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ORIGINAL PAPER

# Downy mildew ($Pl_8$ and $Pl_{14}$) and rust ($R_{Adv}$) resistance genes reside in close proximity to tandemly duplicated clusters of non-TIR-like NBS-LRR-encoding genes on sunflower chromosomes 1 and 13

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**Abstract** Nucleotide binding site-leucine rich repeat (NBS-LRR) proteins are encoded by a ubiquitous gene family in sunflower and frequently harbor disease resistance genes. We investigated NBS-LRR-encoding resistance gene candidates (RGCs) flanking the downy mildew resistance genes $Pl_8$ and $Pl_{14}$ and the rust resistance gene $R_{Adv}$, which map on the NBS-LRR clusters of linkage groups 1 and 13 in sunflower genome. We shotgun sequenced bacterial artificial chromosome (BAC) clones proximal to $Pl_8$, $Pl_{14}$, and $R_{Adv}$ and identified seven novel non-Toll/interleukin-1 receptor (TIR)-like NBS-LRR RGCs, which clustered with previously identified RGCs of linkage group 13 but were phylogenetically distant from the TIR- and non-TIR-NBS-LRR-encoding superfamilies of sunflower. Six of the seven predicted RGCs have intact open reading frames and reside in genomic segments with abundant transposable elements. The genomic localization and sequence similarity of the novel non-TIR-like predicted RGCs suggests that they originated from tandem duplications. RGCs in the proximity of $Pl_8$ and $R_{Adv}$ were likely introgressed from silverleaf sunflower genome, where the RGC cluster of linkage group 13 is duplicated in two independent chromosomes that have different architecture and level of recombination from the respective common sunflower chromosomes.

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## Introduction

Downy mildew and rust, caused by the oomycete *Plasmopara halstedii* (Farl.) Berl. & de Toni and the fungus *Puccinia helianthi* Schwein, respectively, are responsible for severe economic losses in cultivated sunflower (*Helianthus annuus* L.). More than 12 races of downy mildew have been recorded, but *P. halstedii* race 4 is predominant in the United States (Gulya et al. 1997); whereas, for rust four prevailing races are present in North America and shifts in virulence occur frequently (Gulya et al. 1997). Pyramiding genes for resistance to downy mildew and rust, primarily introgressed from wild *Helianthus* species, has dramatically diminished yield losses of commercial hybrids (Gulya et al. 1997). Fourteen genes for resistance to downy mildew, denominated $Pl_1$–$Pl_{13}$ and $Pl_{Arg}$ (DuBle et al. 2004; Rahim

et al. 2002) have been described so far, and an additional resistance gene, $Pl_{14}$, is described in this study, the allelic relationships of which remain to be elucidated. Seven genes for rust resistance, $R_1$–$R_5$, $R_{Adv}$, and $Pu_6$, have been reported, while additional genes derived from wild accessions have been also identified (Lawson et al. 1998; Quresh and Jan 1993; Yang et al. 1989).

Genetic mapping of downy mildew and rust resistance genes has elucidated the localization of disease resistance genes in sunflower genome and facilitated marker assisted introgression into elite germplasm. The downy mildew resistance genes $Pl_1$, $Pl_2$, $Pl_6$, and $Pl_7$ are clustered on linkage group 8 (Bert et al. 2001; Brahm et al. 2000; Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997) of the public sunflower genetic map (Yu et al. 2003), whereas $Pl_5$ and $Pl_8$ map on linkage group 13 (Bert et al. 2001; Yu et al. 2003) and $Pl_{Arg}$ (DuBle et al. 2004; Wieckhorst et al. 2010) and $Pl_{14}$ map on linkage group 1. $R_1$ and $R_{Adv}$ genes for rust resistance have also been mapped on linkage groups 8 and 13, respectively (Lawson et al. 1998; Slabaugh et al. 2003; Yu et al. 2003), in proximity to the aforementioned downy mildew resistance genes (Fick and Zimmer 1975; Slabaugh et al. 2003; Yu et al. 2003).

Notably, several resistance gene candidates (RGC), encoding nucleotide binding site-leucine rich repeat (NBS-LRR) proteins; the most widely represented resistance protein family (McDowell and Woffenden 2003; McHale et al. 2006), were also mapped on linkage groups 8 and 13 harboring the downy mildew and rust resistance genes (Bouzidi et al. 2002; Gedil et al. 2001; Gentzbittel et al. 1998; Radwan et al. 2004, 2003, 2008; Slabaugh et al. 2003). NBS-LRR-encoding RGCs evolve dynamically producing new resistance specificities and are commonly clustered in plant genomes (Hulbert et al. 2001; Leister 2004; Meyers et al. 2003; Michelmore and Meyers 1998; Monosi et al. 2004; Richter and Ronald 2000). Identification of 783 NBS-LRR homologs from common and wild *Helianthus* species and mapping of 167 NBS-LRR loci in sunflower genome revealed that the two largest clusters, comprised 54 and 27 RGCs, coincided with the disease resistance genes on linkage groups 8 and 13, whereas smaller NBS-LRR-encoding clusters were mapped on linkage groups 1, 4, 9, and 15 (Radwan et al. 2008). Duplicated restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers have been identified on linkage groups 8 and 13 (Radwan et al. 2003, 2008; Slabaugh et al. 2003) raising the possibility of genome duplication.

Yet, only RGC clusters harboring $Pl_1$, $Pl_6$, and $R_1$ genes on linkage group 8 have been thoroughly studied in sunflower (Bouzidi et al. 2002; Gedil et al. 2001; Slabaugh et al. 2003). In this study, we developed two plant populations segregating for $Pl_8$, $R_{Adv}$, and $Pl_{14}$ to shed light into the RGC clusters of linkage groups 1 and 13 that harbor resistance genes for downy mildew and rust. We identified RGC markers tightly linked to $Pl_8$, $Pl_{14}$, and $R_{Adv}$, investigated the genetic relatedness of linkage groups 1 and 13 RGCs with respect to the 783 NBS-LRR-encoding RGCs of sunflower, and laid the groundwork for positional cloning of $Pl_8$, $Pl_{14}$, and $R_{Adv}$. We also mapped the genetic factor controlling downy mildew resistance in IMISUN-1 × 29004, which has not been previously described and was herein designated $Pl_{14}$, and investigated its allelism with respect to other downy mildew genes on linkage groups 1, 8, and 13.

