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# Use of SNP markers by KASP assay for MAS studies in sunflower against *Plasmopara halstedii*

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**Abstract:** Downy mildew is a fungal disease caused by *Plasmopara halstedii* and leads to loss of yield up to 100% in sunflower. Disease control is performed mostly through chemical seed treatment and breeding. Due to the time consuming nature of conventional breeding, it is supported by biotechnological approaches. Marker-assisted selection (MAS) is a strategic approach in molecular breeding using molecular markers. Single nucleotide polymorphisms (SNPs) such as insertions, deletions, and base-pair substitutions are more advantageous than other molecular markers. The abundance and biallelic nature of SNPs in a genome provide flexibility in the choosing of SNPs at the desired loci. Competitive allele-specific PCR (KASP) is a genotyping technology for screening of trait-specific SNP markers. In this study, SNP markers (NSA002867, NSA006138; NSA000052, NSA000354; NSA002220, NSA002251) linked with the downy mildew resistance genes $Pl_{arg}$, $Pl_{13}$, and $Pl_8$, respectively, were analyzed via KASP in three parental crosses (RHA-419 × Colombi, RHA-419 × P64LC53, RHA-419 × Oliva) for $Pl_{arg}$, one parental cross (HA-R5 × P64LC53) for $Pl_{13}$, one parental cross (P64LC53 × HA-89) for $Pl_8$, and 140 $F_2$ individuals. According to the allelic discrimination results, NSA002867 and NSA006138 markers were discriminative in all crosses for $Pl_{arg}$, NSA000354 marker was discriminative for $Pl_{13}$, and NSA002220 and NSA002251 markers were discriminative for $Pl_8$. This study has revealed the potential use of SNP markers in combination with KASP assay for MAS studies, particularly downy mildew resistance in sunflower.

**Key words:** Downy mildew, KASP, MAS, SNP, sunflower, resistance genes

## 1. Introduction

Sunflower (*Helianthus annuus* L.) is a very important oil and protein source as it contains high amounts of oil (Hu et al., 2010). While sunflower represents 11% of oilseed worldwide, it supplies 47% of vegetable oil production in Turkey, according to reports by the Food and Agriculture Organization of the United Nations (FAO) released in 2014.

Downy mildew, a common disease caused by *Plasmopara halstedii*, negatively affects the yield in sunflower production (Tozlu, 2008). The appearance of white cottony masses on the underside of leaves in infected plants is the main symptom of this disease (Force et al., 2015). Downy mildew has a great economic impact on sunflower crops, causing crop loss up to 100% (Apaydın et al., 2008). Disease control is performed mostly by chemical seed treatment. Mefenoxam is generally used to control downy mildew in sunflowers. However, this approach cannot be effective for a long time because the pathogen develops immunity to chemicals. Accumulation of chemicals in the soil leads to a carcinogenic effect on living beings also (Gascuel et al., 2015). One disease control approach is biological control, which is based on using a microorganism as a biocontrol agent. It is applied as a seed treatment or via foliar application (Umesha et al., 1998). However, biocontrol agents are not sufficient and 100% effective for disease control. Therefore, alternative and environmentally friendly solutions are required in agriculture. With the developing technology, biotechnological applications have improved to support traditional breeding, which causes loss of money and time. Molecular breeding, a biotechnological approach in agriculture, makes it possible to identify genetically resistant hybrids and select the best offspring. This way, the hybrid with the desired traits will spread throughout the agricultural area (Gascuel et al., 2015).

Compared to conventional methods, marker-assisted selection (MAS) is a promising application of DNA markers in molecular breeding. Molecular markers can be used for MAS to enhance the efficiency of plant breeding

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(Kim et al., 2016). Molecular markers in a genome occur by some mutations, such as structural mutations (inversions, translocations, duplications, and deletions) and point mutations (base pair substitutions, insertions, and deletions) (Force et al., 2015). Single nucleotide polymorphisms (SNP) are point mutations found in a genome as insertions, deletions, or base pair substitutions. SNPs are very advantageous in comparison with other DNA markers. An abundance of SNPs in a genome provides flexibility in choosing SNPs at the desired loci. Another advantage of SNPs, in comparison with gel-based markers, is the rapid processing of large populations without needing intensive labor. In addition, due to the biallelic nature of SNP markers, they are very suitable for diploid sunflowers (Lai et al., 2005; Thomson, 2014).

