Characterization of Post-haustorial Resistance to Sunflower Broomrape

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ABSTRACT

The development of durable resistance to broomrape (*Orobancha cumana* Wallr.) in sunflower (*Helianthus annuus* L.) requires detailed characterization of the genetic and physiological bases of resistance. The objective of the present study was to map accurately the resistance gene, and to characterize the mechanism of resistance to broomrape observed in a sunflower inbred line (PHSC1102). PHSC1102, which was consistently resistant against race F and race G populations of broomrape, was crossed with PHSC1201, which was susceptible to races F and G. A mapping population of 150 F₂ genotypes was phenotyped by evaluating F_{2:3} families for resistance to broomrape races F_{GV} and G_{TK}. The use of single nucleotide polymorphism (SNP) markers mapped the *OrsII* gene to linkage group 4 (LG4) of the sunflower genome. Mini-rhizotron and histological studies of the resistant line revealed that the attachment of broomrape to host roots was similar in both the resistant and susceptible lines and that the resistance was observed at a late stage, i.e., after tubercle development. Interestingly, the resistance of the PHSC1102 line was associated with the production of phenolic compounds, which were hypothesized to restrict the parasite's growth. This research provides novel and valuable information about the host-parasite interactions between sunflower and broomrape.

Sunflower broomrape is a holoparasitic (i.e., a plant not capable of photosynthesis and sustaining itself entirely through parasitism) weed that is widely distributed in sunflower production areas of the Old World. The species has been reported to occur in most of the sunflower producing regions of Spain and France, around the Black and Caspian Seas; in China, and, recently, in some parts of Africa (Fernández-Martínez et al., 2015).

The *O. cumana*-sunflower system generally involves a qualitative gene-for-gene interaction, in which a dominant resistance gene in the host interacts with a dominant avirulence gene in the parasite, which help eliminate the aboveground symptoms of parasitization (Rodríguez-Ojeda et al., 2013). This system differs from other interactions involving parasitic members of the Orobanchaceae, in which resistance is generally governed by horizontal genetic mechanisms (Pérez-Vich et al., 2013).

Resistance mechanisms can operate at the pre-attachment, pre-haustorial, or post-haustorial stage of *Orobanche*-host plant interactions (Pérez de Luque et al., 2009). At the pre-attachment stage, the most common resistance mechanisms involve low-level secretions of germination stimulants, germination inhibitors, and inhibitors of radicle development (Höniges et al., 2008). Resistant sunflower germplasm that exhibit low-level secretions of germination stimulants (Labrousse et al., 2001) or of 7-hydroxylated simple coumarins have been reported (Serghini et al., 2001). Pre-haustorial mechanisms operate between the initial contact of broomrape with the host root and the establishment of effective vascular connections (Pérez de Luque et al., 2009). Different mechanisms, such as lignification, suberization, protein crosslinking, and callose accumulation, which contribute to the development of physical barriers that impede penetration by parasitic structures, have been reported (Echevarria-Zomeño et al., 2006; Letousey et al., 2007). The production of chemical compounds, such as phenolics, which are toxic to broomrape, has also been described in sunflower (Echevarria-Zomeño et al., 2006). Post-haustorial

mechanisms operate after vascular connections between broomrape and its sunflower host have been established and nodules are visible (Pérez de Luque et al., 2009). Necrosis and subsequent death of broomrape nodules at several stages of development have been reported in sunflower line LR1 (Labrousse et al., 2001; Zélicourt et al., 2007; Louarn et al., 2016). Louarn et al. (2016) detected 17 quantitative trait loci (QTL) associated with resistance to broomrape races F and G in a segregating recombinant inbred line (RIL) population derived from a cross between sunflower lines HA89 and LR1 (a line derived from an interspecific cross with *Helianthus debilis*), which indicated that resistance to broomrape was polygenic.

One source of resistance to sunflower broomrape (i.e., System II resistance) was developed by Pioneer Hi-Bred International in 2008. The mechanism underlying this resistance was thought to be post-haustorial because the resistant genotypes exhibited underground broomrape tubercles that did not emerge under field conditions. In a patent application, Hassan et al. (2008) reported that the trait was controlled by a gene named *BrsII*. In this manuscript, the nomenclature *OrsII* will be used instead of *BrsII* for consistency with other broomrape resistance genes. The gene was mapped to linkage group 4 (LG4) of the sunflower genetic map (Hassan et al., 2008). The objective of the present work was to map the *OrsII* gene with higher resolution, and to characterize the mechanism of resistance.

MATERIALS AND METHODS

Plant Materials, Mapping Population, and Phenotypic Evaluation

Four sunflower germplasms were used in the present study: three F₈ inbred lines (PHSC1201, PHSC1102, and PHSC0933), which were developed by Pioneer Hi-Bred,

and population B117, which was obtained from IAS-CSIC. Line PHSC1201 was resistant to sunflower broomrape races A to E (*Or5* resistance gene) and susceptible to races F and G. Line PHSC1102 was the donor of resistance gene *Or5H* and was developed from the original donors of the resistance described by Hassan et al. (2008). Line PHSC0933 carried the *Or7* resistance gene, which provides resistance to races A to G (Duriez et al., 2017). These three inbred lines were produced by Pioneer Hi-Bred using its own germplasm so that it is not possible to establish any relationship with public lines. Meanwhile, B117 was a landrace population of confectionery sunflower collected in Spain (Pérez-Vich et al., 2018), which is susceptible to all sunflower broomrape populations evaluated so far.

Four sunflower broomrape populations were used in the present study: SP, which was collected from the Guadalquivir Valley of Spain and classified as race F_{GV}; GT, which was collected in Turkey and classified as race G_{TK}; IP2, which was collected in Romania and classified as race G_{RO}; and RP4, which was collected in Russia and classified as race G_{RU}. Race nomenclature follows Martín-Sanz et al. (2016). The three race G populations were chosen because of their high degree of virulence, which was representative of the current predominant level of virulence in Eastern Europe, whereas race F_{GV} was the predominant race in Southern Spain.