## Materials and methods

### Population development

One hundred and seventy $F_{2:3}$ families were developed from ZENB8, a proprietary oilseed B-line of Advanta Semillas S.A.I.C. (Balcarce, Argentina) susceptible to downy mildew and rust, and RHA340, a public oilseed R-line conferring downy mildew resistance to *P.halstedii* races 2, 3, and 4 and rust resistance to *P.helianthi* race 4 (Miller and Gulya 1988). RHA340 was developed from HA89 after backcrossing with *H. argophyllus* 415 and carries a single dominant gene, $Pl_8$, for resistance to downy mildew introgressed from *H. argophyllus* (Miller and Gulya 1988, 1991), and a novel gene for fertility restoration (Gustavo Abratti 2008, personal communication). In addition, 286 $F_{2:3}$ families were developed from IMISUN-1 (PI 607927), an oilseed line resistant to imazethapyr and imazamox herbicides (Al-Khatib and Miller 2000), and 29004, a proprietary line of Advanta Semillas S.A.I.C. that was found resistant to *P. halstedii* races 330 and 730 in Argentina and race 710 in France. Notably, 29004 was derived from HA-R4 (PI 650755), a plant introduction resistant to *P. helianthi* races 1, 2, 3, and 4, Verticillium wilt, and *P. halstedii* races 2 and 3 (Gulya 1985). The $F_1$ plants of ZENB8 × RHA340 and IMISUN-1 × 29004 were self-pollinated in the greenhouse. $F_2$ seed was planted in one field location at Oran, Argentina in 2003. $F_2$ plants were self-pollinated and $F_3$ seed was harvested from each individual plant. The $F_{2:3}$ families were subsequently evaluated for resistance to downy mildew and rust.

### Disease resistance evaluations

Twenty seedlings for each $F_{2:3}$ family of ZENB8 × RHA340 and IMISUN-1 × 29004 were infected with zoosporangia of *P. halstedii* race 4 (race 730) using the whole seed immersion technique (Mouzeyar et al. 1993) to

evaluate downy mildew resistance in growth chambers. Ten days after infection, seedlings were evaluated for disease resistance after being exposed to water-saturated atmosphere for 24 h. Seedlings were considered susceptible to downy mildew when sporulation was observed on the cotyledons. In the case of scarce sporulation, seedlings were transplanted to pots and disease progress was monitored. Rust resistance was evaluated with 20 plants for each of the 170 $F_{2:3}$ families of ZENB8 × RHA340, which were inoculated (1 ml/plant) 2 weeks after emergence with suspended uredospores (50,000 spores/ml) collected from naturally infected plants. The Argentinean isolate collected was equivalent to the American *P.helianthi* race 4, as indicated by the virulence of differential lines (P386, HA89, CM90, MC69, MC29, HA-R1, HA-R2, HA-R3, HA-R4, HA-R5) according to the system proposed by Kochman and Goulter (1985). Next, plants were placed in chambers at 18°C and 100% humidity for 24 h, and were transferred to the greenhouse at 25°C and 13 h of light. Scoring for the presence of pustules on leaves, necrosis and chlorosis was conducted 12–13 days after inoculation, using 0–4 rating that classifies 0, 1, and 2 as resistant plants, and 3 and 4 as susceptible (Yang et al. 1986).

Downy mildew and rust phenotypes were derived from the number of susceptible plants (*ss*) divided by the total number of plants evaluated (*RR*, resistant; *Rs*, segregating; and *ss*, susceptible) for each $F_{2:3}$ family. The proportions of susceptible plants were converted to codominant marker scores; thus, $F_{2:3}$ families with 100 and 0% proportions were respectively scored as susceptible (*ss*) and resistant (*RR*), while the remaining proportions were considered segregating (*Rs*) families.

## Genotypic evaluation and linkage mapping

Leaf tissue was collected from $F_2$ plants prior to flowering in 2003 at Oran, Argentina. DNA was extracted using a modified CTAB protocol (Webb and Knapp 1990). DNA markers on the sunflower public genetic map linkage groups 1 and 13 (Tang et al. 2002; Yu et al. 2003) were amplified for IMISUN-1 × 29004 and ZENB8 × RHA340, respectively. Previously developed RGC markers mapping on linkage groups 1 and 13 (Radwan et al. 2008) and newly developed markers for sequence-tagged sites localized on linkage group 13 (RGC266, RGC267, RGC269, RGC270, and RGC271) were screened with bulk segregant analysis (Michelmore et al. 1991) using DNA of parental lines and bulks of susceptible and resistant families. RGCs segregating for downy mildew and rust resistance flanking $Pl_8$, $Pl_{14}$, and $R_{Adv}$ genes on linkage groups 1 and 13 were identified (ESM 1). These RGCs were genotyped as insertion-deletion (INDEL) or single-strand conformational polymorphism (SSCP) markers (Radwan et al. 2008). Genetic maps of linkage group 13 in ZENB8 × RHA340 and linkage group 1 in IMISUN-1 × 29004 (Fig. 1) were constructed with Joinmap 3.0 software (Van Ooijen and Voorrips 2001) and centiMorgan (cM) distances were derived using Kosambi's mapping function.