In MAS studies, various microarray-based assays are used for high-throughput analysis. The competitive allele-specific PCR (KASP) genotyping assay is a novel, homogeneous, and fluorescence-based application that enables the identification and computation of SNPs, such as insertions and deletions (He et al., 2014). KASP is suitable for SNP genotyping at 90% and it is more powerful and cost-effective than other approaches. Moreover, it is reliable, repeatable, and flexible in terms of the number of samples as well as the number of SNPs (LGC, https://www.lgcgroup.com/products/kasp-genotyping-chemistry). Recently, KASP was applied to wheat (Chhetri et al., 2017; Tan et al., 2017), soybean (Shi et al., 2015), maize (Semagn et al., 2014), rice (Thomson et al., 2014), and peanut (Khera et al., 2013) using SNP markers in MAS studies for crop improvement. In addition to these studies, KASP was used to discriminate *Plasmopara halstedii* pathotypes based on the polymorphism of pathogenicity effectors (Gascuel et al., 2016).

Sunflowers have *Pl* resistance genes against *Plasmopara halstedii*. $Pl_{arg}$, $Pl_{13}$, and $Pl_{8}$, which are known to remain active in agriculture, have been reported as effective against many races of *P. halstedii* (Jocić et al., 2010; Forrest et al., 2014; Qi et al., 2017). $Pl_{arg}$ and $Pl_{13}$ loci were mapped on LG1, and a $Pl_{8}$ locus was mapped on LG13 (Gascuel et al., 2015). The aim of the study was to identify the SNP markers related to $Pl_{arg}$, $Pl_{13}$, and $Pl_{8}$ by scanning the sunflower gene pool. Additionally, the use of SNP markers with KASP will be spread in the agricultural area, which will enable resistant sunflowers to be distinguished from susceptible ones. In the short term, the SNP markers analyzed with KASP assay in this study will help MAS studies control downy mildew in sunflower breeding programs. In addition, the study can contribute to increasing sunflower yield in the long term by spreading sunflower lines resistant to downy mildew.

## 2. Materials and methods

### 2.1. Plant materials

Based on the phenotypic evaluations and the scoring of disease symptoms on sunflower genotypes by Trakya Agricultural Research Institute, RHA-419, HA-R5 and P64LC53 were reported as resistant genotypes for $Pl_{arg}$, $Pl_{13}$, and $Pl_{8}$, respectively. Moreover, RHA-419 and HA-R5 were reported by different studies to include $Pl_{arg}$ and $Pl_{13}$, respectively (Dußle et al., 2004; Mulpuri et al., 2009; Qui et al., 2017).

Twenty-three $F_2$ individuals derived from the cross of RHA-419 × Colombi, twenty-six $F_2$ individuals derived from the cross of RHA-419 × Oliva, and thirty-eight $F_2$ individuals derived from the cross of RHA-419 × P64LC53 were analyzed for $Pl_{arg}$ . In the seed sector, the Colombi genotype is listed as tolerant for downy mildew (https://www.syngenta.com.tr/product/seed/colombi). Oliva is recorded as resistant to $Pl_{6}$ races of *P. halstedii*; however, the $Pl_{6}$ gene has been overcome by *Plasmopara halstedii* (http://www.may.com.tr/urun/oliva-cl). HA-89 was recorded as susceptible by Mulpuri et al. (2009).

Thirty $F_2$ individuals derived from the cross of HA-R5 × P64LC53 were analyzed for $Pl_{13}$. Twenty-three $F_2$ individuals derived from the cross of P64LC53 × HA-89 were analyzed for $Pl_{8}$. The sunflower breeding material (parents and their $F_2$ individuals) used in this study, linkage groups, resistance genes, and selected SNP markers are shown in Table 1.

### 2.2. Genomic DNA isolation

Doyle and Doyle's (1987) method was applied to plant samples for genomic DNA isolation. The quantity of genomic DNAs was checked with a Qubit 2.0 fluorometer and genomic DNA was imaged via agarose gel electrophoresis.

### 2.3. SNP markers and KASP assay

SNP markers were obtained from the National Sunflower Association, (NSA; https://www.sunflowernsa.com) as part of the Research Projects in National Program 301, 2006–2011. SNP markers linked with $Pl_{arg}$, $Pl_{13}$, and $Pl_{8}$ were analyzed with KASP in the sunflower gene pool. Two SNP markers (NSA002867, NSA006138) linked with $Pl_{arg}$, two SNP markers (NSA000052, NSA000354) linked with $Pl_{13}$, and two SNP markers (NSA002220, NSA002251) linked with $Pl_{8}$ were selected from the linkage groups 1 and 13, respectively, as shown in Figure 1.

