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ORIGINAL PAPER

Fine mapping of the sunflower resistance locus Pl_{ARG} introduced from the wild species *Helianthus argophyllus*

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Abstract Downy mildew, caused by *Plasmopara halstedii*, is one of the most destructive diseases in cultivated sunflower (*Helianthus annuus* L.). The dominant resistance locus Pl_{ARG} originates from silverleaf sunflower (*H. argophyllus* Torrey and Gray) and confers resistance to all known races of *P. halstedii*. We mapped Pl_{ARG} on linkage group (LG) 1 of (cms)HA342 × ARG1575-2, a population consisting of 2,145 F_2 individuals. Further, we identified resistance gene candidates (RGCs) that cosegregated with Pl_{ARG} as well as closely linked flanking markers. Markers from the target region were mapped with higher resolution in $NDBLOS_{sel}$ × KWS04, a population consisting of 2,780 F_2 individuals that does not segregate for Pl_{ARG}. A large-insert sunflower bacterial artificial chromosome (BAC) library was screened with overgo probes designed for markers RGC52 and RGC151, which cosegregated with Pl_{ARG}. Two RGC-containing BAC contigs were anchored to the Pl_{ARG} region on LG 1.

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the major oilseed crops cultivated worldwide in 25 mio. ha (FAOSTAT 2008). *Plasmopara halstedii* (Farl.) Berl. & de Toni is a soil-, seed- and wind-borne pathogen causing downy mildew in sunflower, which can survive up to 10 years in the soil in the form of oospores (EPPO/CABI 1997). Downy mildew is a common sunflower disease responsible for significant yield loss and can be controlled by fungicides and cultivation of resistant hybrids. Several *Pl* loci (Pl_1–Pl_{13}, Pl_{ARG}, Pl_v–Pl_z) have been described, which confer resistance to one or more races of *P. halstedii* (Dußle et al. 2004; Miller and Gulya 1991; Molinero-Ruiz et al. 2002b, 2003; Mulpuri et al. 2009; Rahim et al. 2002; Vranceanu and Stoenescu 1970; Vranceanu et al. 1981; Zimmer and Kinman 1972). The *Pl* loci were discovered in wild *H. annuus* ecotypes, as in the case of Pl_6 (Miller and Gulya 1991), or were introduced from other wild *Helianthus* species. Pl_5 was introgressed from *H. tuberosus* (Vranceanu et al. 1981) and Pl_7 from *H. praecox* (Miller and Gulya 1991), while Pl_8 and Pl_{ARG} both originated from *H. argophyllus* (Miller and Gulya 1991; Seiler et al. 1991). Two types of resistance are known: *P. halstedii* is limited to the roots in seedlings with type I resistance but in seedlings with type II resistance it grows through the hypocotyls and

a slight sporulation can be observed on the hypocotyls (Mouzeyar et al. 1994). Sackston (1990) called the phenomenon observed in type II resistance "cotyledon limited infection" (CLI).

In the last decade, new races of *P. halstedii* were discovered in the cultivation areas of sunflower (Gulya et al. 1991; Molinero-Ruiz et al. 2002a; Tourvieille de Labrouhe et al. 2000). Delmotte et al. (2008) investigated 24 isolates collected in France between 1966 and 2006 and concluded that the biology of *P. halstedii*, the multiple introductions of the pathogen into France and the selection pressure caused by the use of host resistance genes may have caused the spread of new races of *P. halstedii*. Gulya (2007) reported the existence of at least 35 pathotypes. Several races developed tolerance to metalaxyl and mefenoxam, the only effective fungicides available (Albourie et al. 1998; Molinero-Ruiz et al. 2005; Molinero-Ruiz and Melero-Vara 2003; Spring et al. 2006). Therefore, investigation of the structure and functionality of *Pl* loci is necessary in order to exploit them effectively and durably in plant breeding.

Pl loci were initially considered single independent genes (Miller and Gulya 1991), but genetic mapping and segregation studies indicated that some loci consist of clusters of resistance genes with different specificities (Vear et al. 1997). Mouzeyar et al. (1995) mapped the first *Pl* locus (Pl_1) on group 1 of the CARTISOL map (Gentzbittel et al. 1995), which corresponds to LG 8 of the publicly available simple sequence repeat (SSR) map constructed by Tang et al. (2002). Roeckel-Drevet et al. (1996) and Vear et al. (1997) mapped Pl_2 and Pl_6, respectively, and found that both loci are located in the same genomic region as Pl_1. Similarly, it was shown that LG 13 carries Pl_5 and Pl_8 (Bert et al. 2001; Radwan et al. 2003). Dußle et al. (2004) showed that Pl_{ARG} is located on LG 1 and is unlinked to all previously mapped *Pl* loci. Pl_{ARG} was introgressed from *H. argophyllus* and mediates resistance to all known races of *P. halstedii* (Seiler et al. 1991, G. Seiler, personal communication). Recently, Mulpuri et al. (2009) mapped Pl_{13} on LG 1, but it is unlinked to Pl_{ARG} and has its position at the lower end of LG 1.

Many plant disease resistance (*R*) genes have been discovered and cloned in the recent years. Five major classes of *R*-genes are known, the largest of which is the NBS-LRR class, consisting of the TIR-NBS-LRR and non-TIR-NBS-LRR subclasses (Dangl and Jones 2001). Most of the known *R*-genes form tightly linked gene families (Hulbert et al. 2001).