## Physical mapping and BAC sequence annotation

We designed overgo probes for RGC203, RGC251, and RGC260 and screened a large-insert bacterial artificial chromosome (BAC) library developed from the digestion of high molecular weight genomic DNA of HA383 (Miller and Gulya 1995) with the *Hin*dIII restriction endonuclease. The BAC library was constructed at the Clemson University Genomics Institute according to standard protocols (Choi and Wing 2000; Peterson et al. 2000) and is publicly available. It consists of 202,752 clones with average length of 125.5 Kb and provides an 8.3× coverage of HA383 genome (http://www.genome.clemson.edu/). Positive BAC clones, identified by high-density filter hybridization using the RGC203, RGC251, and RGC260 overgo probes, were verified by colony PCR with RGC-specific primers. High information content fingerprinting (HICF) was conducted with snapshot labeling (Luo et al. 2003) followed by capillary electrophoresis using an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Contigs were built from fingerprinted clones using FPC software (Soderlund et al. 1997).

BAC-end sequences (BESs) were also obtained from both ends of each positive clone insert with T7 and M13 primers (ESM 2) using an ABI 3700 XL DNA Analyzer (Applied Biosystems, Foster City, CA). Phred and Cross_Match were used for quality trimming and removal of vector sequences, respectively (Ewing and Green 1998; Ewing et al. 1998). The sequence redundancy of the BESs was evaluated using ClustalW (Larkin et al. 2007) and mined simple sequence repeats (SSRs) using SSRIT (Temnykh et al. 2001). DNA markers developed from BESs confirmed the location of the contigs built from fingerprinting and facilitated selection of BAC clones for shotgun sequencing. In addition, BLASTN, BLASTX, and GO database searches were conducted with PLAN (He et al. 2007) to identify putative BESs functions.

The BAC clones P339N08, P102A12, and P408L01, which were identified, respectively with the probes RGC203, RGC251, and RGC260, were shotgun sequenced to 8.5× depth (Fig. 1). DNA isolated from these BAC clones was randomly sheared, subcloned into plasmid vectors and sequenced with T7 and Sp6 primers. Phred and Cross_Match were used for quality trimming and removal of vector sequences (Ewing and Green 1998; Ewing et al. 1998). Sequences were assembled into contigs with Phrap and CAP3 (Huang and Madan 1999), and Consed (Gordon