A first experiment was designed to evaluate the resistance of line PHSC1102 against a diverse set of sunflower broomrape populations. For that, the three inbred lines and population B117 were evaluated against the four broomrape populations mentioned above. Nine plants for each combination of sunflower line and broomrape population were grown in pots in a greenhouse and evaluated by counting and weighing the emerged and underground broomrape structures (Fig. 1).

For the genetic mapping study, plants of lines PHSC1201 and PHSC1102 were crossed by manual emasculation and pollination. A population of 150 F₂ plants from a single F₁ plant was used for genotyping, and the phenotypic evaluation of broomrape resistance was conducted using F_{2:3} families (evaluation of individual F₃ plants from each family) against broomrape populations F_{GV} and G_{TK}. The F₂ plants were not evaluated for broomrape resistance to maximize F₃ seed production through the self-fertilization of individual F₂ plants. F₃ plants from the 150 F_{2:3} families were evaluated for resistance to race F_{GV} in the field, using two replications of nine plants each, and for race G_{TK} in pots under open-air conditions, based on 12 plants from each family, in both cases using parental lines as controls. The evaluation of F_{2:3} families with additional broomrape populations was not feasible because of limited number of F₃ seeds available. Broomrape resistance was evaluated by counting emerged broomrape shoots at the end of flowering of sunflower. In the pot evaluation, the height of underdeveloped, emerged broomrape shoots was also measured. Shoot height is easier to measure than shoot weight when evaluating large populations and is similarly associated with System II resistance. No underdeveloped, emerged shoots were observed in the field experiment.

For all experiments, sunflower seeds were germinated on moistened filter paper in small pots (7 × 7 × 7 cm) that contained a 1:1 (by volume) mixture of sand and peat that was carefully mixed with 50 mg broomrape seeds of the corresponding population. The plants were incubated in a growth chamber for 20–25 d at 25 °C/20 °C (day/night) under a 16-h photoperiod. For the pot experiments, plants were transplanted to larger pots that contained 5 L of a 2:1:1 (by volume) soil mixture of sand, silt, and peat and 8 g controlled-release NPK fertilizer (Nutricote 15-9-10 (2MgO)+ME; Jcam Agri. Co., Ltd, Tokyo, Japan). For the field experiments, F_{GV}-inoculated plants were transplanted to an infested field plot, in which only race-F_{GV} experiments had been conducted since 1999.

A basal application of 500 kg ha⁻¹ of 8-15-15 NPK fertilizer was made before transplanting. The plants in all experiments were irrigated as required to avoid water stress. The soil, pots, and remaining plants were sterilized in a large capacity (868 L) steam sterilizer (Sterivap 9612-1ED, BMT Medical Technology, Zábřdovice, Czech Republic) after the pot experiments.

Plant Genotyping

The mapping population consisted of 150 F₂ plants. Genomic DNA was extracted from lyophilized leaf tissue of each parent and F₂ plant using a modified cetyl trimethylammonium bromide (CTAB) protocol (Kidwell and Osborn, 1992). The extracted DNA was quantified using an ND 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The parental lines of the population were genotyped using an Infinium single-nucleotide polymorphism (SNP) array (Illumina, San Diego, CA, USA), which is designed to target polymorphic SNPs (Bachlava et al., 2012). Genome studio ver. 2011.1 (Illumina) was used to make SNP calls, and map positions were assigned according to the consensus genetic map of Bowers et al. (2012). The SNP markers that were polymorphic between the two parental lines were then used to genotype the F₂ population using the polymerase chain reaction (PCR)-based KASP (Kompetitive allele specific PCR) genotyping assay (KBioscience, Hoddesdon, UK).

Marker Analysis and Map Construction

All markers were analyzed for the expected segregation ratios of 1:2:1 for codominant markers and 3:1 for dominant markers using the Chi-square test, and only the markers that exhibited Mendelian segregation were used for the analysis. Linkage analysis and genetic distances were calculated using MapDisto 1.7 (Lorieux, 2012), with a minimum

log of odds (LOD) score of 4.0 and maximum recombination fraction of 0.2 used to identify linkage groups. The “order sequence” command was used to establish the initial order of markers in each linkage group, and the “ripple order,” “check inversions,” and “drop locus” commands were used to refine and validate the final order of the markers. The Kosambi function was used to convert recombination fractions to genetic distances.

The *OrsII* gene was mapped by inferring the F₂ genotypes from the phenotypes of their F₃ progenies. The F₂ individuals were classified as *OrsII OrsII* if all their F₃ progenies exhibited a resistant phenotype (i.e., System II resistance), *OrsII orsII* if their F₃ progenies segregated for resistance and susceptibility, or *orsII orsII* if all their F₃ progenies exhibited a susceptible phenotype. Linkage was considered significant if the LOD score was >10.0. The linkage group map in which the *OrsII* gene was located was reconstructed in MapDisto using the commands described above, and the map of the linkage group that contained *OrsII* was drawn using MapChart (Voorrips, 2002).

