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BSA-seq mapping reveals major QTL for broomrape resistance in four sunflower lines

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Abstract Broomrape (*Orobanche cumana*) is a parasitic weed that causes substantial yield losses in sunflower. In this study, four biparental genetic populations comprised of between 96 and 150 F_3 families were phenotyped for resistance to broomrape race G. Bulk segregant analyses (BSA) combined with genotyping-by-sequencing (GBS) technology was used to verify previously identified genes and map new resistance QTLs. Resistance had a polygenic basis, and numerous QTLs were found in all mapping populations. Contributing components to resistance that were common to all populations mapped to two major QTLs on chromosome 3, which were designated *or3*.1 and *or3*.2. QTL *or3*.1 was positioned in a genomic region where a previous broomrape resistance gene *Or5* has been mapped, while QTL *or3*.2 was identified for the first time in the lower region of the same chromosome. Following the identification of major QTLs for resistance using the BSA-seq approach, all plants from three F_2 populations were genotyped using newly developed CAPS markers nearest to the QTL peak for *or3*.2, which confirmed the association of these markers with broomrape race G resistance. The results of this study will bring us a step closer to understanding the mechanisms underlying resistance of sunflower to highly virulent broomrape races, and the molecular markers developed herein will be highly useful for sunflower breeding programs.

Keywords Sunflower · Broomrape · QTL · Race G · GBS · BSA

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Introduction

Broomrape (*Orobanche cumana*) is a non-photosynthetic plant that parasitizes sunflower roots, leading to severe yield losses. Sunflower broomrape is believed to have evolved in Russia from *O. cernua*, which parasitizes wild species in the sunflower family, *Asteraceae*. It has subsequently spread to other sunflower growing regions in central and western Europe, Asia (Antonova 2014), and Tunisia (Amri et al. 2012). Broomrape is completely dependent on its hosts; after establishing a connection with sunflower roots, the parasite depletes the host of nutrients and consequently hampers growth, development, and especially yield

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(Molinero-Ruiz et al. 2015). Depending on the intensity and timing of infestation, yield reductions can vary from 5 to 100% (Manschadi et al. 1996; Grenz et al. 2005; Fernández-Aparicio et al. 2009). At physiological maturity, a single broomrape plant can produce and disperse up to 100,000 seeds (Chater and Webb 1972), which can retain viability for up to 20 years (Parker 2013). The minute seeds are easily dispersed by wind and water, but are also often introduced into new areas as contaminants of sunflower seeds (CABI 2018).

A number of biotypes of *O. cumana* have been reported that differ in virulence to sunflower cultivars. Based on responses to a set of sunflower lines with five different resistance genes, Vrânceanu et al. (1980) classified biotypes in Romania into physiological races, and designated them A-E. Using the same differential line set, broomrape biotypes from other regions were classified into races as well. According to Shindrova (1994), races A-E were also found in Bulgaria. In Spain, *O. cumana* was first detected on confectionery sunflowers in 1958 in the province of Toledo. Later reports showed that the parasite had expanded into the central and southern regions of Spain (González-Torres et al. 1982) and was comprised of three races that were assumed to be different from those described in Eastern Europe (Melero-Vara et al. 1989). Subsequently, a more virulent biotype F was identified in Spain (Alonso et al. 1996; Molinero-Ruiz et al. 2008), Romania (Vrânceanu and Păcureanu 1995), Turkey (Kaya et al. 2004), and Serbia (Maširević et al. 2012). Škorić and Păcureanu (2010) reported the presence of a new *O. cumana* biotype (designated race G) that infests sunflower cultivars resistant to race F. In Russia, the most virulent races of the parasite (G and H) have been reported from many regions of sunflower cultivation, including Rostov, Voronezh, Volgograd, Saratov, Orenburg, Stavropol, and Krasnodar (Antonova 2014).

Plant pathogens evolve rapidly. Thus, new, more virulent populations arise frequently and overcome formerly resistant crop varieties. Consequently, breeders are constantly searching for new resistance genes. Historically, resistance to *O. cumana* in sunflower was primarily vertical and race-specific (Škorić and Păcureanu 2010; Molinero-Ruiz et al. 2015). Genes *Or1*, *Or2*, *Or3*, *Or4*, and *Or5*, which provide resistance to races A, B, C, D, and E, respectively, are single dominant genes (Vrânceanu et al. 1980). For race F, resistance was found in germplasm of both cultivated and wild sunflower (Vrânceanu and Păcureanu 1995; Jan et al. 2002; Fernández-Martínez et al. 2004; Velasco et al. 2012), and depending on the source has been reported to be controlled by a single dominant gene designated Or_6 (Vrânceanu and Păcureanu 1995; Pérez-Vich et al. 2002), two recessive genes (Akhtouch et al. 2002, 2016), two partially dominant genes (Velasco et al. 2007) or multiple quantitative trait loci (QTLs) (Pérez-Vich et al. 2004a; Louarn et al. 2016). With respect to race G, preliminary results imply that resistance is conferred by a single dominant gene (Velasco et al. 2012). In contrast, Imerovski et al. (2016) reported that resistance to races higher that F can be controlled by a single recessive gene.

To ensure stabile sunflower production in areas affected by broomrape, identification and mapping of new broomrape resistance genes are needed. The objective of the present study is to: (i) identify promising resistant lines from germplasm collections of the Institute for Field and Vegetable Crops, Novi Sad, Serbia (IFVCNS); (ii) verify resistance and compare resistance spectra of a subset of lines in multi-environmental trials in regions with the most virulent broomrape populations; (iii) determine the mode of inheritance of resistance to *O. cumana* in the most promising inbred lines; (iv) identify QTLs conferring broomrape resistance in sunflower inbred lines; and (v) develop molecular markers for the transfer of these QTLs into elite sunflower lines.

Materials and methods

Preliminary trials and identification of lines with high and stable resistance to *O. cumana*

A sunflower germplasm collection consisting of 300 accessions that are maintained by the IFVCNS was screened for *O. cumana* resistance under field conditions in the summer of 2011. Screening was conducted in naturally infested fields at four locations: Serbia, Spain, and two regions in Romania (Constanta and Tulcea) (Fig. S1a). The selected locations had been used for resistance screening in the past, and historical data on the responses of differential lines at these sites was available prior to the trials reported here (Fig. S1b). The lines were sown in a completely randomized design. An experimental unit consisted of two rows, with 24 plants per row ($n = 48$). Within each plot, a susceptible hybrid Labud was included as a check in every tenth row.