Sources of NSA002867 and NSA006138 SNP markers linked with $Pl_{arg}$ are RHA-419 and RHA-420 genotypes. RHA-419 and RHA-420 genotypes were recorded, including $Pl_{arg}$. NSA000052 and NSA000354 SNP markers were generated from ORS965 and ORS716 polymorphic microsatellites, respectively, both of which are in linked group 1 (Lai et al., 2005). NSA002220 and NSA002251 SNP markers were developed from the loci of single-strand conformation polymorphism markers by the NSA and derived from a RHA-340 resistance source (Qui et al., 2017; NSA, https://www.sunflowernsa.com) (Table 2).

**Table 1.** Breeding material and SNP markers used in this study.

| Linkage group | Gene | SNP markers | Crosses | | Number of $F_2$ |
|---|---|---|---|---|---|
| | | | Resistant | Susceptible | |
| LG 1 | $Pl_{arg}$ | NSA002867 NSA006138 | RHA-419 | Colombi | 23 |
| | | | RHA-419 | Oliva | 26 |
| | | | RHA-419 | P64LC53 | 38 |
| | $Pl_{13}$ | NSA000052 NSA000354 | HA-R5 | P64LC53 | 30 |
| LG 13 | $Pl_8$ | NSA002220 NSA002251 | P64LC53 | HA-89 | 23 |

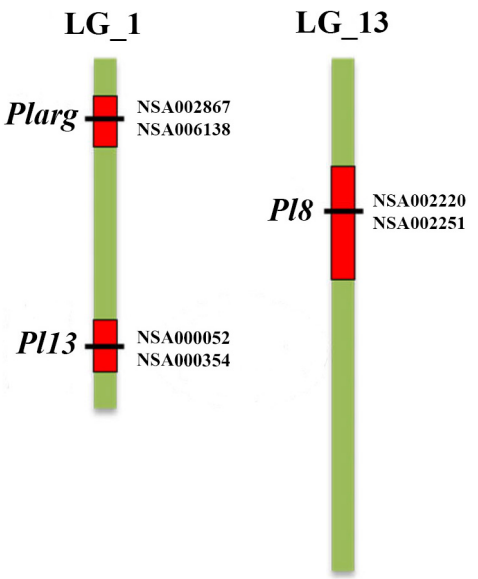


**Figure 1.** Genetic linkage map of $Pl_{arg}$, $Pl_{13}$, and $Pl_8$ and related SNP markers (NSA, https://www.sunflowernsa.com; Gascuel et al., 2015).

The SNP markers used in this study, closely linked to $Pl_{arg}$, $Pl_{13}$, and $Pl_8$ on chromosome arms 1 and 13, were converted to KASP assays and SNPs were scanned in sunflower breeding materials. SNP primer sequences, including forward-1 for allele 1, forward-2 for allele 2, and common primers are given in Table 3.

MicroAmp Optical 96-well Reaction Plates (Thermo Fisher, Waltham, MA, USA) and its adhesive covers were used for reaction media. The KASP kit used includes a master mix and an assay mix. The assay mix contains the allele-specific 2 forward primers and 1 reverse primer. The master mix includes FRET cassettes, which contain fluorescent dyed molecules (FAM and HEX dyes) in addition to PCR components such as reaction buffer, Taq polymerase enzyme, and dNTPs. The master mix should be kept away from the light because of the unwanted reaction between FRET cassettes labelled with dyes. KASP assays were run with 10.28 µL of reaction system, including a 10 µL of master mix (Global Genetik Ltd., İstanbul, Turkey), 0.28 µL of assay mix, and 10 µL of 2 ng/µL genomic DNA. The thermocycling conditions for KASP were 94 °C for 15 min, followed by 10 cycles at 94 °C for 20 s, and decreased from 61 °C to 57 °C by 0.4 °C per cycle, followed by 26 cycles at 94 °C for 20 s and 57 °C for 1 min. KASP fluorescent endpoint readings were performed at 30 °C for 1 min by using the Applied Biosystems StepOnePlus Real-Time PCR Systems (Foster City, CA, USA) (Table 4).

## 3. Results and discussion

SNP markers (NSA002867, NSA006138; NSA000052, NSA000354; NSA002220, NSA002251) linked with $Pl_{arg}$, $Pl_{13}$, and $Pl_8$, respectively, were analyzed via KASP in the sunflower gene pool.