Histological Characterization of Resistance Mechanism

A modified version of the mini-rhizotron system, described by Pérez de Luque et al. (2005), was used to dissect the phenotypic components of resistance and for histological study. Sunflower seedlings (3 cm radicle length) of the PHSC1201, PHSC1102, and PHSC0933 inbred lines were transferred to square Petri dishes (15 × 15 cm) that were filled with vermiculite, with one plant per mini-rhizotron, and then covered with Whatman® glass microfiber filter paper grade GF/A (GE Healthcare Life Sciences, Little Chalfont, UK). After 7–9 d, inoculation was performed by replacing the glass microfiber paper with inoculated glass microfiber paper, on which *O. cumana* seeds (~10 mg) had been homogeneously spread and then conditioned with 2-(N-morpholino) ethanesulfonic acid (MES; 0.3 mM concentration) in darkness at 20 °C for 10 d. The two broomrape

populations used to phenotype the mapping population (races F_{GV} and G_{TK}) were also used in this experiment. After inoculation, the rhizotrons were sealed with parafilm, covered with aluminum foil to exclude light, placed vertically in trays with Universal liquid fertilizer (Compo GmbH, Munster, Germany), and then incubated in a phytotron at 25 °C/22 °C (day/night) with a 14-h photoperiod. Infection status was monitored daily using a binocular stereoscopic microscope (Leica M80; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany), and images were captured using a digital camera (Leica IC80 HD, Leica Microsystems). Ten plants per genotype and broomrape race combination were subjected to physiological characterization of resistance and to produce material for histological analysis. After 21 d of incubation, 200 broomrape seeds that were in close vicinity (<3 mm) of host roots were studied in each rhizotron using a stereoscopic microscope at 30X magnification. Broomrape germination was assessed as the percentage of seeds with a germ tube, and attachment percentage was determined by counting the broomrape seedlings that were in contact with host roots. The successful penetration of the parasite into the host root tissues was calculated as the percentage of contacted seeds that had initiated penetration. Finally, the number of broomrape tubercles per host plant, their dry weight, and the percentage of necrotic tubercles were recorded. The resulting data were subjected to an analysis of variance (ANOVA) using InfoStat Software v. 2010. Seedlings and tubercles of *O. cumana* were sampled with the corresponding attached parts of sunflower roots 7, 14, and 21 d after inoculation for histological observation.

For staining, the samples were fixed overnight at 4 °C in 4% formaldehyde in phosphate-buffered saline (PBS; pH 7.3) solution. After washing in PBS (3 × 15 min), the fixed samples were stored in 0.1% formaldehyde in PBS at 4 °C. The stored samples were submerged for 24 h in a solution of 30% sucrose in PBS, transferred to a beaker with a freezing matrix, and then frozen at -20 °C inside a cryostat. Finally, 7 µm-thick

sections were cut using a cryostat microtome (Leica CM3050 S; Leica Biosystems Nussloch GmbH, Nussloch, Germany) and attached to adhesive-treated microscope slides (polysine slides; Menzel GmbH & Co KG, Braunschweig, Germany).

Fifty tubercles of each genotype, which were collected at 21–25 d post-inoculation from different mini-rhizotrons, were subjected to histological observation using an Olympus BX61 microscope (Olympus, Hamburg, Germany) that was equipped with an epifluorescence condenser and a Hoechst/DAPI longpass filter set configured at an excitation filter of 340–380 nm. To develop a general overview of the host-parasite interaction, fresh sections were first prepared and photographed. Meanwhile, to detect tannins, lignin, and suberin, other sections were stained using 0.05% toluidine blue O (TBO) in PBS (0.1 M, pH 5.5) for 2 min and then rinsed with water for 1 min (Bordallo et al., 2002). To determine H₂O₂ and peroxidase activity, fresh samples were stained with 3,3-diaminobenzidine (DAB) using a modified version of the procedure described by Thordal-Christensen et al. (1997). Briefly, fresh samples were submerged in DAB solution (1 mg/ml, in distilled water, pH 3.8) for 2–3 h, washed using a 1:1:1 mixture of lactic acid, glycerol, and water for 1 h, hand-cut using a razor blade, and then mounted on slides using the 1:1:1 mixture of lactic acid, glycerol, and water. Finally, the accumulation of phenolic compounds in fresh, hand-cut samples was observed using epifluorescence (340–380 nm).

RESULTS

Characterization of Resistance in Parental Inbred Lines

The inoculation of PHSC1102 plants with seeds of sunflower broomrape populations F_{GV}, G_{TK}, G_{RO}, and G_{RU} resulted in the absence of or extremely low emergence of broomrape shoots, with a highest mean of 0.5 broomrape shoots per sunflower plant observed for broomrape population G_{RU} (Table 1). Meanwhile, the highest mean of emerged broomrape shoots for line PHSC1201 was 9.7, which was observed for broomrape population G_{RU}. Except for population G_{TK}, the number and weight of emerged broomrape shoots on lines PHSC1201 and PHSC1102 were significantly different. In all cases, the emerged broomrape shoots on line PHSC1102 were clearly underdeveloped, characterized by reduced plant growth and the absence of regular flower development (Fig. 1), indicating that the parasite was not able to complete its life cycle on this line. The highest mean broomrape shoot weight on line PHSC1102 was recorded for the emerged G_{RU} broomrape shoots (67.5 mg). This revealed a limited broomrape growth on PHSC1102, as shown by the mean broomrape shoot weight of the G_{RU} population on line PHSC1102, as shown by the mean broomrape shoot weight of the G_{RU} population on line PHSC1201 (5799.2 mg) or on the B117 population (5319.0 mg). PHSC1102 plants were also characterized by the presence of underground nodules and shoots. The mean number of underground structures in line PHSC1102 ranged from 4.7 (population G_{RO}) to 56.9 (population G_{RU}), whereas that in line PHSC1201 ranged from 0.1 (population G_{TK}) to 7.4 (population G_{RU}). Differences for the mean number of underground structures in lines PHSC1102 and PHSC1201 were significant for all the broomrape populations (Table 1). It is important to note that the underground nodules and shoots generally lacked necrosis at the time of evaluation, with the exception of the apical part of the shoot (Fig. 1) but

became completely necrotic later in sunflower maturity. Line PHSC0933, which carried *Or7*, was completely resistant to all broomrape populations.