Before physiological maturity, plants were scored for resistance to broomrape, with the aim of identifying highly resistant lines (i.e., genotypes that showed no signs of broomrape infestation or had a very low number of attached parasitic plants). Therefore, scoring was carried out using an arbitrary scale as follows: 0 = no broomrape infestation; 1 = up to 5% broomrape incidence observed with no effect on development and yield; 2 = up to 50% of plants infested; 3 = between 50 and 90% infestation; and 4 = 90–100% infestation and plants were undeveloped with severe yield losses. To identify the most stable lines, we compared performance across the four locations. Resistance of promising lines was validated by subsequent experiments (below).

Multi-environmental field tests of resistant lines

To validate the resistance of promising cultivars, we conducted multi-environment trials with lines that showed high resistance in the preliminary screens. Multi-environmental testing was carried out over two consecutive years (2016 and 2017), at eight locations across Europe (Fig. S1, Table S1). In addition to the fields in Spain and Romania that were used in the 2011 trial, we included testing sites in Ukraine and Russia; the Serbia test site was excluded due to low discriminatory power. At each site, four resistant genotypes (HA-267, LIV-10, LIV-17 and AB-VL-8) as well as their F_1 hybrids were tested. To confirm that these four genotypes had superior resistance to any public line, we employed the differential line set kindly provided by National Agricultural Research and Development Institute (NARDI) Fundulea, Romania, as well as an additional race E resistant IFVCNS line and line P-96, which is resistant to Spanish population of broomrape (Table S2). A randomized complete block design with four replicates and ten plants per row was used. Each test row was surrounded by the susceptible check Labud. At crop maturity, the number of emerged broomrape plants per host plant was recorded. Broomrape incidence was expressed relative to that of the susceptible control.

To determine genotypic differences and genotype × environment interactions, a combined analysis of variance was performed with genotype and environment as fixed factors. Data were transformed to an approximately normal distribution by arcsin square root transformation. Each site in a given year was considered to be a separate environment. Because G × E interactions were significant, we conducted a GEE biplot analysis (Yan et al. 2000; Yan et al. 2001; Yan and Kang 2002), a method based on principal component analysis (PCA) which considers both genotype (G) and GE interaction effects and graphically displays GE interactions (Yan and Kang 2002). Analyses were performed using R package "gge" (versions1.4.) (Wright 2018).

Genetic studies of four biparental populations developed from resistant lines. Mapping population development

The four resistant lines (HA-267, LIV-10, LIV-17 and AB-VL-8) were used for development of segregating populations. Since higher genetic differentiation between parental lines facilitates mapping, we used simple sequence repeat (SSR) markers to screen a subset of IFVCNS lines that were highly susceptible to broomrape at all testing sites during 2011 field trials; the lines showing the highest level of genetic differentiation compared to the resistant lines were then chosen as susceptible parents for development of mapping populations. HA-267 was crossed with OD-DI-82, and LIV-10 and LIV-17 were crossed with HA-26-PR. Segregating F_2 populations with 150, 106 and 89 individuals, respectively, were obtained by self-pollination of an individual F_1. The F_2 plants were advanced to produce F_3 families, which were phenotyped as described below. The fourth population included in the study was developed from a cross between AB-VL-8 and L-OS-1; this population was previously used to map the broomrape resistance gene $or_{AB\text{-}VL\text{-}8}$ (Imerovski et al. 2016). Though all of the lines originate from IFVCNS germplasm, there is no known genetic relationship between HA-267, LIV-10, LIV-17, and AB-VL-8. Resistance in AB-VL-8 is thought to be controlled by a single recessive gene that maps to LG 3 (Imerovski et al. 2016), whereas inheritance of resistance in HA-267, LIV-10, and LIV-17 has not been previously reported.

Parasite population

Broomrape seeds were originally collected in Serbia from sunflower hybrids known to be resistant to race E. This broomrape population was designated as LP12BSR and was used in a previous study as well (Imerovski et al. 2016). Differential line tests showed that LP12BSR infects the Romanian differential line for race F (i.e., line LC1093), which is known to carry the

dominant gene *Or6* (Imerovski et al. 2016). From this, we infer that the broomrape biotype is race G.

Phenotypic evaluation of F_3 families

To infer genotypes of F_2 individuals, F_3 families were tested under greenhouse conditions. Each F_3 family consisted of 15 to 20 individuals. Sunflower seeds were sown in 9-dm^3 elongated pots filled with mixture of sand, perlite, and peat (Klasmann-Deilmann Substrate 1), in which broomrape seed was homogeneously mixed. Plants were grown for 7 weeks under a temperature regime of 24/18 °C and a 16-h photoperiod. In the fifth and sixth weeks of the experiment, plants were watered with fertilizer Fitofert (N.P.K. – 12.4.6 + micronutrients) solution. Resistance was determined following Pérez-Vich et al. (2004a). Briefly, we assessed broomrape infestation by counting the number of emerged broomrape shoots around each sunflower plant. To minimize escapes, resistant plants were carefully uprooted to observe any non-emerged broomrape and nodules or stalks (Fig. 1a). Based on these results, we then calculated broomrape incidence, defined as the number of resistant plants per F_3 family over the total number of plants evaluated per family. F_3 families were scored as resistant if all plants in the family were resistant, segregating if the family had both resistant and susceptible plants, or susceptible if all plants in the family were susceptible. After the greenhouse test, phenotypes of families scored as non-segregating were confirmed under field conditions (Fig. 1b). These families were later used for bulk segregant analyses; hence, it was critical that the phenotypes were accurate.

DNA extraction and bulked segregant analyses via genotyping-by-sequencing (GBS)

DNA was isolated from leaf samples of F_2 plants (each representing an F_3 family phenotyped for resistance to broomrape race G) using a CTAB-based method (Permingeat et al. 1998). Individual leaf samples were collected from F_2 plants and snap frozen in liquid nitrogen. The quality of DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and was quantified with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

Following the phenotyping of the F3 families, we used selective bulked segregant analyses (BSA) coupled with genotyping-by-sequencing (GBS) to genotype the four mapping populations (Win et al. 2017). Within each mapping population, F_2 plants that produced F_3 families with no segregation for resistance or susceptibility were selected and contrasting DNA bulks were prepared. At least ten plants were included in each bulk, as highlighted in Table 1. The GBS libraries were prepared according to Poland et al. (2012), with some modifications. Briefly, genomic DNA was double-digested with enzymes *MspI*/*PstI-HF*, followed by ligation with a barcode adapter and a common Illumina sequencing adapter. After PCR enrichment, samples were quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and equal amounts were pooled together. An agarose gel-based size selection step was performed to retain amplified fragments between 300 and 500 bp in length. We then performed a depletion step to reduce the fraction of repetitive sequence by treating the enriched libraries with Duplex-Specific Nuclease (Todesco et al. in preparation). The libraries were sequenced on the Illumina HiSeq v4 (125 bp, paired-end reads).