Initially, SNPs were genotyped in parents and SNPs that showed polymorphism between the parents were then genotyped in the $F_2$ generation. Analysis with NSA002867 containing A/G base pair substitution indicated that RHA-419 is heterozygous, with alleles 1 and 2 exhibiting luminescence (1.0256 and 0.1637 frequency), and that Colombi is homozygous with respect to allele 1 (A1: 1.2939, A2: 0.0963 frequency), as shown in Figure 2a1. While 10 of the 23 $F_2$ individuals were heterozygous (A1: 0.3227–0.7174; A2: 0.1456–0.3000 frequency ranges), 10 $F_2$ individuals were homozygous (A2: 0.3513–0.4182 frequency ranges) and 3 $F_2$ individuals were homozygous and susceptible character after crossing with Colombi (A1: 0.0758–0.1103 frequency ranges). Analysis with NSA006138 containing a -/A single base deletion revealed that RHA-419 was heterozygous, with allele 1 and 2 luminescence (0.1446 and 0.3454 frequency) and that Colombi was homozygous with respect to allele 2 (A1:-

**Table 2.** Sources of SNP sequences for $Pl_{arg}$, $Pl_{13}$, and $Pl_{8}$ (NSA).

| Gene | SNP marker name | SNP source | SNP Sequences |
|---|---|---|---|
| $Pl_{arg}$ | *NSA002867* | RHA-419 | CGCTTCAATTAGGCTTTCGGGTAAAGTGGGGGCCACCTCAGGGTGTTCTTTTATAGCAG GACGAACTTGTGTGCGCTCTAAAAATGTTTCCTTTACTTGTTTCAGCTTTTTATACACCGA TTCTGCTTGTTCTTCGTCGCAGCTGAAATCAAAGTGCTTCTTTGCAAG[**A/G**]GCAAACGAC TCACTCCTGTCGATTTTATTTTTCGCTAGAGAAGAACCAACCCAACACTAAAACATGAAAA AAATCATGTCGGTAAATACGTTAAATATTTTCAAAAAAAGAGAGCATTCAGTTTGTTAATA GATATTAAATTTAAATACCAGTGATATCATGAAAGCCTGT |
| | *NSA006138* | RHA-420 | AACATAACTAATTAAACGTTAACACTTAAGCTGGTTTCCAGTTCCAATTGAAATATTCGCA AAAACTCTTTGTTCTACAAACATGATTAGTAATTTAGTTCAATTTCCATTTTTTTCAGAAGA ACGCTGACGTCATAACAAACAACCCTAAAAAATAACAAAACCGAACATTTCACACAGAAA [-/**A**]TCAGATCTTAAAACAAACACTAACAGATCTCAACACATAATGAAACCTAATCTTACA AAAATAAAAAGAAATCTCAACGAAACTAACAATTATAAGAACATTGAAAGAAGACTA |
| $Pl_{13}$ | *NSA000052* | ORS965 | GCGATTGTTTTCGTCCACCGAGATTCTTCGCTTCATAAAACAATTAAGAGAAAGTGGGATAG ATAGATAGATCGGAGCAAGAAAGATGACCAGAGAATCTTGATTTCAAGAAAAGTTAACGGT TATCAGTCACCCA[**T/C**]TAAAACACATACACCATTTAACCTTATATTATATGTCATCTCTTTAC ATGCATATGTATATATATATGTATATATATATGTTGAGAATTGAGGTTGAAGATTGGCATCTT GAAGAAAAGAAAATGGTTGCTGTCTGTGTGATCTCTGAGAATTAAAAGTTTTAAATAGTTGTT AAAAGGCAATTTTGCCGGTAAAGGAGAATTACTAAATGCAGAATGGTCCTTGGAGATGGAGT GATGTACATAATATGCCCC |
| | *NSA000354* | ORS716 | ATCCCCACATGGAATTCGGCGCTGGTAACTTCATCTTCTATCTCATCGTCGAAGCTTGCCAATCT ATCATCCCTGCAATGTCAACCACACCTTCCTACTCCTCGTCCGGCGCTCACTTATCGTCGAAGCA AGCGTTCATCGCTTCCCATAGTAATCTCTCTCTCTCTCTCTTCTTTTTTCATTCATTTGCCTTATAC CATAGGTCTTCTGTCTTTAATTAGCATTTCAACTGAAACCAAATTACTATC[**T/C**]GTCCGTACAAT TAGCTAGGTTTCTGATTTTATTCATGTCCACAACTTAACTTAAACAGGTGGTCGTG |
| $Pl_{8}$ | *NSA002220* | RHA-340 | TTTAGGAAAACAAAAGATGCAAACAAGAAGAGGTCTTTGGAGTCAAGAACTTGTCCTTAT TACCATGAGTTAAGTAAATTATACAA[**T/C**]CAAGAGAAGCTAGCATCATTGTCCAACTCAG CCGGAGAGGAGCTACTCGCGACCAAAATTACACCGGAAAACTGATGATGTGTGTTCACA GATCAATGGTATATGAGTTCAGAATGATCGAGGCCTAATAACTAGACTTCCAATGCGCGG GTGTGTCTTTTTACTACTATATTTTGGTACATTTT |
| | *NSA002251* | RHA-340 | AAGTATTGGGACATTGTATGAAGCAGCAGGCAAGCTTGACCCCATGCAGCATTTATCTCA TCCCACTCAACCTAACAAACAAAGATTTAAACAAGAAAACATCTTGACAGCAAGCAAGCC CACCAATTTGATTATAAGTGCAAGCAACATACTGGAATTTTAGGCAGTCTTCCCAGCCTG AAATTGTTAATTGTTCC[**A/G**]AATTCTCCATCATACCAAATTGGAAATGCATCATTCAGGAC ATTCGTTCTCTTCAGAAGTTCTAAATGTGCTTGTGATACTTCTATTTTGGCCAATATTGCAT CTCTCTCTTCCTGCCAGCCACAACAAACAGAGTTTAATATTACAAAGGTCAAAGAAAGCA ACTAACTGCCTGCCTAACATATAATATTATATCTGATTCCATGGCCACTGCAAATAAATG |