Knowing that the resistance genes Pl_1, Pl_2, Pl_6, Pl_7 on LG 8 (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997) and Pl_5, Pl_8 on LG 13 (Bert et al. 2001; Radwan et al. 2003) are clustered, we may as well assume that Pl_{ARG} might also coincide with a cluster of resistance genes. Our study aims at the fine mapping and map-based cloning of the Pl_{ARG} locus. Our objectives were: (1) to identify markers flanking Pl_{ARG} and find recombinants in the target region, (2) to carry out phenotypic analyses with different races of *P. halstedii* and elucidate the genetic structure of Pl_{ARG}, (3) to define markers as starting points for a map-based cloning approach, (4) to select bacterial artificial chromosome (BACs) for sequencing to identify candidate genes for Pl_{ARG}, and (5) to investigate whether Pl_{ARG} is a single gene or a complex resistance gene cluster.

Materials and methods

Plant material

In the present study, we used five resistant inbred lines: ARG1575-2, RHA419, RHA420, RHA443 and 79ARGMTP. ARG1575-2 is a homozygous resistant inbred line that carries the Pl_{ARG} locus and mediates resistance to all known races of *P. halstedii* (Seiler et al. 1991). To our knowledge, there is no report about virulent races of *P. halstedii* that overcome the Pl_{ARG} resistance. ARG1575-2 was derived by crossing *H. argophyllus* accession 1575 (PI 468651) with cmsHA89 followed by two generations of backcrossing with cmsHA89 and five selfing generations (Seiler et al. 1991). The homozygous resistant inbred lines RHA419, RHA420 and RHA443 carry Pl_{ARG} and are all derived from ARG1575-2. RHA419 and RHA420 were derived from the cross RHA373 × ARG1575-2 (Miller et al. 2002), whereas RHA443 is an $F_{6:7}$ restorer line selected from the cross RHA426/RHA419//RHA377/AS4379 (Miller et al. 2006). 79ARGMTP carries a downy mildew resistance on LG 1, which is likely to be Pl_{ARG}, and was developed from the cross of *H. argophyllus* MPHE-92 with *H. annuus* FS20-6-2 at INRA, Montpellier (Vear et al. 2008a).

Three susceptible inbred lines, HA342, $NDBLOS_{sel}$ and KWS04 were used in this study. The susceptible inbred line HA342 was available both in the normal as well as in the PET1 cytoplasmic male sterility (CMS) cytoplasm. HA342 is derived from a single BC_1F_4 plant from the cross HA89*2/Pervenets and has high oleic seed content (Miller et al. 1987). $NDBLOS_{sel}$ is an inbred line selected from the germplasm pool ND-BLOS (Roath et al. 1987) and was used for QTL mapping of *Sclerotinia* midstalk rot resistance (Micic et al. 2005). KWS04 is proprietary line of KWS SAAT AG (Einbeck, Germany) and is used for the development of hybrid varieties.

Development of mapping populations

To fine map the target Pl_{ARG} region on LG 1, we developed a segregating population from the cross of (cms)HA342

and ARG1575-2, which comprised of two subpopulations with a total of 2,145 F_2 individuals. The subpopulation cmsHA342 × ARG1575-2 consisted of 1,065 F_2 individuals, whereas the subpopulation HA342 × ARG1575-2 consisted of 1,080 F_2 individuals. In this paper (cms)HA342 × ARG1575-2 refers to the whole population of 2,145 F_2 individuals. We also developed population $NDBLOS_{sel}$ × KWS04 comprising of 2,780 F_2 individuals to increase the mapping resolution of the targeted genomic region. $NDBLOS_{sel}$ and KWS04 are both highly polymorphic lines and susceptible to *P. halstedii*; therefore $NDBLOS_{sel}$ × KWS04 did not segregate for Pl_{ARG}. DNA was isolated from dried leaves of each F_2 plant and parental lines using the CTAB extraction method (Doyle and Doyle 1990).

Resistance tests

Initially, we evaluated resistance to downy mildew in a subset of 183 $F_{2:3}$ families of the subpopulation cmsHA342 × ARG1575-2 using the whole seedling immersion test (Gulya 1996) with a suspension of *P. halstedii* race 730 at a concentration of 40,000 spores/ml. Next, we evaluated only informative lines which were selected as described below. The resistance of F_2 plants was investigated by testing 16–40 F_3 seedlings per F_2 individual. Seedlings were considered susceptible when high fungal sporulation was evident on the cotyledons and resistant when no sporulation or only spurious sporulation was observed on the cotyledons. Because of the occurrence of CLI in plants which carry the resistance gene Pl_{ARG}, progenies with ambiguous phenotype were re-tested using 2–10 $F_{3:4}$ families. F_2 plants were classified as homozygous susceptible, homozygous resistant or heterozygous according to the phenotypes of F_3 or F_4 families and the goodness-of-fit of observed segregation ratios was tested.

Linkage mapping analyses

We developed an anchor map of LG 1 for 475 F_2 individuals of cmsHA342 × ARG1575-2 using seven polymorphic codominant SSR markers of the public sunflower linkage map (Tang et al. 2003; Tang et al. 2002), which were identified by Dußle et al. (2004). To increase the density of makers in the target Pl_{ARG} region, 22 additional markers were screened for polymorphism among (cms)HA342, ARG1575-2, $NDBLOS_{sel}$ and KWS04. Thirteen SSR markers (Tang et al. 2002, 2003; Yu et al. 2003), six single nucleotide polymorphism (SNP) markers (Lai et al. 2005a) and three resistance gene candidates (RGCs) (Radwan et al. 2008) were analyzed using a LI-COR DNA-Analyzer 4300 (LI-COR Biosciences, Bad Homburg, Germany) or the single strand conformation polymorphism (SSCP) method (Slabaugh et al. 1997). HT211 and RGC151 were converted into cleaved amplified polymorphic sequence (CAPS) markers and were resolved with 3.0% agarose gels after digesting with *Taq*I and *Rsa*I, respectively.