The GBS reads were demultiplexed using an in-house Perl script that also trims off adapter read-through, and discards reads shorter than 50 bp following the trimming step (Owens et al. 2016). The remaining reads were aligned to the reference genome of *Helianthus annuus* (HanXRQr1.0-20151230e; http://www.heliagene.org) using "NextGenMap" (Sedlazeck et al. 2013). Calling variants in the repetitive fraction of the sunflower genome is challenging because of the high fraction of TE repeats (i.e., > 80% of the genome), so we ignored reads that aligned entirely to annotated TEs. Alignments were converted to binary format using "SAMtools" (version 0.1.19) (Li et al. 2009). Read group information was added using "Picardtools" (version 1.114) (http://broadinstitute.github.io/picard). Genotyping was performed using the

Fig. 1 **a** Resistant and susceptible plant after uprooting in the greenhouse. **b** Confirming phenotypes of non-segregating F_3 families in field conditions. **c** BSA-seq QTL analysis in populations HA-267/OD-DI-82, LIV-10/HA-26-PR, LIV-17/HA-26-PR, and AB-VL-8/L-OS-1. The significance threshold (FDR ≤ 0.1) is indicated by the horizontal red line. **c** Overlapping QTLs on chromosome 3. **d** Boxplots based on genotyping data of AB-VL-8/L-OS-1, LIV-10/HA-26-PR, and LIV-17/HA-26-PR F_2 populations. "RR" indicates plants homozygous for the resistant parent allele, "RS" plants heterozygous at this locus, and "SS" plants homozygous for the susceptible parent allele. Genotype classes which do not share the same superscript letter have significantly different mean fractions surviving (Tukey–Kramer HSD, $p < 0.01$)

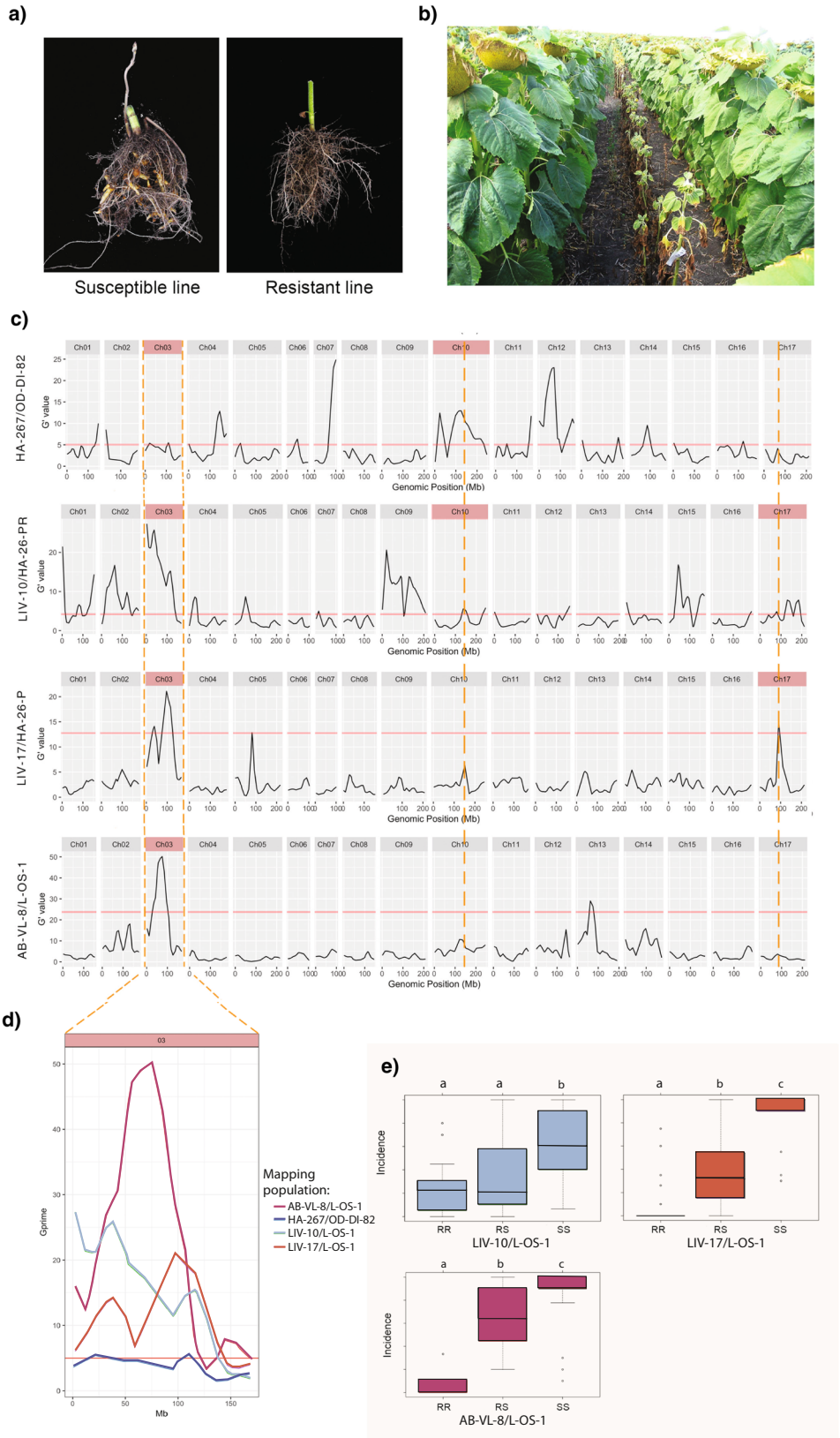
a)
Susceptible line
Resistant line
b)
c)
HA-267/OD-DI-82
LIV-10/HA-26-PR
LIV-17/HA-26-P
AB-VL-8/L-OS-1
G' value
Genomic Position (Mb)
Ch01 Ch02 Ch03 Ch04 Ch05 Ch06 Ch07 Ch08 Ch09 Ch10 Ch11 Ch12 Ch13 Ch14 Ch15 Ch16 Ch17
d)
Gprime
Mb
Mapping population:
AB-VL-8/L-OS-1
HA-267/OD-DI-82
LIV-10/L-OS-1
LIV-17/L-OS-1
e)
Incidence
RR RS SS
LIV-10/L-OS-1
LIV-17/L-OS-1
AB-VL-8/L-OS-1