0.0024, A2: 0.3921 frequency), as shown in Figure 2.b1. While 10 of the 23 $F_2$ individuals were heterozygous and resistant (A1: 0.1003–0.3688; A2: 0.0817–0.2587 frequency ranges), 10 $F_2$ individuals showed homozygous resistance character (A1: 0.4077–0.6107 frequency ranges) and 3 $F_2$ individuals were homozygous and susceptible after crossing with Colombi (A2: 0.3204–0.3785 frequency ranges).

Based on greenhouse and field observations, Oliva is regarded as susceptible concerning $Pl_{arg}$. Analysis with NSA002867 demonstrated that Oliva was homozygous with respect to allele 1 (A1:1.2419, A2:0.0890 frequency) and RHA-419 was heterozygous, with allele 1 and 2 exhibiting luminescence (1.0027 and 1.6153 frequency), as shown in Figure 2a2. While 17 of the 26 $F_2$ individuals were heterozygous and resistant (A1: 0.5199–0.7240; A2: 0.2253–0.2908 frequency ranges), 4 $F_2$ individuals were homozygous and resistant (A1: 1.1918–1.2681 frequency ranges) and 5 $F_2$ individuals were homozygous and susceptible (A2: –0.0100 to 0.0002 frequency ranges) after crossing with Oliva. Analysis with NSA006138 proved that Oliva was homozygous with respect to allele 2 (A1: –0.0124, A2: 0.3761 frequency) and that RHA-419 was heterozygous, exhibiting allele 1 and 2 luminescence (0.1262 and 0.3256 frequency), as shown in Figure 2b2. While 17 of the 26 $F_2$ individuals were heterozygous and resistant (A1: 0.2451–0.3244; A2: 0.1736–0.2811 frequency ranges), 4 $F_2$ individuals were homozygous and resistant