Genetic Mapping

A total of 188 SNP markers that were polymorphic between the two parental lines were used to genotype the F₂ population. The SNPs were distributed throughout the species' 17 chromosomes. The *Or_{SH}* genotypes of the F₂ population were deduced by evaluating the F₃ phenotypes, and the gene was mapped accordingly. The F₂ genotypes with progenies that were uniformly resistant were assumed to possess the *Or_{SH} Or_{SH}* genotype, whereas F₂ genotypes with progenies that were uniformly susceptible were assumed to possess the *or_{SH} or_{SH}* genotype, and the F₂ genotypes with F₃ progenies that segregated for resistance/susceptibility were assumed to possess the *Or_{SH} or_{SH}* genotype. The *Or_{SH}* gene was mapped to the upper part of linkage group 4 of the sunflower genome, close (0.7 cM) to SNP marker M-4_30.40 (Fig. 2).

Characterization of Resistance Mechanisms

Table 2 contains a summary of the characterization of resistance performed in mini-rhizotron experiments to identify the mechanism of resistance acting in PHSC1102. The only statistical difference ($P < 0.05$) detected between lines PHSC1201 and PHSC1102 was the weight of broomrape tubercles, when infected with broomrape races F_{GV} and G_{TK}. The broomrape development on the resistant and susceptible lines was clearly different (Fig. 3). The data suggested that the mechanism of *Or_{SH}* resistance was post-haustorial, acting at the stage of tubercle development. The control line (PHSC0933), which harbored the *Or7* resistance gene, exhibited no tubercle development for any of the broomrape races.

Histological analyses were conducted on sections of infected roots of the parental lines of the mapping population that had attached broomrape seedlings. No differences were apparent in either the fresh or TBO-stained specimens, suggesting that the resistance was not the result of physical barriers, such as lignin or suberin layers. Meanwhile, DAB staining, which was performed to detect H₂O₂ and peroxidase activity, was inconclusive because strong staining was not observed for any of the genotypes. However, when investigating the accumulation of phenolic compounds using a fluorescence microscope (340–380 nm), intense blue fluorescence was observed inside parasite nodule cells and neighboring host tissue in incompatible interactions, whereas no fluorescence was detected in the compatible interactions of genotype PHSC1201 (Fig. 4). None of the tubercles from line PHSC1201 exhibited fluorescence, whereas 85% of the nodules from line PHSC1102 exhibited intense fluorescence. This suggested that phenolic compounds were involved in limiting the growth of sunflower broomrape shoots attached to the roots of sunflower line PHSC1102.

DISCUSSION

The genetic resistance of sunflower to broomrape typically follows a gene-for-gene interaction, in which a dominant gene for host resistance interacts with a dominant avirulence gene in the parasite (Rodríguez-Ojeda et al., 2013). In such interactions, dominant resistance is usually complete (i.e., both the dominant homozygote and heterozygote are fully resistant), thereby resulting in a complete absence of symptoms (Fernández-Martínez et al., 2015). However, in *Orobanch*e-host parasitization systems, resistance is generally horizontal, rather than vertical (Pérez-Vich et al., 2013). Complete monogenic dominant resistance in sunflower has been reported against all the broomrape

313 races described so far, including races A to E (Vranceanu et al., 1980), race F (Pacureanu-
314 Joita et al., 2004), and race G (Velasco et al., 2012). Exceptions, in which resistance to
315 sunflower broomrape is controlled by other genetic systems, including control by two
316 independent dominant genes (Pacureanu-Joita et al., 2008), one dominant and one
317 modifying gene (Velasco et al., 2007), one recessive gene (Imerovski et al., 2016), and
318 two recessive genes (Akhtouch et al., 2002), have also been reported, but even in these
319 cases, resistance was characterized by the complete absence of emerged shoots of the
320 parasite. No studies have investigated the mechanisms underlying the resistance
321 mechanisms in resistant donor genotypes, but the absence of broomrape tubercles is an
322 indicator of either pre-attachment or pre-haustorial resistance mechanisms. Incomplete
323 resistance, which is characterized by a reduced number of parasitization structures, has
324 also been reported, although, in those cases, the trait was reported to be quantitatively
325 inherited (Pérez-Vich et al., 2005; Louarn et al., 2016). Accordingly, System II resistance,
326 which is characterized by the drastic reduction in broomrape growth at the post-haustorial
327 stage by a gene having a major effect, is notably different from resistance sources
328 described in previous studies.

329 Previous studies on physiological mechanisms that underlie the genetic resistance
330 of resistant sunflower genotypes have revealed several mechanisms that occur at different
331 stages of parasitism (Labrousse et al., 2001; Echevarría-Zomeño et al., 2006; Letousey et
332 al., 2007; Zélicourt et al., 2007; Louarn et al., 2016). In those studies, necrosis of
333 broomrape nodules and developing shoots were observed to occur as a result of defensive
334 mechanisms. However, the necrosis of parasite structures observed in the present study
335 occurred very late, after tubercle development. It is likely that this observation was
336 associated with the relatively delayed activation of resistance in line PHSC1102, when
337 compared to other resistance sources. In cases of monogenic control of resistance, e.g.,

the resistance provided by *Or5* against broomrape race E, the responsible locus may actually be a cluster of recognition-dependent resistance genes that encode proteins that contain both a nucleotide-binding site (NBS) domain and leucine-rich repeats (LRRs; Lu et al., 2000; Radwan et al., 2008).

In the present study, no physical barriers (e.g., lignin, suberin, or callose) were observed to impede the penetration by broomrape into the host. This suggests that the resistance is the result of another type of defensive mechanism. The intrusive parasite cells easily reached the host cortex and endodermis, and finally the vascular cylinder, which resulted in the development of tubercles and shoots. The data revealed that phenolic compounds were involved in the resistance of sunflower line PHSC1102. Other studies have also reported the association of phenolics with broomrape resistance in sunflower, although at earlier stages. Echevarría-Zomeño et al. (2006), for example, reported that phenolic compounds, together with other resistance mechanisms, such as suberization and protein cross-linking at the cell wall, were involved in the incompatible reaction of race F broomrape and the resistant sunflower line HE-39999. In that study, the authors determined that resistance was achieved by impeding penetration by the parasite. This differs from the mechanism underlying the resistance of line PHSC1102, in which the resistance mechanisms act after vascular connections between the parasite and host have been established.