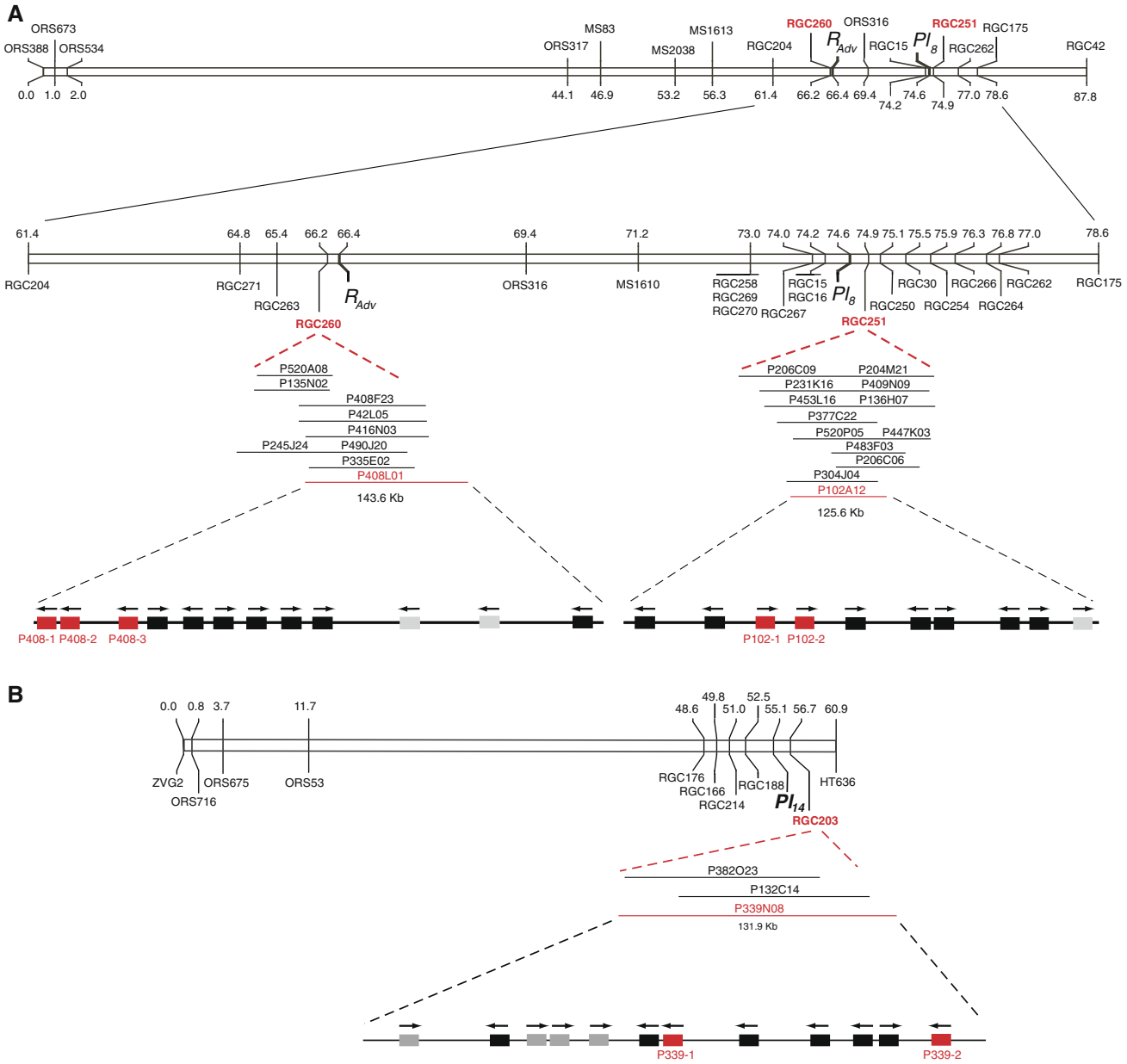



**Fig. 1** Genetic maps and structural annotation of shotgun sequenced BAC clones that flank the resistance genes $Pl_8$, $R_{Adv}$, and $Pl_{14}$. **a** Genetic map of linkage group 13 in ZENB8 × RHA340. Positive BAC clones were identified with the overgo probes RGC251 and RGC260 that are closely linked to $Pl_8$ and $R_{Adv}$ and were assembled into contigs. The BAC clones P102A12 and P408L01 were shotgun sequenced. **b** Genetic map of linkage group 1 in IMISUN-1 × 29004. Positive BAC clones were identified with the overgo probe RGC203 that maps near $Pl_{14}$, and were assembled into contigs. The BAC clone P339N08 was shotgun sequenced and its structural annotation is graphically represented. Putative RGCs mined from the BAC clones P102A12, P408L01, and P339N08 are shown in *red*, retroelements are shown in *black* and other annotated peptides are shown in *gray*

et al. 1998) was used for finishing the sequence assemblies. We predicted gene structures using both ab initio and similarity methods. Putative genes were identified using Genscan (Burge and Karlin 1997), FGENESH (Salamov and Solovyev 2000) and GeneMark.hmm (Lomsadze et al. 2005) with dicot (*Arabidopsis*, tomato, tobacco) training sets and GenomeScan (Yeh et al. 2001) using the RGC203, RGC251, and RGC260 peptides. Putative functions of the predicted genes were assigned with BLASTN, BLASTX, and GO database searches using PLAN (He et al. 2007), and searches with TIGFAMs (Haft et al. 2003), PROSITE (Hulo et al. 2007) and InterProScan (Zdobnov and Apweiler 2001).

### Phylogenetic analysis

We collected sequences of 783 previously identified NBS-encoding sunflower RGCs (Radwan et al. 2008) and putative RGCs present in P339N08, P102A12, and P408L01 BAC clones (Fig. 2). The NBS domains of RGCs, consisting approximately of 495–516 bp, were aligned using ClustalW (Larkin et al. 2007) and 100 maximum likelihood trees were obtained with PHYML (Guindon and Gascuel 2003) using GTR as substitution model, number of invariable sites equal to zero and gamma distribution equal to *e*. A bootstrap consensus tree that does not account for branch lengths was constructed with PHYLIP (Felsenstein 1989) and drawn with Interactive Tree Of Life (iTOL) (Letunic and Bork 2007). In the bootstrap consensus tree, branch lengths represent the frequency of each branch. One of the 100 maximum likelihood trees is also provided for comparison of branch lengths that correspond to evolutionary distance (ESM 3). The P-loop/Kin-1a, Kin-2, and GLPL motifs, which appeared to be the most conserved in the NBS domain, were appended to the bootstrap consensus tree of the 790 sunflower RGCs regardless of the presence of putative stop codons within the NBS-coding region (Fig. 3).

## Results

### Downy mildew and rust resistance phenotyping

The two oilseed inbred lines RHA340 and ZENB8, and 170 ZENB8 × RHA340 $F_{2:3}$ families were phenotyped for resistance to *P. halstedii* race 4 and *P. helianthi* race 4. RHA340 was predicted to be homozygous for dominant alleles for resistance to both downy mildew ($Pl_8$) and rust ($R_{Adv}$), whereas ZENB8 was predicted to be homozygous for recessive alleles. RHA340 and ZENB8 were included in the downy mildew and rust resistance assays as controls, and had consistently 0 and 100% proportions of susceptible plants. Despite previous reports of limited sporulation on the cotyledons (Bert et al. 2001; Gedil et al. 2001; Mouzeyar et al. 1994), symptoms of systemic infection were observed on the cotyledons of RHA340. RHA340 × ZENB8 $F_{2:3}$ progeny segregated as 31 homozygous resistant ($Pl_8Pl_8$) to 74 heterozygous ($Pl_8pl_8$) to 49 homozygous susceptible ($pl_8pl_8$) for downy mildew resistance phenotypes, and 40 homozygous resistant ($R_{Adv}R_{Adv}$) to 73 heterozygous ($R_{Adv}r_{Adv}$) to 42 homozygous susceptible ($r_{Adv}r_{Adv}$) for rust resistance phenotypes. The observed ratios were not significantly different from the expected

