Candidate gene analysis and identification of TRAP and SSR markers linked to the *Or5* gene, which confers sunflower resistance to race E of broomrape (*Orobanche cumana* Wallr.)

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ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr.) is a root holoparasitic angiosperm considered as being one of the major constraints for sunflower production in Mediterranean areas. Breeding for resistance has been crucial for protecting sunflowers from broomrape damage. The *Or5* gene, which confers resistance to race E of broomrape, has been efficiently used for years in sunflower commercial programs to control broomrape. Despite its importance in sunflower breeding, little is known about the nature of the *Or5* gene. The objective of this study was to explore different strategies to determine the nature of this gene. These include a map-based cloning strategy and a candidate gene approach.

Key words: candidate genes – *Orobanche cumana* – race E – SSR– sunflower resistance – TRAP.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important annual oilseed crops in the world. *Orobanche cumana* Wallr. (sunflower broomrape), a holoparasitic angiosperm that infects sunflower roots, is regarded as one of the most important constrains in sunflower production in Spain and most countries of southern and eastern Europe and the Middle East (Parker, 1994). Control of this parasite remains extremely difficult, as thousands of tiny seeds produced by one single broomrape plant can be easily dispersed by wind, water, animals, humans, machinery or attached to sunflower seeds. Although the use of herbicides is being considered as a promising control measure, at present genetic resistance offers the most effective and feasible control against *O. cumana*. However, the introduction of new resistance sources has been frequently followed by the appearance of new pathogenic races overcoming the resistance. In a classic study in the late 1970s, Vrânceanu et al. (1980) identified five pathogenic races in Romania, named A through E, with a set of sunflower differentials carrying the dominant resistance genes *Or1* through *Or5*, which provided an accumulative resistance to the five successive races. Races B and C were identified at that time in Spain in addition to populations of *O. cumana* of higher virulence than those reported in Romania (Melero-Vara et al., 2000). Besides, race F which overcomes the resistance gene *Or5*, was identified in the mid 1990s in Spain (Alonso et al., 1996). Resistance to this new race was found in germplasm of both cultivated and wild sunflower (Sukno et al., 1999; Fernández-Martínez et al., 2000).

The host-parasite system of sunflower-*O. cumana* described for races A to E appears to follow the gene-for-gene model. The previously mentioned study of Vrânceanu et al. (1980) already established the monogenic and dominant inheritance of resistance to sunflower broomrape races A to E, which was later confirmed by other authors (Ish-Shalom-Gordon et al., 1993; Sukno et al., 1999). However, the nature of the *Or1* to *Or5* genes involved in this interaction is not known. Molecular mapping studies have revealed that the *Or5* gene conferring resistance to race E of broomrape is located on a telomeric region of linkage group (LG) 3 of the sunflower genetic map (Lu et al., 2000; Tang et al., 2003a; Pérez-Vich et al., 2004). Recently, Letousey et al. (2007) have identified different genes differentially expressed in resistant genotypes when infected with race E of broomrape. Among them, a sunflower defensin gene may play a major role in *Orobanche* cell death (de Zélicourt et al., 2007) during the incompatible reaction.

The aim of this study was to investigate the nature of the *Or5* gene through a candidate gene approach, and to identify markers close to the gene in order to facilitate map based cloning strategies.

MATERIALS AND METHODS

Plant material, phenotypic evaluation for race E resistance and RFLP linkage map construction

The sunflower lines used to generate the F_2 mapping population were P-96, an inbred line resistant to races E and F of broomrape developed from cultivated sunflower of Yugoslavian origin, and P-21, a Genetic Male Sterile (GMS) line of sunflower, which is highly susceptible to broomrape. The development of the F_2 and F_3 populations from the cross P-21 x P-96 and the F_3 phenotypic evaluation for disease reactions to race E of broomrape are described in Pérez-Vich et al. (2004). From the P21 x P-96 population, an RFLP linkage map comprising 17 LGs was constructed, in which the *Or5* gene was mapped to LG 3 (Pérez-Vich et al., 2004).

SSR marker analysis

Genomic DNA from four samples of the F_2 population P21 x P-96 and the parental lines were screened with LG 3 previously mapped simple sequence repeats (SSRs) (Tang et al., 2003b; Yu et al., 2003), identified by ORS and CRT prefixes, in order to find polymorphic SSR markers and map them in the P-21 x P-96 population. PCRs were performed as described by Tang et al. (2002), and the amplification products were resolved by electrophoresis on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1x TBE buffer with ethidium bromide incorporated in the gel.