In another study, Lozano-Baena et al. (2007) characterized the mechanisms of resistance of *Medicago truncatula* (accession SA4327) against crenate broomrape (*Orobancha crenata*) and reported mechanisms with some similarity to the mechanisms underlying resistance in line PHSC1102. The authors reported that, even though the parasite attached to SA4327 roots without apparent difficulty, most of the parasitic structures turned necrotic and died before emerging and that phenolic compounds were

released at the infection point, where host cells made contact with parasite tissues and xylem vessels. In both *M. truncatula* (SA4327) and *H. annuus* (PHSC1102), the accumulation and secretion of phenolic compounds occur at a later stage of infection. This suggests that the host poisons the broomrape structures, thereby inhibiting parasite development, as demonstrated in the present study by differences in the growth of parasites structures in sunflower lines PHSC1102 and PHSC1201. In the present study, fluorescence was observed in the *O. cumana* tubercle and in the sunflower root tissue that was connected to the tubercle but not in tissues that were located further from the vascular connections. This suggests that the host root produces the phenolic compounds at the infection point. In *M. truncatula*, the broomrape tubercles that developed on SA4327 became necrotic (Lozano-Baena et al., 2007). However, this was not observed in sunflower line PHSC1102.

Despite remarkable differences in the size and weight of broomrape tubercles attached to PHSC1201 and PHSC1102, no tubercle necrosis was observed in the mini-rhizotron study. In the pot experiments, necrosis was observed to occur very late, at the time of sunflower maturity (Fig. 1). There are several examples of phenolics providing disease resistance in crops. In some cases, phenolics have been demonstrated to inhibit spore germination of phytopathogenic fungi, whereas, in other cases, phenolic compounds have been demonstrated to inhibit mycelial growth (Lattanzio et al., 2006). The results of the present study suggest that phenolic compounds are involved in the delayed parasite development observed in the PHSC1102-*O. cumana* system. However, it remains unclear whether the necrosis observed in broomrape plants at the end of the sunflower cycle is a consequence of phenolic compounds or just the result of sunflower maturity.

Several genes conferring resistance to sunflower broomrape have been mapped in sunflower. *Or5*, which confers resistance to broomrape race E, has been mapped to a telomeric region of LG3 (Lu et al., 2000; Tang et al., 2003; Pérez-Vich et al., 2004); and Imerovski et al. (2013; 2016) identified SSR markers on LG3 that were strongly associated with resistance genes (*Or2*, *Or3*, *Or6*, and *orab-vl-8*) that conferred resistance to races B, C, F, and higher than F, respectively. Meanwhile, Duriez et al. (2017) mapped *Or7*, which confers resistance to race F, to LG7. A number of resistance-related quantitative trait loci (QTL) have also been identified in lines with oligogenic (Pérez-Vich et al., 2004) and polygenic (Louarn et al., 2016) resistance mechanisms. In the present study, a major gene controlling post-haustorial resistance to broomrape was mapped to LG4, confirming the previous results of Hassan et al. (2008). The position of the gene has been more accurately determined in the present research, with markers very close to the gene (Fig. 2). No other broomrape resistance genes have been reported to date in LG4. The defensive genes mapped on that chromosome corresponded to *Pl17*, *Pl19*, *Pl27*, *Pl28*, and *Pl29*, which are downy mildew resistance genes located in the interval between 2.18–8.51 Mb in the reference genome of XRQ inbred line (Qi et al., 2015; Zhang et al., 2017; Pecrix et al., 2018), and were located upstream of *OrSH*.

The development of durable broomrape resistance in sunflower will require detailed characterization of the genetic and physiological bases of resistance sources, which are needed to set up gene pyramiding strategies based on physiology-based criteria. Ideally, gene pyramiding should include genes that combine different mechanisms of resistance to establish barriers that cannot be easily overcome by the parasite (Rubiales, 2018). Several studies have been conducted in sunflower, in regards to pyramiding resistance genes for downy mildew (Qui et al., 2015; 2017), but little research has been conducted on gene pyramiding strategies for broomrape resistance. The present study

characterized a novel resistance mechanism for broomrape resistance, which involved the impairment of broomrape growth after the establishment of vascular connections. Because of the presence of a different physiological mechanism relative to other resistance sources, the *Or_{SH}* gene is a good candidate for gene pyramiding strategies, along with other promising genes, such as *Or7* and *Or_{deb2}*, both of which provide resistance to race G populations of sunflower broomrape.

ACKNOWLEDGMENTS

We are grateful to Placida Nieto and Alberto Merino, for excellent technical support, and to Dr. Alejandro Pérez de Luque, for valuable advice in the development and interpretation of the histological study.

REFERENCES

- Akhtouch, B., J. Muñoz-Ruz, J.M. Melero-Vara, J.M. Fernández-Martínez, and J. Domínguez. 2002. Inheritance of resistance to race F of broomrape (*Orobancha cumana* Wallr.) in sunflower lines of different origin. *Plant Breeding* 121:266-269.
- Bachlava, E., C.A. Taylor, S. Tang, J.E. Bowers, J.R. Mandel, J.M. Burke, and S.J. Knapp. 2012. SNP discovery and development of a high-density genotyping array for sunflower. *PLoS ONE* 7:e29814.
- Bordallo, J.J., L.V. Lopez-Llorca, H.B. Jansson, J. Salinas, L. Persmark, and L. Asensio. 2002. Colonization of plant roots by egg-parasitic and nematode-trapping fungi. *New Phytol.* 154:491-499.