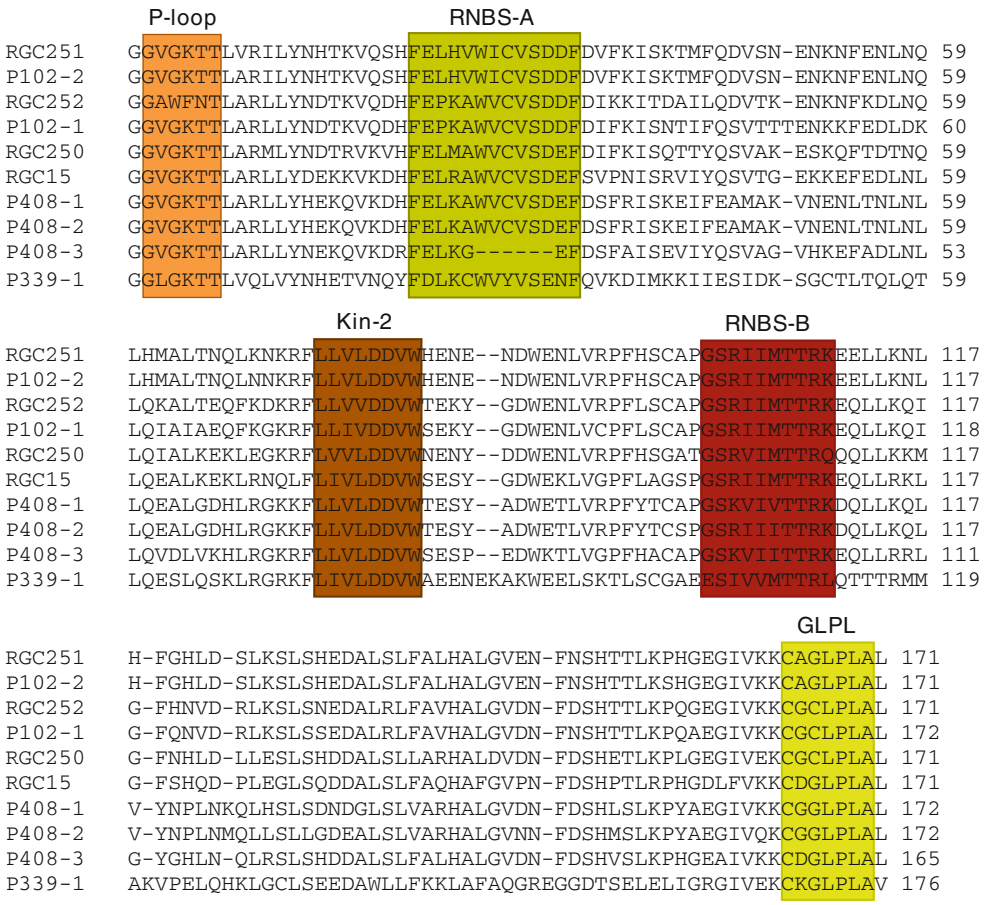
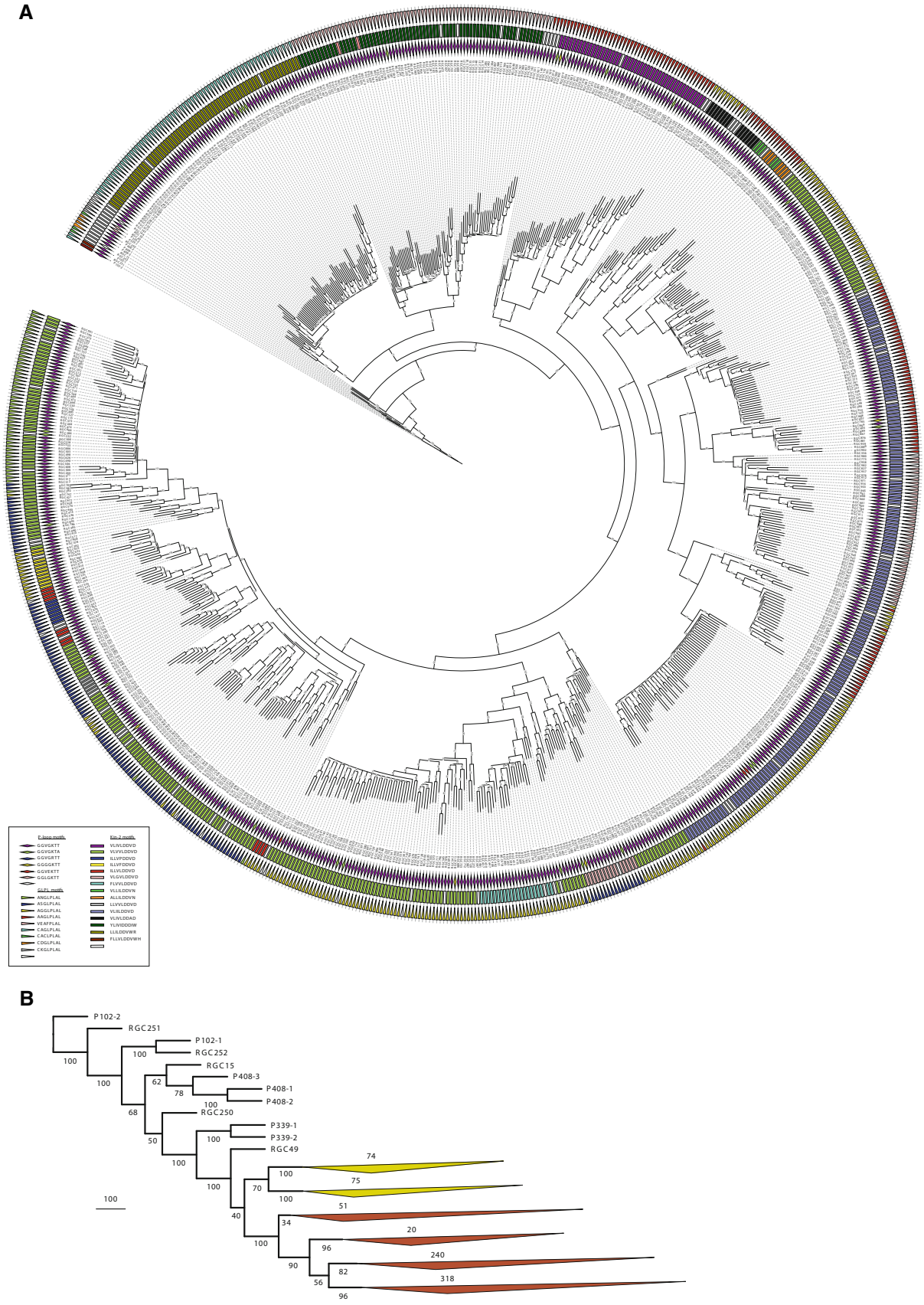



**Fig. 2** Alignment of the NBS domain of putative RGCs mined from BAC clones P102A12, P408L01, and P339N08 and RGC loci of linkage group 13. The conserved non-TIR motifs (P-loop/Kin-1a, RNBS-A, Kin-2, RNBS-B, and GLPL) are highlighted. The putative pseudogene identified in P339N08 is not included


A
B
P102-2
RGC251
P102-1
RGC252
RGC15
P408-3
P408-1
P408-2
RGC250
P339-1
P339-2
RGC49
100
62
78
68
50
70
40
34
96
90
56
82
74
75
51
20
240
318