Table 1 Reaction of HA-267, LIV-10, LIV-17, and HA-26-PR and their F1 and F3 populations to broomrape population LP12BSR. The results from the previous study (Imerovski et al. 2016) are also included for comparative purposes

Line/population	Resistant	Segregating	Susceptible	Total
HA-26-PR	0	0	20	20
OD-DI-82	0	0	18	18
HA-267	20	0	0	20
LIV-10	19	0	0	19
LIV-17	17	0	0	17
AB-VL-8	20	0	0	20
F_1 of LIV-10/HA-26-PR	0	0	19	19
F_1 of LIV-10/HA-26-PR	0	0	20	20
F_1 of LIV-17/HA-26-PR	0	0	18	18
F_1 of AB-VL-8/HA-26-PR	0	0	20	20
F_3 of HA-267/HA-26-PR	32[a]	105	13[b]	150
F_3 of LIV-10/HA-26-PR	12[a]	81	13[b]	106
F_3 of LIV-17/HA-26-PR	26[a]	46	17[b]	89
F_3 of AB-VL-8/HA-26-PR	11[a]	53	32[b]	96

[a] Plants included in the resistant bulk

[b] Plants included in the susceptible bulk

"HaplotypeCaller" and "GenotypeGVCFs" commands in GATK (version 3.7) (Van der Auwera et al. 2013), collectively genotyping all pools together. All raw demultiplexed data were deposited in NCBI's Sequence Read Archive (Table S3).

To identify SNPs that were over-represented in each bulk (resistant vs susceptible), the vcf files were analyzed using the R package "QTLseqr" (version 0.6.4) (Mansfeld and Grumet 2018). First, "QTLseqr" was used to remove low confidence SNPs resulting from low coverage, as well as SNPs that may be in repetitive regions and thus have inflated read depth. Although we ignored reads in known TEs, annotated TEs encompass only one third of the reference genome, which is much lower than the circa 80% expected (Natali et al. 2013). Thus, additional filtering was performed according to recommendations by Mansfield and Grumet (2018) using the following criteria for each pair of pools: (1) total reference read proportion of a SNP between 20 and 80%; (2) sample read depth between 30 and 100; and (3) genotype quality (GQ) score > 30. The number of SNPs before and after filtering is reported in Table S4. We then determined statistical significance of QTLs using the approach proposed by Magwene et al. (2011). A modified G statistic for each SNP was calculated based on observed and expected allele depths and smoothed using a tricube smoothing kernel. This smoothed G′ statistic reduces noise while also accounting for linkage disequilibrium between SNPs. Furthermore, as G′ is close to being log-normally distributed, p values can be estimated for each SNP using non-parametric estimation of the null distribution of G′. This provides a clear and easy-to-interpret result, as well as means for multiple testing corrections (Mansfield and Grumet 2018). G′ at each SNP was calculated with a smoothing window size of $W = 20$ Mb to identify genomic regions that showed G′ peaks, which indicate the possible existence of a QTL. The significance threshold of G′ was estimated using a false discovery rate (FDR) approach according to the analytical procedure proposed by Magwene et al. (2011) and Yang et al. (2013), and QTLs were detected based on FDR ≤ 0.01. Since there is no nomenclature for naming QTLs in sunflowers, each QTL was assigned a name that included the chromosome to which it mapped; if multiple QTLs mapped to the same chromosome, they were numbered consecutively.

Conversion of SNPs into PCR-based CAPS markers

SNP markers underlying major QTL *or3.2* within crosses LIV-10/HA-26-PR, LIV-17/HA-26-PR, and AB-VL-8/L-OS-1 were converted into cleaved amplified polymorphic sequence (CAPS) markers (Table S5). Primers were designed with Primer 3 (http://frodo.wi.

Table 2 Analysis of variance for relative broomrape incidence in 19 sunflower lines in eight environments (combination season-location)

Source	DF	SS	MS	F value
Genotype (G)	18	93.12	5.173	114.161*
Environment (E)	7	5.83	0.832	18.363*
G × E	126	24.81	0.209	4.601**
Residuals	437	14.09	0.045	
Total	588			

* Significant at 0.0001 level of probability

** Significant at 0.001 level of probability

mit.edu/primer3/) (Rozen and Skaletsky 2000). Genotyping of the individual plants from each F_2 population was carried out using a C1000 Touch Thermal Cycler (Bio-Rad, CA, USA). The PCR reaction was performed in 20 μl of reaction system containing 25 ng of template DNA, 15 mM $MgCl_2$, 1× PCR buffer, 2.5 mM dNTP, 15 ng primer, 1 U Taq DNA polymerase. After initial heat denaturation at 94 °C for 4 min, the reaction mixture was subjected to amplification for 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1.5 min. A final extension was performed for 5 min at 72 °C. Restriction digestions were conducted in 20 μl volumes with appropriate enzymes (Table S5) according to the manufacturer's instructions. Digests were resolved on 2% agarose gels in TAE buffer for 15 min with a 50 bp DNA molecular weight standard (Invitrogen), and visualized by ethidium bromide (EtBr) staining. After genotyping of the three F_2 populations, one-way analysis of variance (ANOVA) and post hoc Tukey–Kramer honestly significant difference (HSD) tests of mean broomrape incidence of each of the genotype categories were done using multcomp package (version 1.4-8) (Hothorn et al. 2008).

Results

Preliminary testing of IFVCNS germplasm and identification of resistant lines

Germplasm screening performed on the 300 lines at four locations in 2011 revealed a broad range of responses to broomrape (Fig. S2a–d). The testing sites differed in the discriminating power. A total of 148 and 76 lines were completely resistant in Serbia and Spain, respectively, indicating lower virulence of broomrape biotypes in these regions. The testing site in Tulcea, Romania had