TRAP analysis

The TRAP analysis focused essentially on telomere-associated TRAP markers, especially those identified on LG 3 (Hu, 2006). Assays followed the updated procedures described by Hu (2006). Totally 3 fixed primers (TeloRCN, TeloR, and QGA7H07L) designed against *Arabidopsis*-type telomere repeat sequences and EST homologous to *Arabidopsis* homeobox genes (Hu et al., 2005; Hu, 2006) as well as six arbitrary primers (Ga3-800, Ga5-800, Trap13-800, Sa4-700, Sa12-700, and Trap03-700) labeled with either IR (infrared) 700 or IR 800 dyes were used to generate TRAP markers. The TRAP markers were designated by the combination of the code of the fixed primer involved, the code of the labeled arbitrary primer, and the fragment size in base pairs.

Candidate gene analysis

Twelve defense-related genes known to be involved in different metabolic pathways (phenylpropanoids, jasmonate, ethylene) and/or resistance mechanisms against microorganisms (Table 1) were used for the candidate gene approach. Primers from these genes were designed by Letousey et al. (2007) and screened for polymorphisms in the P-21 x P-96 population. PCRs were performed as described by Yu et al. (2003) for INDEL markers, using the annealing temperatures described in Letousey et al. (2007) for each specific primer pair. The amplification products were resolved by electrophoresis on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1x TBE buffer with ethidium bromide incorporated in the gel.

Molecular mapping of polymorphic loci and linkage map construction

Polymorphic SSR, TRAP, and candidate gene markers were genotyped in the F_2 population P-21 x P-96 developed by Pérez-Vich et al. (2004). The RFLP map of Pérez-Vich et al. (2004), comprising 17 LGs and spanning a 1,144.4 cM distance, was used to map the SSR, TRAP, and candidate gene loci. Linkage maps were constructed using the software MAPMAKER/EXP version 3.0b (Whitehead Institute, Cambridge, MA, USA) (Lander et al., 1987). The LG nomenclature follows that of the reference map of Tang et al. (2002). Linkage group maps were drawn using the MapChart software (Voorrips, 2002).

Table 1. Primer pairs used for the candidate gene analysis (Letousey et al., 2007)

Gene	Product	Function	PCR primers
ef1-α	Elongation factor 1α (control gene)	Translation	5' GTCAGCACGCTCTTCTCGCT 5' GAGACTCGTGGTGCATCTCA
ubq	Ubiquitin	Proteolytic cofactor	5' TGTGAAGACGTTGACTGGAA 5' CGCAGACGAAGAACAAGGTG
chit.	Chitinase (PR3 protein)	Antifungus	5' CTGCAGTGTCAGCAGCTGAT 5' CTGCACCAGATGGGCGATTT
pal	Phenylalanine-ammonia-lyase	Phenylpropanoid synthesis	5' GTTATGGTGCACTACTGGAT 5' TCCGACAACAATGCAAGTACA
c4h	Cinnamate-4-hydroxylase	Phenylpropanoid synthesis	5' ATGGGTCAGCGTAACCTAGT 5' AAGCTCTGAGCTAATCGACT
lox	Lipoxygenase	JA synthesis	5' GTAGGCGTTGGTGGTATTGTT 5' AATCTTCATCCGCAGGTACAT
HACS.1	ACC synthase	Ethylene synthesis	5' ACCGGATTGTTATGAGCGGT 5' TACGAGCGATGCTCACAACA
ACCO1	ACC oxydase	Ehylene synthesis	5' GATCATTACAAGAAGTGTATGG 5' AGAAGCTGTAGACCGCTAAC
def.	Defensin	Antifungus, marker gene of SA pathway	5' GTGAGAAGGCAAGCCAGACA 5' TCAAGGTTTGGCTGTCGCCT
PR5-1	PR5 protein	Antifungus, marker gene of JA pathway	5' CACAGCAGGAGCCCGTATAT 5' TATGCATCAGGACATCTGGTC
sco	Carbohydrate oxidase	H_2O_2 production, antifungus	5' GTCATGCTTGATCTCGTCAA 5' ACTCGGTGTTAAGCTGAACT
HaAC1	Aldoketo reductase	Polyol synthesis, marker gene of SA pathway	5' CTCGCTACCAAATATGGGAT 5' TCTTCGAGCTTCTCCATCAT

RESULTS

SSR marker analysis

Nine SSR markers mapping to LG 3 (ORS10, ORS202, ORS665, ORS949, ORS1036, ORS1114, ORS1222, CRT392, and CRT314) were screened for polymorphisms in four F_2 individuals and the parental lines P-21, and P-96. Three of them (ORS949, CRT392, and CRT314) were polymorphic (Fig. 1). These polymorphic loci were genotyped in the F_2. ORS949 and CRT314 mapped to LG 3 (LOD score of 7 and maximum recombination frequency of 0.30) (Fig. 2). CRT314 and ORS949 mapped 9.9 cM and 57.1 cM, respectively, downstream from *Or5*. Two polymorphic loci from CRT392 (CRT392a-100bp, and CRT392b-250bp) mapped to LG 9 (LOD score of 7 and maximum recombination frequency of 0.30). These two CRT392 loci on LG 9 have also been reported by Yu et al. (2003). A third CRT392 locus (CRT392c) segregated in the P-21 x P-96 population, but it was not possible to map it in high resolution agarose gels. This locus may be the one mapped close to *Or5* by Tang et al. (2003a).