Overall, 19 SSR, SNP, CAPS and RGC markers were used for linkage analysis of (cms)HA342 × ARG1575-2, and 10 SSR, SNP and CAPS markers were used to construct the linkage map of $NDBLOS_{sel}$ × KWS04. To investigate the portion of *H. argophyllus* genome in the resistant inbred line ARG1575-2 marker scores of ARG1575-2 and cmsHA89 were compared with 14 codominant SSR and two CAPS loci of LG 1, and 94 randomly distributed SSR markers on the rest of the genome (LG 2–LG 17). We also compared the haplotypes of the resistant lines ARG1575-2, RHA419, RHA420, RHA443 and 79ARGMTP to estimate the size of the introgressed segment of *H. argophyllus* using the same set of 14 codominant SSR and two CAPS markers of LG 1.

The goodness-of-fit test was performed for the H_o hypothesis to observe a segregation ratio of 1:2:1 for all polymorphic markers under study in the mapping populations. Maps for the populations (cms)HA342 × ARG1575-2 and $NDBLOS_{sel}$ × KWS04 were constructed with JOINMAP 4.0 (Van Ooijen 2006) using a LOD threshold of >3.0 and the Kosambi mapping function (Kosambi 1944).

Selecting informative lines carrying recombination events in the target region

We used the strategy outlined in Fig. 1 to identify recombination events in the region of Pl_{ARG}. Only recombinant lines were phenotypically tested for fine mapping. Briefly, 2,145 F_2 individuals of (cms)HA342 × ARG1575-2 were genotyped with ORS610 and ORS371, which flank Pl_{ARG}, and with ORS662, which cosegregated with Pl_{ARG}. 188 recombinant F_2 individuals were analyzed with 16 additional polymorphic markers to increase the marker density in the target region, while $F_{2:3}$ or $F_{2:4}$ progenies of 108 recombinant F_2 individuals were subsequently phenotyped to confirm the genomic location of Pl_{ARG}. Next, ORS509, HT244 and HT446 were used to narrow down the interval harboring Pl_{ARG}. We evaluated 25 homozygous F_4 seedlings for each F_2 individual that had a recombination event in the interval of ORS509, HT244 and HT446 for resistance to *P. halstedii* races 330, 100, 710 and 730 (Fig. 1).

BAC library screening and mapping of BAC end sequences

Overgo probes (Han et al. 2000; Zheng et al. 2006) were designed from the sequence of RGC52 and RGC151, which cosegregated with Pl_{ARG}. The probes were used for high-density filter hybridization to the 8× genome coverage large-insert sunflower BAC library, HA_HBa,

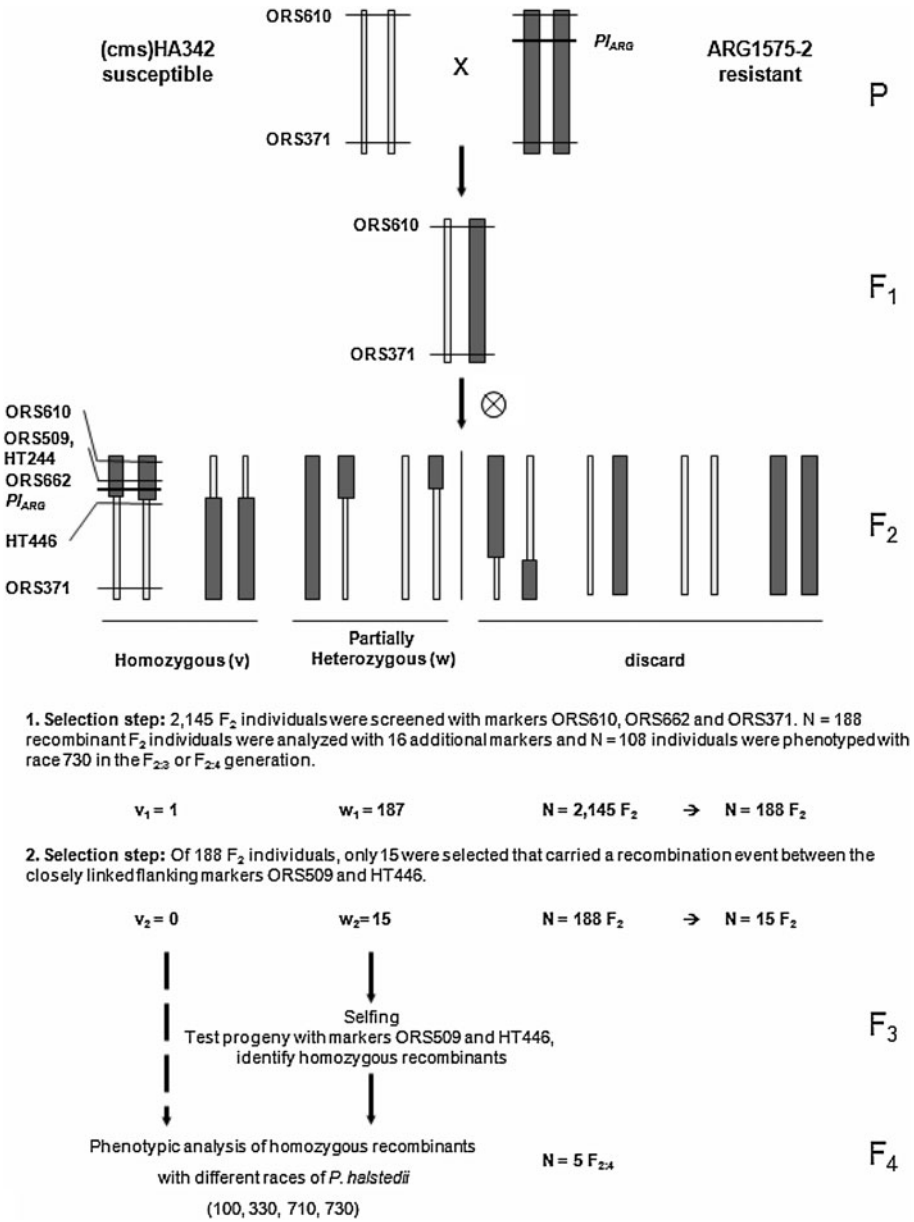

Fig. 1 High resolution mapping strategy for the identification of informative recombinant lines in cross (cms)HA342 × ARG1575-2. The F_2 population consisted of 2,145 lines and was screened in two steps. First, the whole population was screened with markers ORS610, ORS662 and ORS371 and 188 recombinant lines were selected and, subsequently, genotyped and phenotyped. Second, lines with recombination events between the closely linked flanking markers ORS509, HT244 and HT446 were selfed and homozygous recombinant lines were tested with the four *P. halstedii* races 730, 100, 330, and 710