**Table 3.** Sequences of two forward and common reverse KASP primers for each SNP.

| SNP | KASP primers | Primer sequences (without tail sequences) |
|---|---|---|
| [A/G] | NSA002867_T | 5'CGACAGGAGTGAGTCGTTTGCT'3 |
| | NSA002867_C | 5'GACAGGAGTGAGTCGTTTGCC'3 |
| | Common reverse | 5'GCTTGTTCTTCGTCGCAGCTGAAAT'3 |
| [-/A] | NSA006138 | 5'GTTAGTGTTTGTTTTAAGATCTGATTTC'3 |
| | NSA006138_A | 5'ATCTGTTAGTGTTTGTTTTAAGATCTGATTTT'3 |
| | Common reverse | 5'CGCTGACGTCATAACAAACAACCCTA'3 |
| [T/C] | NSA000052_A | 5'ATAATATAAGGTTAAATGGTGTATGTGTTTTA'3 |
| | NSA000052_G | 5'ATAATATAAGGTTAAATGGTGTATGTGTTTTG'3 |
| | Common reverse | 5'AAGAAAAGTTAACGGTTATCAGTCACCCA'3 |
| [T/C] | NSA000354_A | 5'CAGAAACCTAGCTAATTGTACGGACA'3 |
| | NSA000354_G | 5'AGAAACCTAGCTAATTGTACGGACG'3 |
| | Common reverse | 5'GCCTTATACCATAGGTCTTCTGTCTTTAA'3 |
| [T/C] | NSA002220_A | 5'GGACAATGATGCTAGCTTCTCTTGA'3 |
| | NSA002220_G | 5'GACAATGATGCTAGCTTCTCTTGG'3 |
| | Common reverse | 5'GAACTTGTCCTTATTACCATGAGTTAAGTA'3 |
| [A/G] | NSA002251_T | 5'CATTTCCAATTTGGTATGATGGAGAATTT'3 |
| | NSA002251_C | 5'CATTTCCAATTTGGTATGATGGAGAATTC'3 |
| | Common reverse | 5'GTCTTCCCAGCCTGAAATTGTTAATTGTT'3 |

**Table 4.** KASP thermocycling conditions for SNP markers for $Pl_{arg}$, $Pl_{13}$, and $Pl_{8}$.

| Step | Temperature | Time (min) |
|---|---|---|
| Stage 1 (pre-PCR read) | 30 °C | 1:00 |
| Stage 2 (holding stage) | 94 °C | 15:00 |
| Stage 3 (10 cycles) | 94 °C | 00:20 |
| | 61 °C | 1:00 |
| Stage 4 (26 cycles) | 94 °C | 00:20 |
| | 57°C | 1:00 |
| Post-PCR read | 30 °C | 1:00 |
| Holding stage | 4 °C | ∞ |

(A1: 0.4757–0.5603 frequency ranges) and 5 $F_2$ individuals were homozygous and susceptible (A2: –0.0290 to –0.0095 frequency ranges) after crossing with Oliva.

P64LC53 is regarded as susceptible with respect to $Pl_{arg}$. Analysis with NSA002867 revealed that P64LC53 was homozygous with respect to allele 1 (A1:1.2486, A2:0.0942 frequency) and RHA-419 was heterozygous, showing allele 1 and 2 luminescence (0.9949 and 0.1433 frequency) as shown in Figure 2a3. While 18 of the 38 $F_2$ individuals were heterozygous and resistant (A1: 0.7464–0.4745; A2: 0.2414–0.2974 frequency ranges), 8 $F_2$ individuals were homozygous and resistant (A1: 0.3208–0.3857 frequency ranges) and 12 $F_2$ individuals were homozygous and susceptible (A2: 0.9355–1.2698 frequency ranges) after crossing with P64LC53. Analysis with NSA006138 revealed that P64LC53 was homozygous with respect to allele 2 (A1:0.0057, A2:0.3834 frequency) and RHA-419 was heterozygous, with allele 1 and 2 exhibiting luminescence (0.1276 and 0.3276 frequency), as shown in Figure 2b3. While 18 of the 38 $F_2$ individuals were heterozygous and resistant (A1: 0.4636–0.3434; A2: 0.2404–0.2912 frequency ranges), 8 $F_2$ individuals were homozygous and resistant (A1: 0.1575–0.3867 frequency ranges) and 12 $F_2$ individuals were homozygous and susceptible (A2: 0.1491–0.3753 frequency ranges) after crossing with P64LC53.

According to phenotypic evaluations, HA-R5 and P64LC53 are resistant and susceptible with respect to $Pl_{13}$, respectively. Mulpuri et al. (2009) recorded that HA-R5 includes $Pl_{13}$ with simple sequence repeat (SSR) markers. In the analysis of HA-R5 × P64LC53 parents with NSA000052 markers containing a T/C base-pair substitution, no polymorphism was seen between the parents, as shown in Figure 3a. It may be concluded that the NSA000052 marker is not distinctive for the HA-R5 × P64LC53 cross. Analysis with NSA000354 containing