437 Bowers, J.E., E. Bachlava, R.L. Brunick, L.H. Rieseberg, S.J. Knapp, and J.M. Burke.
 438 2012. Development of a 10,000 locus genetic map of the sunflower genome based
 439 on multiple crosses. *Genes Genomes Genet.* 2:721-729.

440 Duriez, P., S. Vautrin, M. Lopez-Sendon, M.C. Boniface, N. Pouilly, H. Bergès, J.
 441 Bazerque, F. Gentoux, C. Paris, J. Piquemal, and S. Muñoz. 2017. Molecular
 442 characterization of the major resistance gene *Or7* controlling resistance to *O.*
 443 *cumana* in sunflower. In: Book of Abstracts of the 14th World Congress on
 444 Parasitic Plants, Pacific Grove, CA, July 25-30, 2017. International Plant Parasitic
 445 Society, p. 22.

446 Echevarría-Zomeño, S., A. Pérez-de-Luque, J. Jorrín, and A. Maldonado. 2006. Pre-
 447 haustorial resistance to broomrape (*Orobancha cumana*) in sunflower (*Helianthus*
 448 *annuus*): cytochemical studies. *J. Exp. Bot.* 57:4189-4200.

449 Fernández-Martínez, J.M., B. Pérez-Vich, and L. Velasco. 2015. Sunflower broomrape
 450 (*Orobancha cumana* Wallr.) In: E. Martínez-Force, N.T. Dunford, and J.J. Salas,
 451 editors, Sunflower oilseed. Chemistry, production, processing and utilization.
 452 AOCS Press, Champaign, IL, p. 129-156.

453 Hassan, E.S.A.R.S., E. Hoefft, Z. Li, and L. Tulsieram. 2008. Genetic markers for
 454 *Orobancha* resistance in sunflower. US Patent Publication US/2008/0178325 A1.
 455 See:
 456 (https://www.lens.org/images/patent/WO/2008083198/A2/WO_2008_083198_A
 457 [2.pdf](#))

458 Höniges, A., K. Wegmann, and A. Ardelean. 2008. *Orobancha* resistance in sunflower.
 459 *Helia* 49:1-12.

460 Imerovski, I., A. Dimitrijevic, D. Miladinovic, B. Dedic, S. Jovic, B. Kovacevic, and D.
461 Obreht. 2013. Identification of PCR markers linked to different *Or* genes in
462 sunflower. *Plant Breeding* 132:115-120.

463 Imerovski, I., A. Dimitrijevic, D. Miladinovic, B. Dedic, S. Jovic, N.K. Tubic, and S.
464 Cvejic. 2016. Mapping of a new gene for resistance to broomrape races higher
465 than F. *Euphytica* 209:281-289.

466 Kidwell, K.K., and T.C. Osborn. 1992. Simple plant DNA isolation procedures. In: J.S.
467 Beckmann and T.C. Osborn, editors, *Plants genomes: methods for genetic and*
468 *physical mapping*, Kluwer Academic Publishers, The Netherlands, p. 1-13.

469 Lattanzio, V., V.M.T. Lattanzio, and A. Cardinali. 2006. Role of phenolics in the
470 resistance mechanisms of plants against fungal pathogens and insects. In: F.
471 Imperato, editor, *Phytochemistry: advances in research*. Research Signpost, India,
472 p. 23-67.

473 Labrousse, P., M.C. Arnaud, H. Serieys, A. Bervillé, and P. Thalouan. 2001. Several
474 mechanisms are involved in resistance of *Helianthus* to *Orobanche cumana* Wallr.
475 *Ann. Bot.* 88:859-868.

476 Letousey, P., A. De Zélicourt, C. Vieira Dos Santos, S. Thoiron, F. Monteau, P. Simier,
477 P. Thalouarn, and P. Delavault. 2007. Molecular analysis of resistance
478 mechanisms to *Orobanche cumana* in sunflower. *Plant Pathol.* 56:536-546.

479 Lorieux, M. 2012. MapDisto: fast and efficient computation of genetic linkage maps.
480 *Mol. Breed.* 30:1231-1235.

481 Louarn, J., M.C. Boniface, N. Pouilly, J. Issakoff, L. Velasco, B. Pérez-Vich, P. Vincourt,
482 and S. Muños. 2016. Sunflower resistance to broomrape is controlled by specific
483 QTLs for different parasitism stages. *Front. Plant. Sci.* 7:590.

484 Lozano-Baena, M.D., E. Prats, M. Teresa Moreno, D. Rubiales, and A. Pérez de Luque.
 485 2007. *Medicago truncatula* as a model for nonhost resistance in legume-parasitic
 486 plant interactions. *Plant Physiol.* 145:437-449.

487 Lu, Y.H., J.M. Melero-Vara, J.A. García-Tejada, and P. Blanchard. 2000. Development
 488 of SCAR markers linked to the gene *Or5* conferring resistance to broomrape
 489 (*Orobancha cumana* Wallr.) in sunflower. *Theor. Appl. Genet.* 100:625-632.

490 Martín-Sanz, A., J. Malek, J.M. Fernández-Martínez, B. Pérez-Vich, and L. Velasco.
 491 2016. Increased virulence in sunflower broomrape (*Orobancha cumana* Wallr.)
 492 populations from southern Spain is associated with greater genetic diversity.
 493 *Front. Plant Sci.* 7:589.

494 Pacureanu-Joita, M., S. Raranciuc, D. Stanciu, E. Sava, and D. Nastase. 2008. Virulence
 495 and aggressiveness of sunflower broomrape (*Orobancha cumana* Wallr.)
 496 populations, in Romania. *Romanian Agric. Res.* 25:47-50.