constructed from HA383 and publicly available through the Clemson University Genomics Institute (CUGI, http://www.genome.clemson.edu). Positive BAC clones were confirmed by colony PCR and plasmids were isolated using the alkaline lysis method (Birnboim and Doly 1979). Plasmid DNA was used for high information content fingerprinting (HICF) (Ding et al. 1999; Luo et al. 2003) and BAC end sequencing (BES). BAC contigs were assembled using the FPC software v.8.5.3 (Soderlund et al. 1997). Inserts of positive BAC clones were sequenced from both ends using the T7 and M13 sequencing primers. Primers were designed from BAC end sequences with Primer3 (Rozen and Skaletsky 2000). To map the assembled BAC contigs in (cms)HA342 × ARG1575-2 and $NDBLOS_{sel}$ × KWS04, the polymorphic markers Co3-4_T7, Co3-2_T7 and Co7-11_M13 were analyzed which were designed from BES P202H01_T7, BES P0323D15_T7 and BES P399D04_M13 (Supplementary Table 1). PCR was carried out in a final volume of 10 µl using 275 nM of each primer (Table 1). In the presence of 1× Taq polymerase buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP and 0.4 U Taq polymerase (Q-Biogene, Illkirch, Germany) PCR was performed under the following conditions: intitial denaturation at 95°C for 5 min, 35 PCR cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1:15 min and an extension at 72°C for 10 min. The PCR products of Co3-4_T7 and Co7-11_M13 were analyzed on 1.5% agarose gels and products of Co3-2_T7 were analyzed on a SSCP gel.

Table 1 Sequences of forward and reverse primers used for anchoring BAC contigs to the sunflower linkage map

Marker name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Co3-2_T7	TCTCACTTTCACCGCACAGT	TGAGCCTTTTCTCAGCATCA
Co3-4_T7	TTTACCGCACGAAAAGGAAT	TCAATTAAAAATGCAAAATAACCA
Co7_11_M13	TTGACCCGCACACACTTTACAT	GTGCAGCTTGTCAGACTTCTTTG

Table 2 Segregation ratios and X^2 values of the codominant SSR markers ORS610, ORS662, ORS053, and ORS371 analyzed in three sunflower populations

	Population		
	cmsHA342 × ARG1575-2 ($N = 1{,}065$)	HA342 × ARG1575-2 ($N = 1{,}080$)	$NDBLOS_{sel}$ × KWS04 ($N = 528$)
ORS610	272:587:206[a]	272:561:247	143:253:131
$X^2_{DF=2}$	19.34 ($p = 0.0001$)	2.79 ($p = 0.2478$)	1.38 ($p = 0.5016$)
ORS662	271:585:209	270:561:249	131:269:128
$X^2_{DF=2}$	17.57 ($p = 0.0002$)	2.45 ($p = 0.2938$)	0.22 ($p = 0.8958$)
ORS053	rec. F_2.[b]	263:569:248	130:266:132
$X^2_{DF=2}$		3.53 ($p = 0.1712$)	0.05 ($p = 0.9753$)
ORS371	272:582:204	268:559:244	Not analyzed
$X^2_{DF=2}$	19.36 ($p = 0.0001$)	3.13 ($p = 0.2091$)	

[a] Numbers are given for the marker genotypes (homozygous parent 1 allele:heterozygous:homozygous parent 2 allele)

[b] Only recombinant F_2 individuals were analyzed

Results

Localization of Pl_{ARG} on LG 1

An anchor linkage map of cmsHA342 × ARG1575-2 was constructed using seven polymorphic SSR markers. ORS1182, ORS610, ORS1128 and ORS543 cosegregated at the upper end of LG 1, whereas ORS662, ORS959 and ORS371 mapped 0.5, 3.6 and 5.2 cM from the upper end, respectively. $F_{2:3}$ or $F_{2:4}$ families of 183 F_2 individuals that produced sufficient seeds were evaluated for resistance to downy mildew. We observed 25 homozygous resistant, 114 segregating, and 44 homozygous susceptible progenies, which differs significantly from the expected 1:2:1 Mendelian segregation ratio of Pl_{ARG} ($X^2 = 15.01$, DF = 2, $p \leq 0.0006$). Pl_{ARG} cosegregated with ORS662 (map not shown).

Fine mapping of the target region

Population (cms)HA342 × ARG1575-2

To increase the genetic resolution around Pl_{ARG} we genotyped 2,145 F_2 individuals of (cms)HA342 × ARG1575-2 as described in Fig. 1. ORS610 and ORS371 mapped at the upper and lower end of LG 1, respectively, and flank Pl_{ARG}, which cosegregates with ORS662. The three markers were used to screen 2,145 F_2 individuals. All markers showed significant segregation distortion with a lack of the ARG1575-2 allele in the subpopulation cmsHA342 × ARG1575-2, but did not deviate from the expected 1:2:1 segregation ratio in HA342 × ARG1575-2 (Table 2).