◀ **Fig. 3** A bootstrap consensus tree including the 783 previously identified TIR- and non-TIR-NBS-LRR-encoding RGCs of sunflower (Radwan et al. 2008) and seven putative RGCs mined from P102A12, P408L01, and P339N08. **a** The P-loop/Kin-1a, Kin-2 and GLPL motifs for each of the 790 RGCs were identified and represented by *rhombus*, *rectangle*, and *triangle*, respectively. The three shapes were colored to correspond to different motifs as shown in the legend. Shapes were drawn *white* when they differed from all other RGCs. **b** The distinct clades of non-TIR and TIR-NBS-LRRs were collapsed and substituted with *triangles* to draw attention on the putative RGCs identified in P102A12, P408L01, and P339N08. The sides of each *triangle* show the distance to the closest and farthest leaf and the number in each triangle corresponds to the number of RGCs on the collapsed branch

ratio for the segregation of a dominant resistance gene for neither downy mildew ($\chi^2 = 4.4$, $P = 0.10$) nor rust ($\chi^2 = 2.96$, $P = 0.23$). Thereby, it was confirmed that downy mildew and rust in ZENB8 × RHA340 are controlled by the single dominant genes $Pl_8$ and $R_{Adv}$, which were subsequently mapped 8.2 cM apart on linkage group 13 (Fig. 1).

In addition, the inbred lines IMISUN-1 and 29004, and 286 IMISUN-1 × 29004 $F_{2:3}$ families were phenotyped for downy mildew resistance to *P. halstedii* race 4. IMISUN-1 and 29004 had 100 and 0% proportions of susceptible plants, respectively. IMISUN-1 × 29004 $F_{2:3}$ families segregated as 103 homozygous resistant to 135 heterozygous to 48 homozygous susceptible. Homozygous $F_{2:3}$ families were more frequent than expected and the observed ratio was significantly different from the expected ratio for the segregation of a dominant downy mildew resistance gene ($\chi^2 = 22.1$, $P < 0.001$).

IMISUN-1 was inferred to be homozygous for a dominant allele for resistance to downy mildew; while, 29004 was inferred to be homozygous for a recessive (susceptible) allele. The genetic factor controlling downy mildew resistance in IMISUN-1 × 29004 was subsequently designated $Pl_{14}$, and its allelism against previously described downy mildew genes mapped on linkage groups 1, 8, and 13 was initially not known.

## Genetic mapping of downy mildew and rust resistance genes

We screened resistant and susceptible RHA340 × ZENB8 F2 bulks for NBS-LRR single strand conformational polymorphism (SSCP) marker polymorphisms using bulk segregant analysis (Michelmore et al. 1991), and identified 18 polymorphic NBS-LRR loci on linkage group 13, which were subsequently mapped in the RHA340 × ZENB8 $F_{2:3}$ population and localized in proximity of $Pl_8$ and $R_{Adv}$ (Fig. 1). The genomic segment spanning $Pl_8$ and $R_{Adv}$ harbors genes conferring resistance to at least seven races of downy mildew (Bert et al. 2001). Also, a large cluster of NBS-LRR loci on the lower half of linkage group 13, interspersed among the aforementioned resistance genes, was recently confirmed (Radwan et al. 2008). Interestingly, 17 NBS-LRR loci (ESM 1) clustered in a 17.2 cM interval on the distal end of linkage group 13, and five of the 17 (RGC266, RGC267, RGC269, RGC270, and RGC271) have not been previously mapped (Fig. 1). All 18 RGCs (Fig. 1; ESM 1) encoded non-Toll/interleukin-1 receptor (TIR) NBS-LRRs. Only three TIR-NBS-LRRs have been previously identified on linkage group 13 (Radwan et al. 2008), although many of the previously identified NBS-LRR encoding genes could not be phylogenetically classified. RGC251 and RGC15/16 mapped 0.3 cM downstream and 0.4 cM upstream of $Pl_8$, respectively (Fig. 1), which was substantially closer than previously described markers that mapped at least 3.5 cM from the downy mildew resistance gene (Radwan et al. 2004, 2003). RGC260 mapped 0.2 cM upstream of the $R_{Adv}$ locus (Fig. 1), which was slightly farther away than $SCX20_{600}$, a previously mapped dominant sequence characterized amplified region (SCAR) marker (Lawson et al. 1998).

Homozygous resistant and susceptible IMISUM-1 × 29004 F2 bulks were also screened for NBS-LRR SSCP marker polymorphisms. Five NBS-LRR loci were polymorphic (ESM 1) and mapped to the distal end of linkage group 1 (Fig. 1) within a previously mapped cluster of NBS-LRR-encoding loci (Radwan et al. 2008). Therefore, the IMISUN-1 × 29004 $F_{2:3}$ population segregated for a dominant allele ($Pl_{14}$) for resistance to *P. halstedii* race 4, which was independent of $Pl_8$ and other downy mildew resistance genes located on linkage groups 8 and 13. RGC203 mapped 1.6 cM downstream of $Pl_{14}$ and 48.7 cM from RGC52 (Fig. 1), an NBS-LRR locus linked to $Pl_{Arg}$, a downy mildew resistance gene previously mapped to the proximal end of linkage group 1 (DuBle et al. 2004; Wieckhorst et al. 2010). This is the first report on the genetic factor $Pl_{14}$ and its exact location in the sunflower genome. The two markers flanking $Pl_{14}$, RGC203 and RGC188, showed significant deviation from the expected segregation ratio ($\chi^2 = 12.02$, $P < 0.001$ for RGC203 and $\chi^2 = 14.96$, $P < 0.001$ for RGC188), which is in agreement with the observed deviation of phenotypes where homozygous $F_{2:3}$ families were more frequent than expected under the single dominant gene hypothesis.