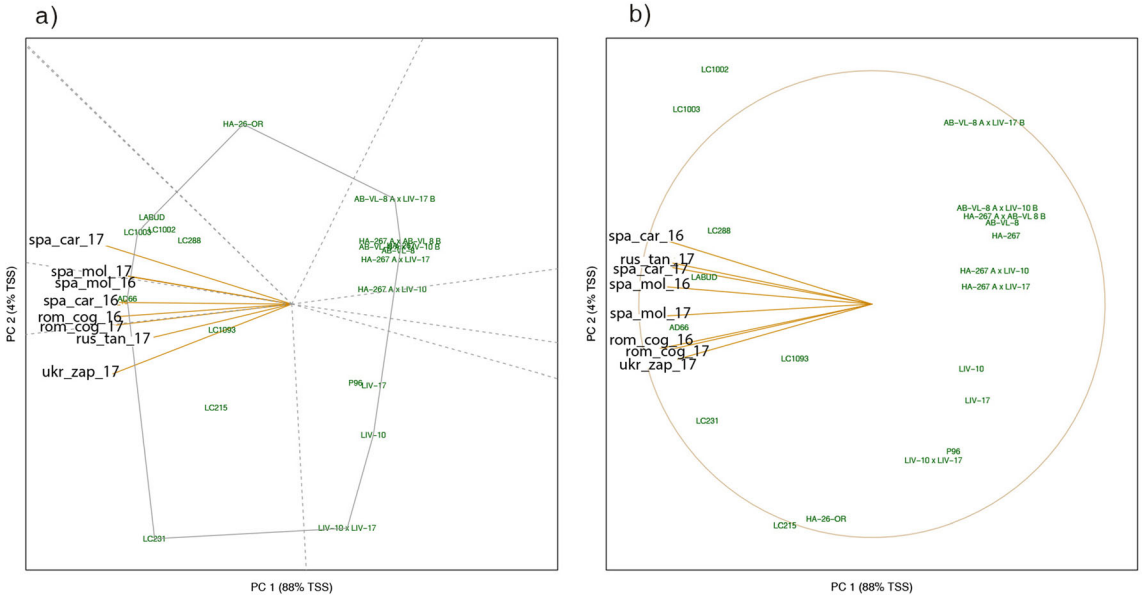

Fig. 2 Unscaled (**a**) and scaled (**b**) biplot GGE based on relative broomrape incidence in 20 sunflower lines in eight environments (combination season–location, Table S1). Gold lines represent environment vectors: spa_car_16 (Carmona, Spain 2016), spa_mol_16 (Molino de Pavia, Spain 2016), rom_cog_16 (Cogealac, Romania 2016), spa_car_17 (Carmona, Spain 2017), spa_mol_17 (Molino de Pavia, Spain 2017), rom_cog_17 (Cogealac, Romania 2017), ukr_zap_17 (Zaporozje, Ukraine 2017), and rus_tan_17 (Taganrog, Russia)

the highest discriminatory power, allowing us to identify the most promising lines. After comparing the germplasm responses across all four sites (Fig. S2e), we identified seven lines that had a score of 0 or 1 at all sites and were hence considered to be resistant. Four of these seven lines (namely HA-267, LIV-10, LIV-17 and AB-VL-8) were chosen for further validation and genetic studies. Lines HA-267 and AB-VL-8 were completely resistant at all testing sites, with no signs of infestation. Lines LIV-10 and LIV-17 were completely resistant in all locations apart from Tulcea, Romania, where the broomrape incidence was less than 5%.

Multi-environmental field trials

A total of 20 lines comprising of four resistant IFVCNS lines (HA-267, LIV-10, LIV-17, and AB-VL-8), their F_1 hybrids, and a set of differential lines were subsequently evaluated across eight different environments during the summers of 2016 and 2017 (Table S2). A combined ANOVA detected significant effects for genotype, environment, and genotype × environment interactions (Table 2). When tested at the same site, line performance was consistent over the years, as shown by the proximity of rom_cog_16 (Cogealac, Romania, 2016) and rom_cog_17 vectors (Cogealac, Romania, 2017), as well as spa_mol_16 (Molino de Pavia, Spain, 2016) and spa_mol_17 (Molino de Pavia, Spain, 2017) vectors in the GGE plot (Fig. 2a). Differences among lines in resistance to broomrape were greatest in spa_mol_17 (Molino de Pavia, Spain, 2017) and ukr_zap_17 (Zaporozje, Ukraine, 2017). The broomrape population in environment ukr_zap_17 (Zaporozje, Ukraine, 2017) was also among the most virulent populations, along with the population present at rom_cog_16 (Cogealac, Romania, 2016), as implied by high-average relative incidence (Table S2).

The partitioning of GE interactions showed that PC1 and PC2 accounted for 92% of GGE variation. The IFVCNS inbred lines HA-267, LIV-10, LIV-17, and AB-VL-8 were stably resistant across all environments. Interestingly, their F_1 hybrids were also resistant, unlike the F_1s of these lines with a susceptible control (see below), indicating that the lines share the same resistance locus, possibly with different alleles. Within the GGE biplot, lines were grouped into five distinct clusters (Fig. 2). The first cluster contained six lines with the best overall resistance (average relative incidence in brackets): inbred lines HA-267 (0.4) and AB-VL-8 (1.5), and hybrids that had either HA-267 or AB-VL8 as one of the parents: HA-267/AB-VL-8 (0.2), HA-267/LIV-17 (3.2), HA-267/LIV-10 (4.8), and AB-VL8/LIV-17 (4.8). The hybrid between HA-267 and AB-VL-8, which performed marginally better than both of its parents, was the highest-ranking genotype throughout the trial. The second cluster included lines LIV-10, LIV-17, differential line P-96, and hybrid LIV-10/LIV-17, which was somewhat isolated at the margin of this group; these lines had a lower overall resistance, but were consistently resistant to Spanish populations of broomrape. Line HA-26-OR was completely isolated from the remaining differential lines, which were placed into the fourth (LC231, LC215and LC1093) and fifth clusters (Labud, LC1002, LC1003, LC288, AD66).

Phenotypic evaluation of four mapping populations

The resistant lines HA-267, LIV-10, LIV-17, and AB-VL-8 were completely resistant to the broomrape population LP12BSR under greenhouse conditions (Table 1). Likewise, the respective susceptible lines were completely infested. The F_1 plants from all four crosses were susceptible, indicating that resistance was recessive.

Based on phenotypes of a minimum of 15 plants, we calculated broomrape incidence for each F_3 family. For populations HA-267/OD-DI-82, LIV-10/HA-26-PR, and LIV-17/HA-26-PR, the frequency distribution of broomrape incidence was non-normal (Fig. S3), with more individuals falling in the resistant category as might be expected if a major effect QTL was present.

BSA-seq QTL analysis in four mapping populations

Illumina high-throughput sequencing yielded on average 3.5 M 125-bp paired-end reads per pool. After trimming and filtering, circa 80% of the reads were to non-TE portions of the sunflower reference genome. Additional filtering in "QTLseqr" resulted in 2369, 3193, 2977, and 2999 high-quality SNPs in populations HA-267/OD-DI-82, LIV-10/HA-26-PR, LIV-17/HA-26-PR, and AB-VL-8/L-OS-1, respectively (Table S4).