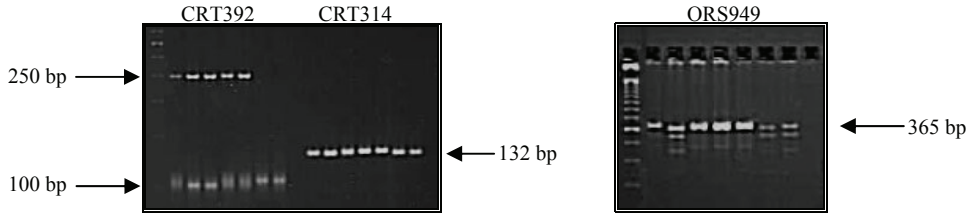

Fig. 1. Amplification profiles of SSR markers CRT392, CRT314, and ORS949 in samples of four F_2 P-21 x P-96 individuals, one P-21 individual, and two P-96 individuals. Mapped polymorphic loci are indicated by arrows. *Lane 1*, 50 bp DNA ladder; *lanes 2-5*, F_2 individuals; *lane 6*, P-21 parental line; *lanes 7-8*, P-96 parental line.

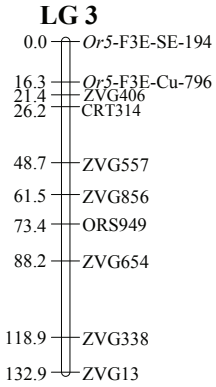

Fig. 2. Linkage map of LG 3 from the P-21 x P-96 population containing the *Or5* gene for resistance to race E of broomrape, as scored in two different phenotypic experiments [F3-race E (SE-194), and F3-race E (CU-796); Pérez-Vich et al., 2004]

TRAP analysis

Six F_2 individuals from the P-21 x P-96 population phenotyped as susceptible to race E of broomrape, and six F_2 individuals from the same population phenotyped as resistant to race E, and selected as homozygous based on the genotype of the closest RFLP marker (ZVG406), were assayed. A fragment of 133 bp in length generated by the primer combination TeloRCN+Sa4-700 (TRAP marker TRC27133) was polymorphic between the resistant and the susceptible groups, and was associated with the resistant phenotype (Fig. 3). The TRC27133 TRAP marker was mapped in previous studies to the upper end of LG 3 (Hu, 2006).

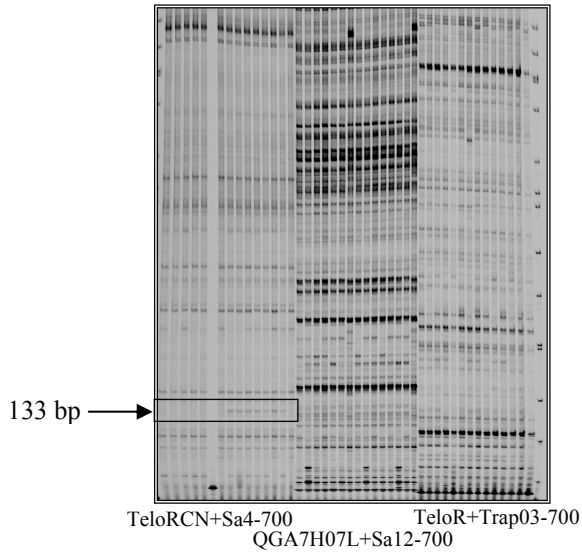

Fig. 3. Gel image generated by primer combinations TeloRCN+Sa4-700 (*left*), QGA7H07L+Sa12-700 (*center*), and TeloR+Trap03-700 (*right*). The TRAP marker TRC27133 polymorphic between susceptible and resistant groups is a fragment of 133 bp of primer combination TeloRCN+Sa4-700, and it is highlighted in a box. *Lanes 1-6*, six susceptible F_2 individuals; *lane 7*, susceptible parental line P-21; *lane 8-13*, six resistant F_2 individuals; *lane 14*, resistant parental line P-96; *lane 15*, susceptible parental line P-21.