## Physical mapping of RGCs linked to $Pl_8$, $Pl_{14}$, and $R_{Adv}$

We designed overgo probes for RGC203, RGC251, and RGC260, the three RGC loci closely linked to $Pl_{14}$, $Pl_8$, and $R_{Adv}$, and screened a large-insert BAC library developed from genomic DNA of HA383, an oilseed sterility maintainer (B) line (http://www.genome.clemson.edu/). High-density filter hybridization with RGC203 revealed 18 positive BAC clones, whereas the probes RGC251 and

RGC260 identified 13 and 9 positive BAC clones, respectively. The selected clones were fingerprinted using high information content fingerprinting (HICF) and the BAC clone ends were sequenced. RGC251-positive BAC clones assembled into two contigs, one of which mapped on linkage group 13 near $Pl_8$, and four singletons. Only 17 BAC clones that consisted this contig are presented (Fig. 1; ESM 2). RGC203-positive BAC clones were assembled into four contigs, three of which mapped on the distal end of linkage group 1 near $Pl_{14}$, and one singleton. One of the three contigs consisting of three BAC clones was selected for further study due to its proximity to $Pl_{14}$ (Fig. 1; ESM 2). RGC260-positive BAC clones were assembled into a single contig, which was tightly linked to $R_{Adv}$ (Fig. 1; ESM 2). It should be noted that since the BAC clones have not been anchored directionality remains unknown.

Overall, 32 high-quality BESs (GenBank accessions HN262612 to HN262643) with mean length 587.0 bp and mean GC content 41.2% were obtained from the 17 RGC251-positive BAC clones (ESM 2). Twelve BESs (GenBank accessions HN262644 to HN262655) with mean length 654.3 bp and mean GC content 39.2% were derived from the 9 RGC260-positive BAC clones (ESM 2). The RGC203-positive BAC clones gave 6 BESs (GenBank accessions HN262656 to HN262661) with mean length 577.0 bp and mean GC content of 33.9% (ESM 2). Pairwise alignments revealed sequence similarities 87–93% of nucleotide identity among the following BESs: P136H07_T7 and P206C09_M13 (87%), P339N08_M13 and P382O23_T7 (98%), and P206C06_T7 and P447K03_T7 (93%).

BLASTN and BLASTX queries identified six BESs, which originated from BAC end sequencing of the positive clones for RGC251 (P136H07_M13, P136H07_T7, P206C06_T7, P206C09_M13, P447K03_T7, M483F03_M13) and RGC260 (P408L01_M13), with putative coiled coil (CC)-NBS-LRR function (ESM 2). Due to the aforementioned BES redundancy this number may not reflect the actual number of unique RGCs in the respective BAC clones. Yet, these BESs did not contain NBS or upstream domains and, therefore, RGCs could not be classified into TIR or non-TIR-NBS-LRR, with the exception of P136H07_M13. The latter included a non-TIR-like 'GLPL' motif, and shared 59–93% identity with the putative non-TIR-NSB-LRR peptides RGC250, RGC252, RGC251, and RGC15 in a 61 bp region covering 35.7% of the NBS domain.

## Isolation of NBS-LRR-encoding genes linked to $Pl_8$, $Pl_{14}$, and $R_{Adv}$

Shotgun sequencing of the BAC clones P339N08, P102A12, and P408L01, which were identified with overgo probes designed for RGC203, RGC251, and RGC260, respectively, yielded three contigs 131.9, 125.6, and 143.6 Kb in length (GenBank accessions HQ222360, HQ222361, HQ222362; Fig. 1). The 125.6-Kb contig of P102A12, contained two RGCs (P102-1 and P102-2), one of which (P102-2) corresponded to RGC251, whereas in P408L01 we identified three tandem RGCs (P408-1, P408-2, and P408-3), one of which (P408-3) resembled RGC260 (Fig. 1). The putative sequence of P408-1 is not complete because this gene was localized on the proximal end of the P408L01 BAC insert. Structural annotation of the P339N08 131.9-Kb contig revealed two putative RGCs (P339-1 and P339-2), one of which (P339-2) resembled RGC203. Gene prediction tools suggested that the NBS domain of P339-2 is truncated due to the presence of two introns. The putative peptide appeared out-of-frame and incorporated several stop codons in the coding region suggesting that, regardless of the validity of the intron prediction, P339-2 encodes a pseudogene. Despite the frequent occurrence of pseudogenes among RGCs (Meyers et al. 2003; Monosi et al. 2004; Radwan et al. 2008), six of the seven putative RGCs had intact open reading frames and likely encode functional NBS-LRR peptides. All seven putative RGCs of P102A12, P408L01, and P339N08 had conserved RNBS-A and Kin-2 motifs (Meyers et al. 1999) of non-TIR-NBS-LRRs (Figs. 2, 3). We also confirmed the presence of LRR domains with a minimum of five repeats in all seven putative RGCs using InterProScan (Zdobnov and Apweiler 2001).

Multiple alignment of NBS domains (P-loop to GLPL motif) using ClustalW (Larkin et al. 2007) showed that the nucleotide and amino acid sequences of P102-1 and P102-2 had 70 and 81% identity, respectively. Similarly, P408-1, P408-2, and P408-3 NBS domains had 69–91 and 77–93% identity, whereas the nucleotide sequence identity of P339-1 and P339-2 was 82%. NBS-LRR loci of linkage group 13 mined from P102A12 and P408L01 had highly similar NBS domains (70–72% nucleotide sequence identity), but were less similar with NBS-LRR loci of linkage group 1 mined from P339N08 (8–39% identity).

Nearly 80, 60, and 30% of the sequence of the P102A12, P408L01, and P339N08 BAC inserts encoded retroelements. In addition to the putative RGCs and retroelements, we also identified seven putative open reading frames with characterized domains; a serine-rich peptide with CCHC-type zinc finger domain in P102A12, a cyclin and a peptide with ubiquitin-associated domain in P408L01, an auxin response factor, an integrase, a helicase and an AMP binding protein in P339N08 (Fig. 1).