Calculation of G′ values using the filtered SNP dataset revealed between 2 and 23 significant QTL peaks for the four mapping populations (Fig. 1c). The full intervals of the regions covered by the major G″ peaks varied in length (Table 3), but were generally very wide, especially for *or2.1*, *or3.1*, and *or9.2* in LIV-10/

Table 3 QTLs conferring broomrape resistance in four sunflower inbred lines identified using BSA-seq

Population	Chromosome	QTL name	Start (Mbp)	End (Mbp)	Length (Mbp)	nSNPs	Max G′	Mean G′
HA267/OD-DI-82	1	*or1.1*	137.4390	147.9332	10.4943	4	9.9794	8.8880
	2	*or2.1*	30.2081	30.2081	0.0000	1	8.5506	8.5506
	3	*or3.1*	18.1367	21.4674	3.3307	19	5.3815	5.2356
	3	*or3.2*	110.2989	112.4449	2.1460	2	5.4834	5.3243
	4	*or4.1*	0.2352	0.6465	0.4113	4	5.2372	5.2054
	4	*or4.2*	126.1653	178.2432	52.0779	83	12.8538	8.8441
	5	*or5.1*	27.1069	27.1071	0.0002	3	5.3648	5.3648
	6	*or6.1*	39.1468	51.4086	12.2618	24	6.3454	5.6771
	7	*or7.1*	70.9522	101.2694	30.3172	43	24.8996	19.3359
	10	*or10.1*	23.9920	23.9920	0.0000	3	12.4100	12.4100
	10	*or10.2*	79.1041	232.7784	153.6743	123	12.9612	8.1386
	11	*or11.1*	53.1993	53.4908	0.2915	2	5.2363	5.2098
	11	*or11.2*	158.5780	168.2981	9.7201	6	11.7651	8.8415
	12	*or12.1*	0.3604	92.9588	92.5984	108	23.0043	13.8116
	12	*or12.2*	157.2201	164.5472	7.3271	6	11.0574	10.5332
	13	*or13.1*	4.0888	6.8097	2.7209	14	6.1122	5.8256
	13	*or13.2*	174.7897	174.7901	0.0004	11	6.7145	6.7144
	14	*or14.1*	86.4114	98.9386	12.5272	7	9.5599	8.2384
	15	*or15.1*	2.5736	4.2684	1.6948	12	5.4267	5.3757
	16	*or16.1*	122.7897	122.7897	0.0000	2	5.8055	5.8055
LIV-10/HA-26-PR	1	*or1.1*	2.2504	14.3038	12.0533	5	21.4839	11.6030
	1	*or1.2*	76.1307	90.1387	14.0080	12	6.6204	6.2625
	1	*or1.3*	117.7858	153.7932	36.0074	56	14.3520	8.5447
	2	*or2.1*	12.6177	142.2760	129.6582	49	16.6965	9.1645
	2	*or2.2*	150.9047	178.9931	28.0885	32	5.8058	5.2061
	3	*or3.1*	2.4917	140.4343	137.9426	137	27.3068	13.0140
	4	*or4.1*	15.8381	34.1895	18.3514	21	8.5755	8.1492
	5	*or5.1*	45.2947	50.8344	5.5397	5	8.6526	7.4514
	9	*or9.1*	1.1200	92.9390	91.8191	56	20.6037	13.5042
	9	*or9.2*	107.6887	209.0578	101.3691	136	13.7825	8.1161
	10	*or10.1*	131.6170	151.2675	19.6505	10	5.5354	5.2356
	10	*or10.2*	232.7784	245.6824	12.9040	36	5.8145	5.3629
	11	*or11.1*	0.2374	3.0316	2.7942	5	4.7694	4.6926
	12	*or12.1*	0.3642	0.3642	0.0000	2	4.2183	4.2183
	12	*or12.2*	155.8697	157.5659	1.6961	4	6.2279	6.2241
	14	*or14.1*	6.2787	19.3705	13.0918	9	7.2344	5.8016
	15	*or15.1*	26.3119	97.8220	71.5101	32	16.8060	10.8367
	15	*or15.2*	134.9130	170.6236	35.7106	18	9.4016	7.6220
	16	*or16.1*	180.4239	188.2721	7.8482	20	4.9512	4.7805
	17	*or17.1*	71.5983	79.4670	7.8686	16	4.8856	4.5936
	17	*or17.2*	130.2588	154.2528	23.9940	19	7.8032	6.8339
	17	*or17.3*	162.1939	194.9531	32.7593	42	7.8122	6.7508
LIV-17/HA-26-PR	3	*or3.1*	31.9755	38.4842	6.5087	4	14.1074	13.9186
	3	*or3.2*	97.1338	127.0655	29.9317	64	21.0583	16.3909
	5	*or5.1*	81.6061	81.6062	0.0001	2	12.8486	12.8486
	17	*or17.1*	90.7412	90.7412	0.0000	1	15.0826	15.0826
AB-VL-8/L-OS-1	3	*or3.1*	27.5752	100.8548	73.2796	79	50.1673	35.5400
	13	*or13.1*	62.1317	76.1960	14.0643	14	29.0140	26.3350

HA-26-PR and *or10.2* in HA-267/OD-DI-82, which exceeded 100 Mb in length. However, as suggested by Win et al. (2017), the causative genes underlying QTL probably reside in genomic regions with the highest G′ values. In some instances, only a single SNP was found in a particular genomic region, as was the case with *or17.1* and *or2*.1. Nonetheless, the G′ values of these peaks were well above threshold, indicating a significant association between these genomic regions and resistance to broomrape.

Resistance in HA-267/OD-DI-82 appeared to be controlled by 20 QTLs dispersed across all chromosomes except 8, 9, and 17. The QTLs with the largest effect sizes were *or7.1* and *or12.1*, followed by *or10.1*, *or10.2*, *or12.2*, *or1.1*, *or4.2*, and *or11.2*.

Resistance in LIV-10/HA-26-PR was controlled by 23 QTLs, which mapped to a total of 13 chromosomes, seven of which had multiple QTLs. The peak corresponding to QTL *or3.1* had the highest G′ value, followed by QTL peaks for *or1.1*, *or9.2*, *or15.1*, *or2.1*, *or1.3*, and *or9.1*. These G′ peaks greatly exceeded the threshold value and represent putative major QTLs. Minor QTLs were distributed throughout the genome, with exception of chromosomes 6, 7, 8, and 13.

Four significant QTLs were segregating in population LIV-17/HA-26-PR. Two distinct QTLs mapped to chromosome 3, whereas the remaining two QTLs were placed on chromosomes 5 and 17.

For AB-VL-8/L-OS-1 GBS, a major QTL was detected on chromosome 3 *(or3.1)*, which is consistent with the results obtained by SSR mapping (Imerovski et al. 2016). An additional QTL on chromosome 13 was also identified. The G′ value suggests that QTL *or13.1* was of less magnitude than *or3.1*.