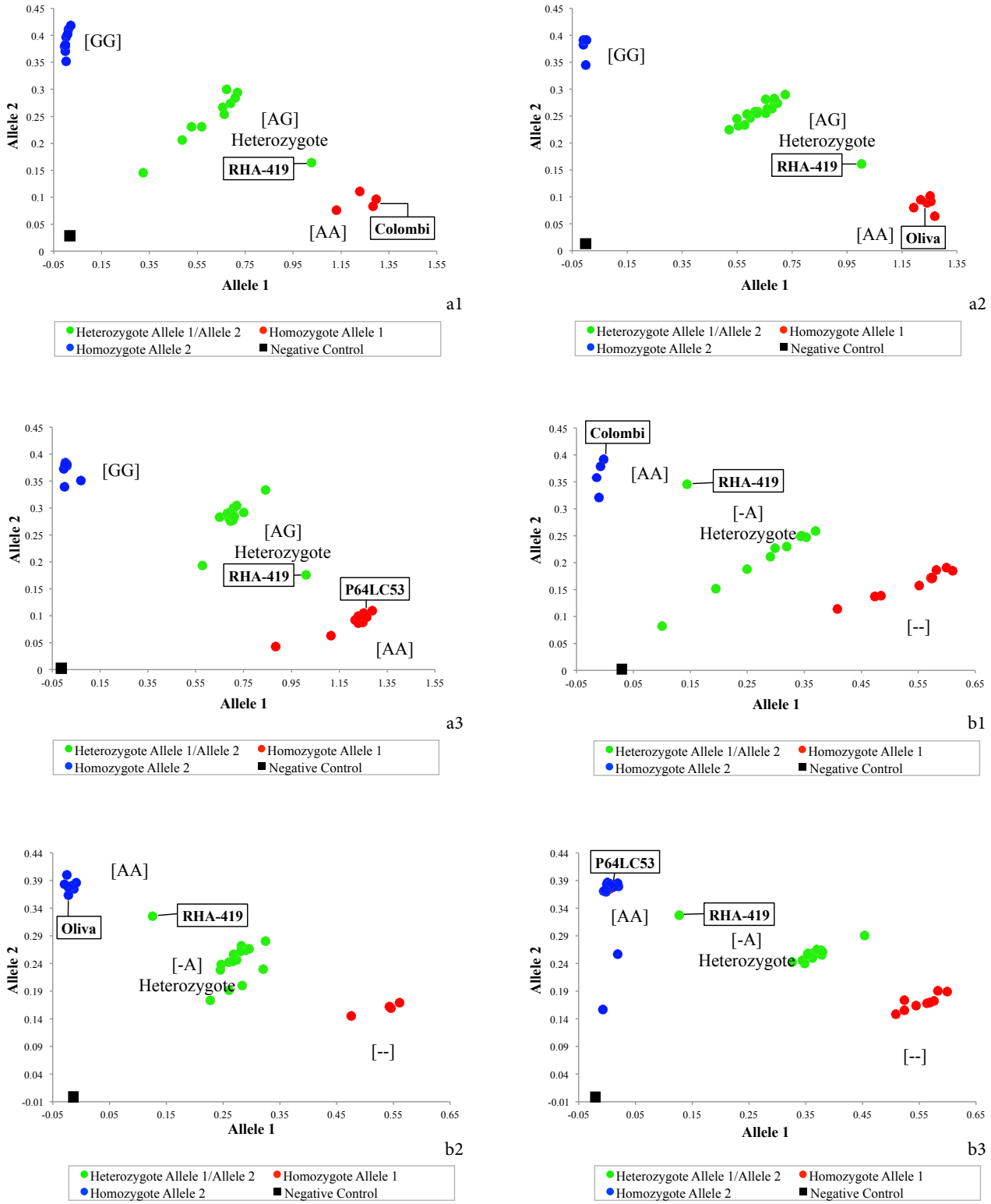


**Figure 2.** Allelic discrimination plot of SNPs (NSA002867, NSA006138) for $Pl_{arg}$ in 3 parental crosses (RHA-419 × Colombi, RHA-419 × Oliva, RHA-419 × P64LC53). a1. RHA-419 × Colombi with NSA002867 for $Pl_{arg}$ a2. RHA-419 × Oliva with NSA002867 for $Pl_{arg}$ a3. RHA-419 × P64LC53 with NSA002867 for $Pl_{arg}$ b1. RHA-419 × Colombi with NSA006138 for $Pl_{arg}$ b2. RHA-419 × Oliva with NSA006138 for $Pl_{arg}$ b3. RHA-419 × P64LC53 with NSA006138 for $Pl_{arg.}$