Candidate gene analysis

From the twelve candidate gene-markers assayed, four of them generated polymorphic loci among F_2 individuals from the P-21 x P-96 population. These corresponded to the candidate genes *ef1-α* (elongation factor 1-α), *chit.* (chitinase, PR3 protein), *c4h* (cinnamate-4-hydroxylase), and *HaAC1* (aldoketo reductase). Dominant polymorphic *ef1-α* (elongation factor 1-α), *chit.* (chitinase, PR3 protein), and

HaAC1 (aldoketo reductase) loci were mapped to LGs 7, 9, and 17, respectively (LOD score of 5 and maximum recombination frequency of 0.30). None of these loci were located on LG 3, where the *Or5* for race E resistance has been mapped. In addition, several QTL for race F resistance have been described in the P-21 x P-96 population (Pérez-Vich et al., 2004), but none of the candidate gene loci mapped in this study co-located with any of them. Finally, a dominant *c4h* (cinnamate-4-hydroxylase) locus was not associated with any of the linkage groups of the P-21 x P-96 map (Pérez-Vich et al., 2004).

DISCUSSION

Different mechanisms may play a role in resistance of sunflower to broomrape. Sources of resistance carrying quantitative or qualitative, or both, resistance mechanisms have been described (Sukno et al., 1999; Pérez-Vich et al., 2004). Qualitative resistance is characterized by dominant race-specific genes, and has played an important role in breeding for resistance in the last fifteen years. The *Or5* gene confers resistance in sunflower to race E, but also to the previous A through D races of less virulence. The nature of this dominant race-specific gene is still unknown. It has been hypothesized that it might be a cluster of resistance (R) genes encoding proteins characterized by the presence of leucine-rich repeat (LRR) motifs and a nucleotide binding site (NBS) N-terminal to the LRR domain (Lu et al., 2000), similar to those NBS-LRR clusters on LG 8 (Bouzidi et al., 2002) and LG 13 (Radwan et al., 2003) conferring resistance to different races of downy mildew. Alternatively, studies focused on the expression patterns of defense-related genes in race E compatible and incompatible reactions, suggested that a salicylic acid-responsive gene, *def.* (defensin) was characteristic of the race E resistant genotype, and that it might play a major role in *Orobanche* cell death (de Zélicourt et al., 2007; Letousey et al., 2007). In order to determine in more detail the nature of the *Or5* gene, different strategies could be used. One is a candidate gene approach, based on the genes hypothesized or related to race E resistance mechanisms. Another approach is a map-based cloning strategy, for which the identification of *Or5* closely linked and flanking markers would be necessary.

This study, combined with the previous results of mapping the TRC27133 TRAP marker (Hu, 2006), and those of identifying SSR markers close to *Or5* (Tang et al., 2003a), indicates that *Or5* is probably located in the TRC27133 to ZVG406/CRT392c marker interval. ZVG406 is the uppermost RFLP marker on LG 3 from the high density RFLP map of Berry et al. (1996), and CRT392c is the uppermost SSR marker on LG 3 described to date (Tang et al., 2003b; Yu et al., 2003). Both ZVG406 and CRT392c markers map 5.1 and 6.2 cM to *Or5* on its centromeric side, respectively. TRC27133 is on the LG 3 telomere, and has been mapped 4.9 cM distal to CRT392 (Hu, 2006). Therefore, *Or5* must be very close to TRC27133, and this marker probably flanks the gene. Unfortunately, we were not able to determine the exact position of TRC27133 in relation to *Or5*, since this marker when run in the entire F_2 P-21 x P-96 population produced bands of different intensities which were not scorable. The TRC27133 marker will be converted into a more useful sequence-tagged-site (STS) marker by cloning and sequencing the 133 bp fragment. The *Or5* marker interval TRC27133 — ZVG406/CRT392c covers a region of about 5-6 cM. This distance may be physically shorter, as suggested by Tang et al. (2003a). Therefore, this set of markers close to *Or5* may provide a foundation for *Or5* map-based cloning strategies.

Another approach to investigating the nature of the *Or5* gene could be a candidate gene analysis. In this study, we have tested genes differentially expressed in resistant genotypes when infected with race E of broomrape. Despite we have been able to map *ef1-α* (elongation factor 1-α), *chit.* (chitinase, PR3 protein), and *HaAC1* (aldoketo reductase) loci to LGs 7, 9, and 17, respectively, none of them co-located to *Or5*. These results were partially expected, since the *chit.* and the *HaAC1* genes play a role in defense responses and *ef1-α* is a housekeeping gene, and dominant race-specific genes such as *Or5* are hypothesized as essentially playing a role in an early stage of the plant-pathogen interaction (i.e. pathogen recognition). Many other candidate genes could be tested, including NBS-LRR type ESTs present in the Compositae Genome Database and the NCBI database.

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