The common theme across all populations was the presence of one or more QTL(s) on chromosome 3, which were especially large in populations LIV-17/HA-26-PR, LIV-10/HA-26-PR, and AB-VL-8/L-OS-1. In LIV-17/HA-26-PR, two highly significant peaks were detected on chromosome 3, the first of which mapped between 31.97 and 38.48 Mb, and the second between 97.13 and 127.06 Mb. For LIV-10/HA-26-PR and AB-VL-8/L-OS-1, unusually wide QTLs were observed in the same region, which might in fact be due to overlap between two closely linked QTLs. With the exception of HA-267/OD-DI-82, the mapping populations had two common QTLs on chromosome 3 — one that stretched from 31.97 to 38.48 Mb and other from 97.13 to 100.85 Mb. Though two QTLs were also mapped in HA-267/OD-DI-82 in close proximity to this region, they were less significant than QTLs *or4*.2, *or7.1*, *or10.1*, *or10.2*, *or11.2*, *or12.1*, and *or12.2*, and did not span the overlapping region that was identified in LIV-17/HA-26-PR, LIV-10/HA-26-PR, and AB-VL-8/L-OS-1. This could be because the QTLs on chromosome 3 were partially masked by the QTLs with much higher peaks. It can therefore be speculated that the overlapping regions underlying QTLs on chromosome 3 represent the most probable location of shared loci providing broomrape resistance in all four lines.

Confirmation of QTL *or3.1* with CAPS marker genotyping

For populations that showed a highly significant G′ peak between 97.13 and 100.85 Mb on chromosome 3 (i.e., LIV-10/HA-26-PR, LIV-17/HA-26-PR, and AB-VL-8/L-OS-1), one SNP per population was converted into a CAPS marker (Table S5). These markers were used for genotyping of the three F_2 populations, with the aim of confirming the QTL that was identified by BSA-seq. There was a statistically significant difference in mean incidence scores between the genotypic classes, i.e., homozygous for resistant parent allele (RR), heterozygous (RS), and homozygous for susceptible parent allele (SS) as illustrated in Fig. 1e and determined by one-way ANOVA [$F_{\text{LIV-10}}$ (2, 106) = 8.8, $p < 0.01$; $F_{\text{LIV-17}}$ (2, 89) = 61.37, $p < 0.01$; $F_{\text{AB-VL-8}}$ (2, 96) = 28.86, $p < 0.01$]. In populations LIV-17/HA-26-PR and AB-VL-8/L-OS-1, post hoc comparisons using the Tukey–Kramer HSD showed that the mean of each genotypic group was significantly different from each other at $p < 0.01$, as indicated by the letters above boxplots. In LIV-10, a significant difference was detected between homozygous susceptible genotypes and other categories, whereas the difference between heterozygotes and resistant homozygous was not statistically significant.

Candidate genes underlying major QTLs on chromosome 3

We further focused on regions of chromosome 3 where QTLs within LIV-10/HA-26-PR, LIV-17/HA-26-PR, and AB-VL-9/L-OS-1 were overlapping, as these represent the most probable genomic regions where the shared locus providing resistance to all four lines would reside. Though HA-267/OD-DI-82 showed two distinct QTLs in a similar region, the area underneath the peaks

was narrow and did not coincide with the QTLs from the other populations, so we excluded it at this point. Exploration of the 6.5-Mb region, the genomic region between 31.9 to 38.48 Mb of chromosome 3, revealed 123 genes, including genes *HanXRQChr03g0065701* (disease resistance protein RPS2-like) and *HanXRQChr03g0065841* (TMV resistance protein N-like) (www.heliagene.org). In the 3.72 Mb overlapping region between 97.13 and 100.85 Mb, 71 genes were revealed, including a putative defense gene *HanXRQChr03g0076321*.

Discussion

Plant parasites are estimated to cause circa US $1 billion in yield losses and to negatively impact the food supply of > 100 million people annually (Gressel et al. 2004; Yoder and Scholes 2010). For sunflower, the extent of yield losses caused by the parasitic plant *O. cumana* (broomrape) depends on both the virulence of the local parasitic population and the susceptibility of the cultivar. Potential losses are greatest in regions with extensive sunflower production, such as Ukraine, Russia, and Romania. While chemical methods for broomrape management are available, high costs limit their application. Hence, growing resistant sunflower varieties remains the most effective and economically and environmentally desirable means of suppressing sunflower broomrape.

Unlike success stories from wheat (Krattinger et al. 2009) and barley (Kleinhofs et al. 2009), where genetic resistance to fungal diseases has been stabile for more than six decades, sunflower resistance to broomrape is much less durable (Alonso et al. 1996; Kaya et al. 2004; Fernández-Martínez et al. 2012; Antonova 2014). Thus, breeding for resistance to *O. cumana* requires constant effort. Extensive previous work has generated a pool of sunflower genotypes that are resistant to broomrape (Sukno et al. 1999; Fernández-Martínez et al. 2000; Kaya et al. 2004; Pérez-Vich et al. 2004b; Christov et al. 2009; Joiţa et al. 2009; Păcureanu et al. 2009; Jocić et al. 2016; Jocković et al. 2018). However, the spectrum of resistance afforded by these different sources is less clear. Because biotypes of *O. cumana* classified as the same race can vary in virulence — for example, race F from Spain appears to be quite different from race F in Romania (Fernández-Martínez et al. 2012; Molinero-Ruiz et al. 2015) — systematic multi-environmental testing of available germplasm is extremely important for identifying genetic sources that offer broad-spectrum resistance.

In the present study, we report on four inbred lines that showed high levels of resistance in all testing sites. Lines AB-VL-8, HA-267, and their hybrid were most resistant and stable across all environments, with an average relative incidence of < 2%. Line HA-267 was particularly important, as it exhibited complete resistance to all broomrape populations, apart from the most virulent population from Zaporozje, Ukraine, where relative broomrape incidence of 3% was recorded. These lines are therefore of high value for developing hybrids that can be grown in all sunflower-producing areas, including the Black sea region, where the most virulent biotypes have been identified (Păcureanu et al. 2009; Antonova 2014). Lines LIV-10 and LIV-17 were slightly less resistant overall but consistently fended off broomrape in Spain, where line P96 is losing effectiveness (Martín-Sanz et al. 2016). Multi-environment testing not only revealed significant genotypic effects, but also significant effects of the environment and genotype × environment interactions, which was expected due to observable differences in virulence of different races. Ukraine (Zaporozje) and Spain (Molino de Pavia) were identified as the most discriminating environments; hence, these locations will be valuable for selecting resistant genotypes in the future.

