of the repetitive sequences in the labelled BAC probe

markers. The higher variation of SSR loci was demonstrated by the present study, in which two out of seven SSR markers tested revealed length polymorphisms between MM and R28.

The three markers identified in this work can be easily obtained (the SSR marker Tom316 requires a simple PCR reaction and visualization on agarose gel) and are not patented, thus can be freely and conveniently applied for MAS in breeding programmes. Furthermore, their co-dominant nature will allow breeders to select directly for the presence of the recessive *ol-2* allele.

In this study, BAC library screening and FISH analyses were used to demonstrate the co-localization of an *ol-2* linked marker with *mlo* orthologue(s) in the pericentromeric heterochromatic region of the short arm of tomato chromosome 4. Amplification of the U3-2 sequence by using BAC DNA as a template was invariably accompanied by the presence of multiple PCR amplification. However, when genomic DNA was used as PCR template, only one PCR fragment was observed. It seems that primer mismatches occur during PCR amplification only with the BAC DNA as template. Multiple PCR amplifications obtained by using the U3-2 primer pair suggests that the BACs

contain repetitive sequences, which are known to occur with very high frequency in heterochromatic regions. The abundance of repetitive sequences also explains the presence of multiple weak hybridization signals obtained with FISH analyses. On the other hand, the presence of discrete PCR fragments with the expected molecular weight for the two-tomato *mlo* ESTs suggests the presence of only one intact *mlo*-like gene sequence. Although heterochromatin has been classically depicted as a gene poor region of the genome, our findings are in accordance with a growing body of evidence which shows that genes resident in heterochromatin can indeed encode essential functions (Nagaki et al. 2004; Rossi et al. 2007).

In tomato, the actual ratio of genetic and physical distance varies considerably depending on the chromosomal region. In the *Tm-2a* region, close to the centromere of chromosome 9, 1 cM corresponds to more than five million base pairs (Pillen et al. 1996), whereas for the chloronerva gene region, located in the euchromatin of chromosome 1, the ratio of genetic to physical distance is only 160 kb per 1 cM (Ling et al. 1999). By determining frequency and distribution of recombination nodules on tomato synaptonemal complexes, Sherman and Stack (1995) observed a much lower frequency of recombination nodules in heterochromatic regions around the centromeres compared to euchromatin. Occurrence of recombination suppression near the centromere is reported also for other plant species (Tanksley et al. 1992; Drouaud et al. 2006). Therefore, the observed clustering of *ol-2* linked markers can well be explained by their localization in the pericentromeric heterochromatin of chromosome 4. The low ratio between genetic and physical distance does not favour *ol-2* positional cloning, as it could require the screening of very large segregating populations and extensive chromosome walking.

In addition to the similarity of *ol-2* and *mlo* resistances for both genetic (recessive) and phytopathological (papilla-based mechanism) characteristics, BAC screening and FISH data in this study indicated the genetic and cytogenetic co-localization of the *ol-2* gene with tomato *mlo*-homologue(s). All of this evidence strongly suggests that *ol-2* is likely a *mlo* homologue. We are currently following a homology-based cloning approach for *ol-2* isolation as an alternative to positional cloning.

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