## Phylogenetic classification of NBS-LRR-encoding genes linked to $Pl_8$, $Pl_{14}$, and $R_{Adv}$

We used NBS domains of sunflower RGCs, which contain highly conserved motifs appropriate for the study of

resistance gene evolution (Plocik et al. 2004), to shed light into their phylogenetic relationships. The TIR- and non-TIR-NBS-LRR-encoding RGCs of sunflower (Radwan et al. 2008) clustered in distant branches of the bootstrap consensus tree (Fig. 3; ESM 3), as expected from the distinct motifs of their respective NBS domains (Fig. 2). We identified two separate clades of non-TIR-NBS-LRRs suggesting the presence of two non-TIR-NBS-LRR-encoding resistance gene families (Fig. 3; ESM 3), as previously observed in the Compositae family (Plocik et al. 2004). The P-loop/Kin-1a is the most conserved motif with six distinct patterns among the 783 TIR- and non-TIR-NBS-LRR-encoding RGCs. For Kin-2 and GLPL motifs 15 and 9 distinct patterns were identified, respectively (Fig. 3). In the non-TIR-NBS-LRR-encoding superfamily, all RGCs shared a common P-loop/Kin-1a motif, whereas two Kin-2 and two GLPL motifs were identified, which coincided with the two distinct non-TIR-NBS-LRR-encoding gene families (Fig. 3).

The seven putative RGCs mined from BAC clones P102A12, P408L01, and P339N08 and four RGCs (RGC15, RGC250, RGC251, and RGC252) mapping on the cluster of linkage group 13 were phylogenetically distant from the two NBS-LRR-encoding superfamilies (Fig. 3; ESM 3). RGC203 or other RGCs mapping on the cluster of linkage group 1 could not be included in the phylogenetic analysis because we are lacking sufficient sequence information on their NBS domains. For the same reason, only 71 of the 167 RGCs mapped in sunflower genome (Radwan et al. 2008) are present on the phylogenetic tree. Therefore, we could only verify the genetic relatedness of newly identified RGCs in P102A12 and P408L01 with previously identified RGCs of the cluster of linkage group 13 (RGC15, RGC250, RGC251, and RGC252). The three TIR-NBS-LRR-encoding RGCs (RGC111, RGC115, and RGC134) (Radwan et al. 2008), which also reside in the cluster of linkage group 13 but do not flank $Pl_8$ and $R_{Adv}$, clustered with the rest of sunflower TIR-NBS-LRRs (Fig. 3).

## Discussion

Seven NBS-LRR-encoding loci in three genomic regions flanking $Pl_8$, $Pl_{14}$ and $R_{Adv}$ genes on linkage groups 1 and 13 were identified. Although further investigation is necessary to test whether any of the six RGCs with intact reading frames are the causal genes for resistance to downy mildew and rust, these results suggest that they have non-TIR-like motifs and are phylogenetically distant from the two non-TIR-NBS-LRR-encoding resistance gene families (Fig. 3; ESM 3). Current knowledge of the genomic localization and sequence similarity of RGCs on linkage group 13 implies that they were derived from tandem duplications as a result of unequal crossing-over; however, the genetic relatedness of the predicted RGCs of linkage groups 1 and 13 suggests that these NBS-LRR-encoding RGCs may have translocated in the sunflower genome. Further study could also determine whether the genomic regions of linkage groups 1 and 13 constitute heterogeneous clusters formed through segmental or ectopic duplication events and how natural selection has shaped these clusters (Leister 2004; Meyers et al. 2005; 2003). The drastically different NBS domains of these novel non-TIR-like RGCs offers an unprecedented opportunity to gain a deeper understanding of the genomic organization, genetic function and evolution of resistance gene clusters in plant genomes.

In addition to the putative RGCs, functional annotation revealed that retrotransposons were ubiquitous among the open reading frames identified in P102A12, P408L01, and P339N08. Repetitive elements are thought to evolve to regulatory regions of downstream genes and facilitate homologous recombination, translocations and gene conversions (Henikoff et al. 1997). In the proximity of resistance genes, transposable elements increase the chance of unequal crossing-over and alter RGCs' expression patterns (Michelmore and Meyers 1998; Richter and Ronald 2000). Thereby, the abundance of Ty1-copia and Ty3-gypsy retrotransposons flanking the seven predicted RGCs may have played an important role in their evolution.

Downy mildew resistance in ZENB8 × RHA340 is conferred by $Pl_8$ gene, which was introgressed from silverleaf sunflower (*H. argophyllus* Torrey and Gray) to RHA340 by phenotypic selection. The introgressed genomic segment harbors the RGC cluster of linkage group 13 and appears to be duplicated in silverleaf sunflower genome (Heesacker et al. 2009). Indeed, the RGC cluster resides on two chromosomes of silverleaf sunflower (ARG7/13-1 and ARG7/13-2), which were probably derived from fusion and duplication of two common sunflower chromosomes (ANN7 and ANN13) (Heesacker et al. 2009). Despite the different chromosomal architecture of common and silverleaf sunflower and the suppressed recombination observed in the genetic map of silverleaf sunflower, the genomic segment flanking $Pl_8$ was successfully introgressed into RHA340. The genetic distance of linkage group 13 RGCs, which were mined from P102A12 and P408L01 or identified previously (RGC15, RGC250, RGC251, and RGC252), from the two sunflower NBS-LRR-encoding superfamilies suggests that these highly similar RGCs may have actually originated from tandem duplication of RGCs introgressed from silverleaf sunflower, and underlines the value of silverleaf sunflower as donor of novel genes with new resistance specificities.

The results of this study are directly applicable to sunflower breeding programs for the marker-assisted introgression of downy mildew and rust resistance genes in elite sunflower germplasm using DNA markers tightly linked to $Pl_8$, $Pl_{14}$, and $R_{Adv}$. Pyramiding disease resistance genes of these RGC clusters can be advantageous because the cluster of linkage group 13 confers broad-spectrum resistance to two unrelated pathogens, *P. halstedii* and *P. helianthi*, whereas both linkage groups 1 and 13 RGC clusters confer resistance to several isolates of *P. halstedii*.

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