Major QTL(s) on chromosome 3 confer resistance to broomrape race G

Building on advances in high-throughput sequencing, numerous genetic mapping strategies have become available that permit rapid identification of large-effect QTLs (Nelson et al. 2018). Yet, despite continued price reductions (Wetterstrand 2018), sequencing costs remain substantial, and the cost of genotyping of numerous individuals is still a limiting factor in many mapping studies. An approach that combines next-generation sequencing technologies with selective genotyping (also referred to as BSA-seq) is a more affordable alternative; it relies on an experimental design proposed by Lander and Botstein (1989), in which individuals from high and low ends of the phenotypic distribution are used for QTL mapping rather than the entire mapping population. In addition to being more budget-friendly, mapping based on selective genotyping is less sensitive to occasional phenotyping mistakes (i.e., “escapes”)

(Schneeberger and Weigel 2011), which makes it a suitable choice for mapping disease resistance. An increasing number of studies suggest that selective genotyping is equivalent or greater in power than conventional QTL mapping (Takagi et al. 2013; Yang et al. 2013; Lambel et al. 2014; Win et al. 2017). Though using larger populations minimizes false positive and false negative results and maximizes detection of minor QTLs, populations of manageable size (e.g., 100–200 individuals) are still sufficient for tagging QTLs of large effect and identifying new DNA markers for regions of the genome shown to contain QTLs (Magwene et al. 2011).

Here, we genotyped pools of samples using GBS and employed a BSA-seq approach to identify QTLs conferring resistance to broomrape in sunflower. The number of identified QTLs ranged from 2 to 23. The common contributing component to resistance in all of the lines was one or more QTLs on chromosome 3, suggesting the existence of the same locus in all tested lines. This conclusion was further supported by the fact that F_1s obtained by mutual crossing of HA-267, LIV-10, LIV-17, and AB-VL-8 were resistant, despite evidence that each resistance locus was recessive. Furthermore, our results are consistent with the presence of two distinct QTLs on chromosome 3. Within *or3.2*, the overlapping region shared between the mapping populations LIV-10/HA-26-PR, LIV-17/HA-26-PR, and AB-VL-8/L-OS-1 stretched from 97.13 to 100.85 Mb. The magnitude of the G′ peak suggested that the effect of this QTL on broomrape resistance was especially large in crosses involving AB-VL-8 and LIV-17. Genotyping of the entire F_2 population using newly-developed CAPS markers from this region validated this hypothesis and demonstrated the utility of the newly developed markers, which will be employed for introgressing resistance QTLs into elite inbred lines via marker-assisted selection. In HA-267, which showed the highest overall broomrape resistance in the multi-environmental trials but the lowest G′ value on chromosome 3, resistance was most likely more influenced by the QTLs located on chromosomes 4, 7, 10, and 12. Alternatively, since major QTLs may interfere with the detection of other QTLs with smaller effects (Li et al. 1997), the QTLs on chromosomes 4, 7, 10, and 12 might have diminished the magnitude of the G′ peak on chromosome 3 in this population.

Previous studies on sunflower resistance to broomrape also reported existence of a resistance locus in the distal region of chromosome 3 (Tang et al. 2003; Pérez-Vich et al. 2004a), which conferred dominant resistance to race E, but only partial resistance to race F. Possibly, *or3.1* in lines LIV-10, LIV-17, HA-267, and AB-VL-8 is in fact the previously mapped *Or5*, which now acts as a "defeated *R* gene". This gene provides only a moderate level of resistance, whereas QTL *or3*.2 (and QTLs on other chromosomes) are required for resistance to races higher than E. The existence of "defeated *R* genes" has previously been reported for rice bacterial blight disease resistance gene *Xa4* which acted as a dominant resistance gene against two strains of *Xanthomonas oryza*e pv. *Oryzae* (CR4 and CXO8). However, when challenged with strain CR6, the same gene acted as a recessive QTL accounting for 50% of resistance (Li et al. 1999). The same study showed that most resistance QTLs mapped to genomic locations harboring previously mapped bacterial blight resistance genes and/or QTLs, suggesting that high levels of resistance can be achieved by pyramiding multiple QTLs, including "defeated" major resistance genes. Other "defeated *R* genes" that may contribute to quantitative disease resistance include those for wheat-powdery mildew (Nass et al. 1981), wheat-yellow rust (Danial et al. 1994), potato-late blight (Stewart et al. 2003), and wheat-stem rust (Zhang et al. 2006). Together, these results suggest that the classification of resistance as either vertical (qualitative) or horizontal (quantitative) is often oversimplified and, in reality, a continuum of scenarios can exist (Nelson et al. 2018).

Candidate genes

The overlapping regions within the major QTLs *or3.1* and *or3.2* contained 123 and 71 genes, respectively, several of which represent promising candidates for future investigation. The presence of disease resistance genes *HanXRQChr03g0065701* and *HanXRQChr03g0065841* in the *or3.1* genomic region and *HanXRQChr03g0076321* in the *or3.2* region will require fine mapping to determine if one of more of them are the cause(s) of resistance to broomrape. Disease resistance genes typically encode proteins that detect microbial pathogens (Tameling and Joosten 2007) through direct or indirect recognition of pathogen-derived effectors (Caplan et al. 2008). However, Li and Timko (2009) showed that effector-based immunity is also involved in responses to parasitic angiosperms. In their study, resistance of cowpea to the parasitic plant

Striga gesnerioides was conferred by *RSG3-301*, a *CC-NBS-LRR* gene. Therefore, it is possible that *R* genes are involved in sunflower resistance to broomrape as well. Further investigations of the disease *R* genes that were identified within *or3.1* and *or3.2* could bring us a step closer to understanding this type of plant-plant interaction.

The pursuit of durable resistance to broomrape

Monogenic resistance is straightforward to introduce into elite germplasm and thus more commonly deployed by breeders. However, resistance can also be achieved via the pyramiding of multiple genes, often with different resistance mechanisms. Such multigenic resistance is likely to be more durable because the parasite has to overcome a variety of defensive mechanisms to successfully infect the host plant (Parlevliet 2002; Zhang et al. 2006). Thus, combining QTLs from HA-267, LIV-10, LIV-17, and AB-VL-8 represents a promising strategy for achieving resistance that will remain effective over a long period of time and across different cultivation areas. The new resistant lines reported in this study, along with markers closely linked to a major QTL on chromosome 3, will enhance breeding schemes using MAS for incorporation of durable resistance to broomrape in sunflower cultivars.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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