In total, we identified 188 F_2 individuals that carried a recombination event between the two flanking markers ORS610 and ORS371. The F_2 individuals were phenotyped and genotyped with 16 polymorphic markers between ORS610 and ORS371. To confirm the genomic position of Pl_{ARG}, we tested $F_{2:3}$ or $F_{2:4}$ progenies of 108 out of the 188 recombinant F_2 individuals (Fig. 2a). The remaining 80 recombinant F_2 individuals could not be tested due to lack of sufficient seed especially for cmsHA342 × ARG1575-2 (Supplementary Fig. 1).

The genetic map of LG 1 in (cms)HA342 × ARG1575-2 spanned 4.2 cM (Fig. 2a). ORS610, ORS543, ORS1128, CRT272, and ORS1182 cosegregated at the upper end of LG 1, whereas Pl_{ARG} cosegregated with ORS662, HT211, RGC52a, RGC52b, RGC151, HT722, and ORS716 0.3 cM from the upper end. ORS509, HT244 on one side and HT446 on the other side flanked Pl_{ARG} at a distance of 0.2 cM and 0.1 cM, respectively. More recombination events were observed below Pl_{ARG} and ORS053, ORS959 and ORS371 mapped 1.9, 2.3, and 3.9 cM from Pl_{ARG}, respectively. Thus, we identified closely linked markers on both sides of Pl_{ARG} to use for fine mapping of the Pl_{ARG} genomic region.

Of the 188 F_2 individuals selected initially (Fig. 1), only 15 F_2 individuals were selected in the second step and revealed a recombination event between the closely linked

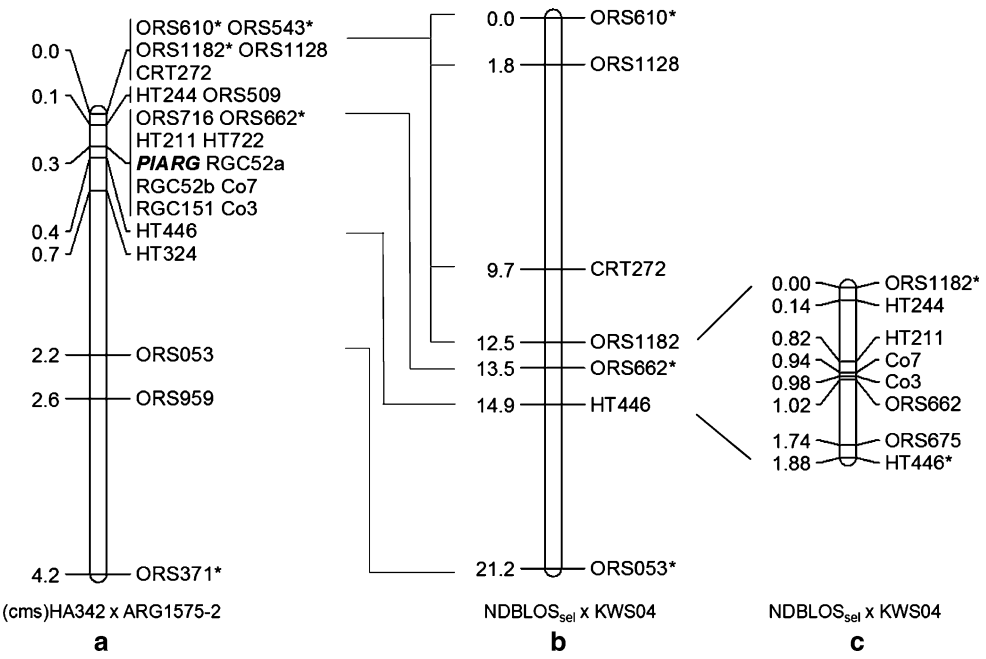

Fig. 2 **a** LG 1 of (cms)HA342 × ARG1575-2 constructed with 2,145 F_2 individuals. **b** Partial LG 1 map of $NDBLOS_{sel}$ × KWS04 constructed with 528 F_2 individuals. **c** Partial LG 1 map of $NDBLOS_{sel}$ × KWS04 constructed with 2,780 F_2 individuals. Markers with an asterisk were screened in all individuals, while all other markers were screened in recombinant lines only. Pl_{ARG} is shown in bold. Maps are not drawn to scale and distances on the left side of the map correspond to centiMorgan

flanking markers ORS509, HT244 and HT446. Five of the 15 recombinant lines produced sufficient seed to develop homozygous recombinant progenies. Four homozygous lines were resistant to *P. halstedii* race 730, and one homozygous line was susceptible to *P. halstedii* race 730 and showed the same pattern of resistance after infection with *P. halstedii* races 100, 330, 710.

Population $NDBLOS_{sel}$ × KWS04

Suppressed recombination was evident in (cms)HA342 × ARG1575-2 comparing with the linkage map of Tang et al. (2002, 2003), which is likely due to the introgression of Pl_{ARG}. To obtain a higher mapping resolution in the target region, we developed the intraspecific population $NDBLOS_{sel}$ × KWS04 of 2,780 F_2 individuals that did not carry an introgression from Pl_{ARG}. Initially, 528 F_2 individuals were screened with ORS610, ORS662 and ORS053 from LG 1. For 198 F_2 individuals a recombination event was detected between ORS610 and ORS053. These individuals were screened with four additional polymorphic SSR markers and resulted in a map based on 528 F_2 individuals (Fig. 2b) that spanned 21.2 cM between ORS610 and ORS053. ORS610, ORS1128, CRT272, and ORS1182 were mapped within 12.5 cM. ORS1182 is the closest flanking marker above Pl_{ARG}. Hence, the target interval (ORS1182–HT446) spans 2.4 cM in $NDBLOS_{sel}$ × KWS04.