497 Pacureanu-Joita, M., C. Veronesi, S. Raranciuc, and D. Stanciu. 2004. Parasite-host plant
 498 interaction of *Orobancha cumana* Wallr. (*Orobancha cernua* Loebl.) with
 499 *Helianthus annuus*. In: G.J. Seiler, editor, Proc. 16th Int. Sunflower Conf., Fargo,
 500 ND, Aug 29-Sept 2, 2004. International Sunflower Association, Paris, France, p.
 501 171-177.

502 Pecrix, Y., C. Penouilh-Suzette, S. Muños, F. Vear, and L. Godiard. 2018. Ten broad
 503 spectrum resistances to downy mildew physically mapped on the sunflower
 504 genome. *Front. Plant Sci.* 9:1780.

505 Pérez de Luque, A., S. Fondevilla, B. Pérez-Vich, R. Aly, S. Thoiron, P. Simier, M.A.
 506 Castillejo, J.M. Fernández-Martínez, J. Jorrín, D. Rubiales, and P. Delavault.
 507 2009. Understanding *Orobancha* and *Phelipanche* host plant interactions and
 508 developing resistance. *Weed Res.* 49:8-22.

509 Pérez de Luque, A., D. Rubiales, J.I. Cubero, M.C. Press, and J. Scholes. 2005. Interaction
 510 between *Orobanche crenata* and its host legumes: unsuccessful haustorial
 511 penetration and necrosis of the developing parasite. *Ann. Bot.* 95:935-942.

512 Pérez-Vich, B., M.R. Aguirre, B. Guta, J.M. Fernández-Martínez, and L. Velasco. 2018.
 513 Genetic diversity of a germplasm collection of confectionery sunflower landraces
 514 from Spain. *Crop Sci.* 58:1972-1981.

515 Pérez-Vich, B., B. Akhtouch, S.J. Knapp, A.J. Leon, L. Velasco, J.M. Fernández-
 516 Martínez, and S.T. Berry. 2004. Quantitative trait loci for broomrape (*Orobanche*
 517 *cumana* Wallr.) resistance in sunflower. *Theor. Appl. Genet.* 109:92-102.

518 Pérez-Vich, B., L. Velasco, J. Muñoz-Ruz, J. Domínguez, and J.M. Fernández-Martínez.
 519 2005. Registration of three sunflower germplasms with quantitative resistance to
 520 race F of broomrape. *Crop Sci.* 46:1406-1407.

521 Pérez-Vich, B., L. Velasco, P.J. Rich, and G. Ejeta. 2013. Marker-assisted and
 522 physiology-based breeding for resistance to root parasitic Orobanchaceae. In:
 523 D.M. Joel, J. Gressel, and L.J. Musselman, editors, *Parasitic Orobanchaceae.*
 524 *Parasitic mechanisms and control strategies*, Springer, New York, p. 369-391.

525 Qi, L.L., Y.M. Long, C.C. Jan, G.J. Ma, and T.J. Gulya. 2015. *PlI7* is a novel gene
 526 independent of known downy mildew resistance genes in the cultivated sunflower
 527 (*Helianthus annuus* L.). *Theor. Appl. Genet.* 128:757-767.

528 Qi, L.L., Z.I. Talukder, B.S. Hulke, and M.E. Foley. 2017. Development and dissection
 529 of diagnostic SNP markers for the downy mildew resistance genes *Pl Arg* and *Pl*
 530 *8* and maker-assisted gene pyramiding in sunflower (*Helianthus annuus* L.). *Mol.*
 531 *Genet. Genomics* 292:551-563.

532 Radwan, O., S. Gandhi, A. Heesacker, B. Whitaker, C. Taylor, A. Plocik, R. Kesseli, A.
 533 Kozik, R.W. Michelmore, and S.J. Knapp. 2008. Genetic diversity and genomic

distribution of homologs encoding NBS-LRR disease resistance proteins in
sunflower. Mol. Genet. Genomics 280:111-125.

Rodríguez-Ojeda, M.I., R. Pineda-Martos, L.C. Alonso, J. Fernández-Escobar, J.M.
Fernández-Martínez, B. Pérez-Vich, and L. Velasco. 2013. A dominant avirulence
gene in *Orobanche cumana* triggers Or5 resistance in sunflower. Weed Res.
53:322-327.

Rubiales, D. 2018. Can we breed for durable resistance to broomrapes? Phytopathol.
Mediterr. 57:170-185.

Serghini, K., A. Pérez-de-Luque, M. Castejón-Muñoz, L. García-Torres, and J.V. Jorrín.
2001. Sunflower (*Helianthus annuus* L.) response to broomrape (*Orobanche
cernua* Loebl.) parasitism: induced synthesis and excretion of 7-hydroxylated
simple coumarins. J. Exp. Bot. 52:2227-2234.

Tang, S., A. Heesacher, V.K. Kishore, A. Fernández, E.S. Sadik, G. Cole, and S.J. Knapp.
2003. Genetic mapping of the *Or5* gene for resistance to *Orobanche* race E in
sunflower. Crop Sci. 43:1021-1028.

Thordal-Christensen, H., Z. Zhang, Y. Wei, and D.B. Collinge. 1997. Subcellular
localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive
response during the barley-powdery mildew interaction. Plant J. 11:1187-1194.

Velasco, L., B. Pérez-Vich, C.C. Jan, and J.M. Fernández-Martínez. 2007. Inheritance of
resistance to broomrape (*Orobanche cumana* Wallr.) race F in a sunflower line
derived from wild sunflower species. Plant Breeding 126:67-71.

Velasco, L., B. Pérez-Vich, A.A.M. Yassein, C.C. Jan, and J.M. Fernández-Martínez.
2012. Inheritance of resistance to sunflower broomrape (*Orobanche cumana*
Wallr.) in an interspecific cross between *Helianthus annuus* and *Helianthus
debilis* subsp. *tardiflorus*. Plant Breeding 121:220-221.