Additional 2,252 F_2 individuals of $NDBLOS_{sel}$ × KWS04 were screened with ORS1182 and HT446 to identify recombinants in this high-resolution mapping population. Altogether, 99 recombinant F_2 individuals were identified in the target region and were screened with HT244, HT211, ORS662 and ORS675. HT211 and ORS662 which cosegregated with Pl_{ARG} in (cms)HA342 × ARG1575-2 were mapped with higher resolution in $NDBLOS_{sel}$ × KWS04 0.2 cM apart (Fig. 2c).

Screening the large-insert sunflower BAC library HA383 and mapping BAC end sequences

Screening the HA383 BAC library with overgo probes designed for RGC52 and RGC151 identified 18 positive BAC clones for RGC52 and 9 positive BAC clones for RGC151. FPC assembles of the 18 BAC clones of RGC52 resulted in two contigs: 11 BACs in contig 2 and 7 BACs in contig 7. BAC clones of RGC151 were assembled all together in contig 3. BES generated 19, 17 and 14 BAC end sequences for contigs 2, 3 and 7. After quality control and vector sequence trimming, the BAC end sequence lengths ranged from 149 to 834 bp. Contig 2 was assigned to LG 16 (W. Gao, unpublished results). Therefore, primers were designed on the BAC end sequences of contig 3 and contig 7 (Supplementary Material Table 1) and amplicons were tested for polymorphism between (cms)HA342 and ARG1575-2 and between $NDBLOS_{sel}$ and KWS04. Marker Co3-4_T7 from contig 3 was polymorphic between (cms)HA342 and ARG1575-2 and marker Co3-2_T7 was polymorphic between $NDBLOS_{sel}$ × KWS04. Marker Co7-11_M13 from contig 7 was polymorphic for both mapping populations. Overall, the 188 recombinant F_2 individuals (ORS610/ORS371) of (cms)HA342 × ARG1575-2 were analyzed with markers Co3-4_T7, and Co7-11_M13 and the 99 recombinant F_2 individuals (ORS1182/HT446) of $NDBLOS_{sel}$ × KWS04 were genotyped with markers

Table 3 Shared haplotypes between cmsHA89 and ARG1575-2, RHA419, RHA420, RHA443, and between FS20-6-2 and 79ARGMTP for markers on LG 1

Marker	cM	Sunflower line						
		cmsHA89	ARG1575-2	RHA419	RHA420	RHA443	FS20-6-2	79ARGMTP
ORS1128	0.0	−	+	+	+	+	−	×
ORS543	0.0	−	+	+	+	+	−	×
ORS610	0.0	−	+	+	+	+	−	×
ORS1182	0.0	−	+	+	+	+	×	×
CRT272	0.0	−	+	+	+	+	−	×
ORS509	0.1	−	+	+	+	+	−	×
ORS716	0.3	−	+	+	+	+	−	×
ORS662	0.3	−	+	+	+	+	−	×
HT211	0.3	−	+	+	+	*n.d.*	−	×
RGC151	0.3	−	+	+	+	*n.d.*	−	×
HT722	0.3	−	+	+	+	+	−	×
HT446	0.4	−	+	+	+	+	−	×
HT324	0.7	+	+	+	+	+	−	×
ORS053	2.2	−	+	+	+	+	−	×
ORS959	2.6	−	+	−	+	−	−	×
ORS371	4.2	−	+	−	+	−	−	×

Marker position in cM corresponds to (cms)HA342 × ARG1575-2 (2,145 F_2 individuals). We used the symbols (+) for markers which have the same alleles as ARG1575-2, (×) for markers which have the same alleles as 79ARGMTP and (−) for marker alleles which differ from genotype ARG1575-2 or 79ARGMTP. *n.d.* Not determined

Co3-2_T7 and Co7-11_M13. Map calculation for the final maps was done for the whole population (cms)HA342 × ARG1575-2 with 2,145 F_2 individuals and $NDBLOS_{sel}$ × KWS04 with 2,780 F_2 individuals. Co3-4_T7 and Co7-11_M13 and consequently contig 3 and contig 7 cosegregated with Pl_{ARG} in (cms)HA342 × ARG1575-2 (Fig. 2a). HT211 and ORS662 flanked contig 3 and contig 7 on LG 1 in $NDBLOS_{sel}$ × KWS04 (Fig. 2c).

Origin of LG 1 in the resistant line ARG1575-2

To explain the suppressed recombination of (cms)HA342 × ARG1575-2, we compared the alleles of ARG1575-2 and cmsHA89 for 14 SSR and two CAPS markers of LG 1. The resistant inbred line ARG1575-2 is expected to carry only 12.5% of *H. argophyllus* genome after two backcrosses with cmsHA89. We found that ARG1575-2 and cmsHA89 carry different alleles in all markers tested on LG 1 with the exception of HT324 (Table 3). We could not investigate whether the HT324 allele of ARG1575-2 originated from *H. argophyllus* or from cmsHA89 because accession 1575 is no longer available. Analysis of 94 SSR markers randomly distributed on LG 2 to 17 revealed that 12.7% of them had shared alleles between ARG1575-2 and cmsHA89. To estimate the size of the introduced segment in other resistant inbred lines carrying Pl_{ARG} we compared the marker alleles of ARG1575-2 and six inbred lines. The haplotypes of RHA419, RHA443 and RHA420 originate mostly from ARG1575-2 (Table 3). A recombination event occurred in RHA419 and RHA443 below Pl_{ARG} in the interval of ORS053 and ORS959. Comparison of 79ARGMTP, which probably also carries Pl_{ARG}, and FS20-6-2 showed no evidence of recombination on LG 1 (Table 3).

Discussion

Wild species are often used to broaden the genetic background of cultivated crops to increase yield (Singh and Ocampo 1997), oil content and quality (Seiler 2007), tolerance to abiotic stress (Miller and Seiler 2003), herbicide tolerance (Al-Khatiba et al. 1998) or to introduce male sterility alleles for developing hybrids (Leclercq 1969). However, wild species are most commonly used as donors of disease resistance genes (Jan et al. 2002, 2004a, b; Kuhl et al. 2001; Ling et al. 2004). The dominant monogenic locus Pl_{ARG} originated from the wild species *H. argophyllus* and is an outstanding source of resistance, because of broad-spectrum resistance against all known races of *P. halstedii*.

Monogenic resistance genes are often not durable. Parlevliet (2002) reported that the presence of many major resistance genes and the occurrence of hypersensitive

response, both of which are typical for *Pl* loci, are characteristics of non-durable resistance. Many major dominant genes for resistance to downy mildew have been described (Pl_1–Pl_{13}) and Radwan et al. (2005) showed that the resistance mechanism of Pl_8 is associated with a hypersensitive response in the hypocotyls. Pl_8 confers till now resistance against all known races of downy mildew, but Pl_6 is an example where the resistance has been overcome by races 304 and 314 (Vear et al. 2007). A few studies have commented on extending the durability of *Pl* loci. Vear et al. (2008b) and Tourvieille de Labrouhe et al. (2008) suggested a combination of monogenic *Pl* loci with quantitative resistance against downy mildew. McDonald and Linde (2002) recommend pyramiding major resistance genes in one variety or growing cultivar mixtures containing genotypes with different major resistance genes. To realize these strategies molecular markers and knowledge of the genetic and functional basis of resistance are required.

Dußle et al. (2004) mapped Pl_{ARG} in the telomeric region of LG 1 using 126 F_2 individuals of cmsHA342 × ARG1575-2. In this study we successfully remapped Pl_{ARG} and defined a new position on LG 1, because a larger mapping population of (cms)HA342 × ARG1575-2 was available and more phenotypic and genotypic data were collected. We enriched the target Pl_{ARG} region with the closely linked flanking markers ORS509, HT244 and HT446 and with the newly identified cosegregating SSR markers ORS716, HT722 and SNP marker HT211 as well as the resistance gene candidates RGC151, RGC52a and RGC52b. The RGCs belong to the NBS-LRR class, but sequence information is not currently available to classify them in the TIR or non-TIR subclasses (Radwan et al. 2008). Here, we present the fine mapping of Pl_{ARG} that will lay the groundwork for map-based cloning of this resistance locus.

Suppressed recombination on LG 1 in cross (cms)HA342 × ARG1575-2

ORS610, ORS1128, CRT272, and ORS1182 cosegregated at the upper end of LG 1 in (cms)HA342 × ARG1575-2. However in $NDBLOS_{sel}$ × KWS04 these markers span a genetic distance of 12.5 cM. Comparison of the marker interval ORS610–ORS053 between the two linkage maps showed that we were able to increase mapping resolution by a factor of ten in $NDBLOS_{sel}$ × KWS04. The same interval spanned 31.5 cM in the publicly sunflower SSR map of LG 1 published by Tang et al. (2003) confirming suppression of recombination in (cms)HA342 × ARG1575-2.

LG 1 of (cms)HA342 × ARG1575-2 can be regarded as an interspecific cross between silverleaf and cultivated sunflower, because no recombination occurred during the cross of ARG1575-2 with cmsHA89 or during the two backcross steps with cmsHA89. During the development of ARG1575-2, resistance to downy mildew was not tested before the BC_2F_5 generation (Seiler et al. 1991). Therefore, the retention of *H. argophyllus* alleles on LG 1 in ARG1575-2 is surprising since the remaining linkage groups (LG 2–17) contain a mixture of *H. annuus* and *H. argophyllus* alleles. This raises the question whether the inheritance of the whole LG 1 occurred randomly or not during the development of ARG1575-2. Other crosses from the original population did not contain Pl_{ARG}, indicating that the discovery of this locus was probably random and at a very low frequency (G. Seiler, personal communication).

Suppressed recombination is often observed in populations that carry wild genome introgressions as in case of *Mla* introduced into cultivated barley from *H. spontaneum* (Wei et al. 1999), *Run1*, the grapevine powdery mildew resistance gene introduced from *Muscadinia rotundifolia* into *Vitis vinifera* (Barker et al. 2005), and *Mi* and *Tm2-a*, both introduced from *Lycopersicon peruvianum* into *L. esculentum* (Ganal et al. 1989; Kaloshian et al. 1998; Messeguer et al. 1991; Seah et al. 2004). Seah et al. (2004) showed that both resistant and susceptible lines carry two *Mi-1* homolog clusters which are separated in the resistant genotypes by 300 Kb while in susceptible genotypes they are separated by a genomic fragment of unknown size. Molecular markers that flanked the resistant and susceptible loci had the same orientation, but markers in between the two clusters had an inverse orientation. Therefore, it was concluded that suppressed recombination may be due to chromosomal inversion. Comparing sequences near the *Mi-1*-homologs between susceptible and resistant genotypes showed blocks of homology, but also regions that have undergone considerable rearrangements (Seah et al. 2007).

In sunflower, comparative genetic linkage maps of *H. annuus*, *H. petiolaris*, *H. anomalus*, *H. deserticola* and *H. paradoxus* were established to study karyotypic evolution and a high rate of chromosomal rearrangements was observed (Burke et al. 2004; Lai et al. 2005b). Heesacker et al. (2009) studied comparative mapping between *H. annuus* and *H. argophyllus* and identified 10 collinear chromosomes, 9 chromosomal rearrangements, 3 putative segmental duplications and 2 putative whole chromosome duplications. LG 1 was collinear between *H. annuus* and *H. argophyllus*; therefore, chromosomal rearrangements are not likely the reason for suppressed recombination, but reduced homology may be a plausible explanation. Suppressed recombination was previously described in sunflower mapping populations of interspecific origin (Burke et al. 2002; Heesacker et al. 2009). However, in an intraspecific mapping population of *H. argophyllus* recombination was also suppressed near Pl_{ARG} so that the reduced homology between silverleaf and cultivated sunflower

seems not to be the sole cause of suppressed recombination (Heesacker et al. 2009).