- Voorrips, R.E. 2002. MapChart: software for the graphical presentation of linkage maps and QTL. J. Hered. 93:77-78.
- Vranceanu, A.V., V.A. Tudor, F.M. Stoenescu, and N. Pirvu. 1980. Virulence groups of *Orobanche cumana* Wallr. differential hosts and resistance sources and genes in sunflower. In: Proc. 9th Int. Sunflower Conf., Torremolinos, Spain, July 8-13, 1980. International Sunflower Association, Paris, France, p. 74-80.
- Zélicourt, A., P. Letousey, S. Thoiron, C. Champion, P. Simoneau, K. Elmorjani, D. Marion, P. Simier, and P. Delavault. 2007. Ha-DEF1, a sunflower defensin, induces cell death in *Orobanche* parasitic plants. Planta 226:591-600.
- Zhang, Z.W., G.J. Ma, J. Zhao, S.G. Markell, and L.L. Qi. 2017. Discovery and introgression of the wild sunflower-derived novel downy mildew resistance gene *Pl19* in confection sunflower (*Helianthus annuus* L.). Thero. Appl. Genet. 130:29-39.

Figure legends

Figure 1. Phenotypic responses of *O_{rsII}*-homozygous and -heterozygous sunflower plants to broomrape infection. A, emerged broomrape on homozygous plant; B, subterranean broomrape nodules on homozygous plant; C, emerged broomrape on heterozygous plant; D, subterranean broomrape nodules on heterozygous plant; E, phenotype of susceptible inbred line (PHSC1201); F, necrosis observed in the apex of broomrape tubercles at 105 d after sunflower sowing. Pictures depict greenhouse-grown sunflower plants that were inoculated with broomrape population G_{TK} at 90 d after sunflower sowing.

Figure 2. Genetic map of sunflower linkage group 4 (LG4). LG4 harbors *O_{rsII}*, which controls post-haustorial resistance to sunflower broomrape in sunflower line PHSC1102.

Figure 3. Phenotypic responses of sunflower lines at 21 d after broomrape infection. A, PHSC1201; B, PHSC1102; C, PHSC0933. Sunflower plants were inoculated with broomrape population G_{TK} in mini-rhizotrons. Small squares on the top left corner show detailed pictures of root-level responses observed with a stereomicroscope at 14 d after infection.

Figure 4. Fresh hand-cut sections of sunflower roots and broomrape tubercles collected at 21 d after inoculation. A, susceptible line (PHSC1201); B, resistant line (PHSC1102). Sections were observed under fluorescence (340-380 nm), with fluorescence indicating the accumulation of phenolic compounds. hc, host cortex; pha, parasite haustorium; pt, parasite tubercle.

Table 1. Resistance of sunflower lines PHSC1201 and PHSC1102, control resistant line PHSC0933, and control susceptible population B117 to several populations of sunflower broomrape.

	FGV		GTK		GRO		GRU	
	N [†]	Wt [‡]	N	Wt	N	Wt	N	Wt
PHSC1201_E [‡]	4.8 ^{b§}	3934 ^b	1.0 ^a	812.2 ^a	4.3.0 ^b	1933.0 ^b	9.7 ^b	5799.2 ^b
PHSC1102_E	0.2 ^a	1.7 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.5 ^a	67.5 ^a
PHSC0933_E	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
B117_E	17.0 ^c	6358.5 ^c	21.6 ^b	4797.0 ^b	9.8 ^c	10308.2 ^b	25.3 ^c	5319.0 ^b
PHSC1201_U [‡]	0.8 ^a	644.2 ^b	0.1 ^a	40.0 ^a	1.1 ^a	606.7 ^a	7.4 ^a	2295.8 ^a
PHSC1102_U	11.2 ^b	110.0 ^a	7.6 ^b	44.8 ^a	4.7 ^b	21.2 ^a	56.9 ^b	1265.0 ^a

[†]N, mean number of broomrape shoots per plant; Wt, mean shoot weight (mg).

[‡] E: Emerged sunflower broomrape structures; _U: Underground sunflower broomrape structures.

[§] Different lowercase letters within columns indicate significant differences (Tukey test, $P < 0.05$)

Table 2. Responses of inbred sunflower lines to *Orobanche cumana* infection in mini-rhizotrons.

Sunflower genotype	<i>O. cumana</i> seed		Germinated seeds that		Contacted radicles that		Mean no. tubercles		Tubercles with necrosis		Tubercle weight (mg)	
	germination (%)		contacted host roots (%)		formed organs (%)	attachment	/ plant		(%)			
	F _{GV}	G _{TK}	F _{GV}	G _{TK}	F _{GV}	G _{TK}	F _{GV}	G _{TK}	F _{GV}	G _{TK}	F _{GV}	G _{TK}
PHSC1201 (<i>Or5</i>)	49.2 ^{b†}	57.0 ^a	13.5 ^a	14.1 ^a	56.2 ^a	52.0 ^a	33.7 ^b	46.2 ^b	26.5 ^a	25.5 ^a	32.7 ^b	46.3 ^b
PHSC1102 (<i>Or_{SH}</i>)	63.2 ^a	51.7 ^a	8.8 ^a	8.8 ^a	52.8 ^a	51.7 ^a	27.2 ^b	40.0 ^b	21.8 ^a	19.9 ^a	0.1 ^a	0.6 ^a
PHSC0933 (<i>Or7</i>)	43.7 ^a	51.2 ^a	7.4 ^a	8.7 ^a	47.5 ^a	51.5 ^a	0.0 ^a	0.0 ^a	-	-	-	-
B117	54.5 ^{ab}	55.2 ^a	11.9 ^a	11.7 ^a	56.0 ^a	52.0 ^a	33.2 ^b	44.5 ^b	26.0 ^a	20.9 ^a	30.0 ^b	35.7 ^b

[†]Different lowercase letters within columns indicate significant differences (Tukey test, $P < 0.05$).