Does the introgressed region influence fertility and segregation distribution in mapping populations?

Eighty-one percent of the cmsHA342 × ARG1575-2 F_2 individuals showed a limited seed set producing less than 10 g of sunflower seed. ARG1575-2 must carry a restorer gene for the PET1 cytoplasm otherwise no seed production would be possible in the progenies. For 25% of the F_2 individuals no seed set was observed. This fits the expected 3:1 segregation of a single dominant restorer gene. Since the backcrossing parent cmsHA89 possesses no restorer gene for the PET1 cytoplasm, the restorer gene must originate from *H. argophyllus*. Compared to the seed production of subpopulation HA342 × ARG1575-2 the seed set in subpopulation cmsHA342 × ARG1575-2 is reduced. The lack of plants homozygous for the ARG1575-2 allele in cmsHA342 × ARG1575-2 could be due to incomplete restoration of the PET1 cytoplasm based on unfavorable effects of the restorer or other genes that prohibit full male sterility. The restorer gene is not closely linked to Pl_{ARG}, because only 64 of 209 F_2 individuals which carry the alleles of ARG1575-2 in the target region had no seed production (data not shown). Abratti et al. (2008) described a monogenic restorer gene for the PET1 cytoplasm originating from *H. argophyllus*. They mapped the *Rf3* gene on LG 7 using the population RHA340 × ZENB8. It was not analyzed if *Rf3* is the restorer gene in ARG1575-2. Another possible cause for the reduced seed set could be the introgressed genomic segment from *H. argophyllus* since reduced seed set has been frequently observed in progenies of interspecific crosses. Chetelat and Meglic (2000) reported a reduced seed set of tomato back-cross lines that carry introgressions from *Solanum lycopersicoides*. Lai et al. (2005b) reported fertility reductions observed in hybrids derived from interspecific crosses in the genus *Helianthus*. Fertility problems in cmsHA342 × ARG1575-2 were also reflected by the significant distortion from the 1:2:1 segregation at loci in the target region. Distorted segregation was not observed in subpopulation HA342 × ARG1575-2, which also had a better seed set, thus, the introgression from *H. argophyllus* itself does not seem to severely influence fertility and segregation ratios in the target region in crosses with the normal cytoplasm.

Fine mapping of the target region Pl_{ARG}

A prerequisite for efficient fine mapping of a given genomic region is the availability of closely linked flanking markers. We identified the closely linked flanking markers (ORS509, HT244 and HT446) at a distance of 0.2 cM above and 0.1 cM below Pl_{ARG}. We identified F_2 individuals which carry a recombination event between the markers to reduce phenotyping work with different races of *P. halstedii* only to genetically informative lines (Bauer and Graner 1995). Ultimately, five recombinant lines were screened with four different races of *P. halstedii*, but no differences in segregation patterns were observed. This may be due to the lack of recombination in the resistance gene cluster or due to extreme physical proximity of clustered resistance genes. In the study of *Mi-1* locus discussed previously, Seah et al. (2007) concluded that lethality of recombination events within the inverted genomic region between the two clusters of *Mi* homologs might explain the observed linkage map contraction. Moreover, another explanation could be that Pl_{ARG} is a single resistance gene mediating resistance against several races of *P. halstedii*, similar to *RPP13*, a single resistance gene that protects *Arabidopsis thaliana* against different isolates of the biotrophic oomycete *Peronospora parasitica* (now known as *Hyaloperonospora arabidopsis*) (Bittner-Eddy et al. 2000). Pl_{ARG} may differ from the previously analyzed *Pl* loci, which clustered on LG 8 (Pl_1, Pl_2, Pl_6 and Pl_7) (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997) and LG 13 (Pl_5 and Pl_8) (Bert et al. 2002; Radwan et al. 2003). Further study is necessary to find out whether Pl_{ARG} is a single resistance gene or a complex locus of genes and characterize the molecular structure of Pl_{ARG}. One plausible approach is the map-based cloning of Pl_{ARG} to solve the question about the structure and to answer to which class of resistance genes Pl_{ARG} belongs.

Radwan et al. (2008) showed that RGCs are landmarks for the identification and isolation of *R*-genes in sunflower. They identified RGCs linked to the downy mildew resistance loci Pl_{ARG} and Pl_8, to the black rust resistance genes R_1 and R_{ADV}, and to Or_5, which protects sunflower against the parasitic weed *Orobanche cumana* Wallr. race E. Recently, Pl_{13} was mapped on the lower end of LG 1 (Mulpuri et al. 2009) where Radwan et al. (2008) mapped several RGCs. We used RGCs cosegregating with Pl_{ARG} to screen the large-insert sunflower HA383 BAC library. Two BAC contigs were identified in the target Pl_{ARG} region on LG 1 with overgo probes of RGC52 and RGC151, which indicates that there is a cluster of RGCs in the target region and Pl_{ARG} may be a part of it. A third contig identified with RGC52 mapped on LG 16 (W. Gao, unpublished results). A similar approach was used by Meyers et al. (1998) who screened a BAC library of lettuce cultivar Diana with a probe designed in the NBS region of RGC2, which co-segregated with the downy mildew resistance gene *Dm3*, and identified several BAC clones. The positive BAC clones revealed 22 distinct members of a resistance gene family and RGC2B was identified as a candidate gene by screening deletion mutants.

$NDBLOS_{sel}$ × KWS04 is a suitable population for fine-mapping BAC end sequences in the target region at a higher resolution compared to (cms)HA342 × ARG1575-2. The aim of this work is to select and sequence BACs that map in the target region using next-generation sequencing technologies.

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