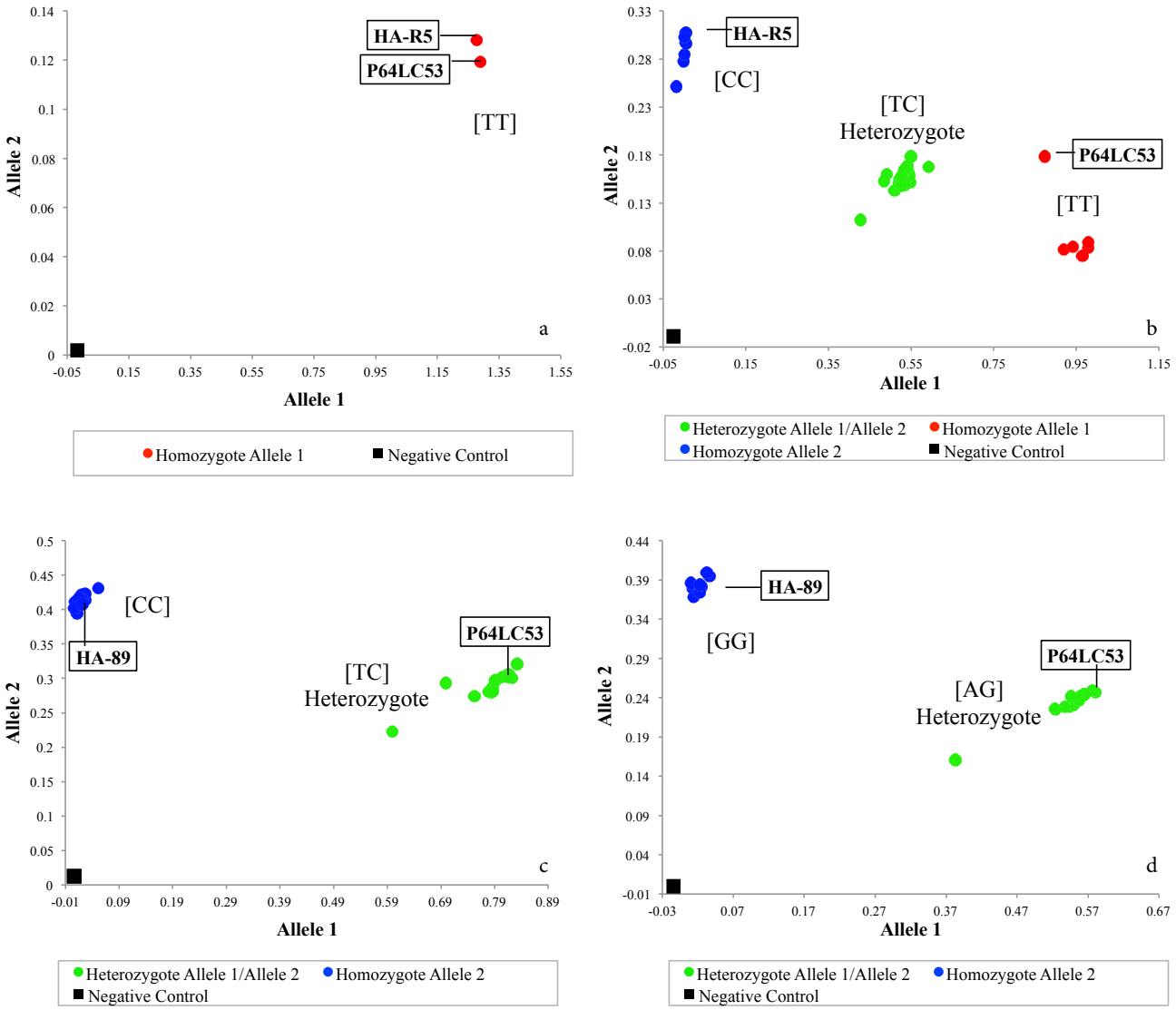


**Figure 3.** Allelic discrimination plot of SNPs (NSA000052, NSA000354; NSA002220, NSA002251) for $Pl_{13}$ and $Pl_{8}$, respectively, in 2 different parental crosses (HA-R5 × P64LC53; P64LC53 × HA-89). a. HA-R5 × P64LC53 with NSA000052 for $Pl_{13}$ b. HA-R5 × P64LC53 with NSA000354 for $Pl_{13}$ c. P64LC53 × HA-89 with NSA002220 for $Pl_{8}$ d. P64LC53 × HA-89 with NSA002251 for $Pl_{8}$.

the T/C base-pair substitution revealed that HA-R5 was homozygous with respect to allele 2 (A1: 0.0043, A2: 0.3075 frequency) and P64LC53 was homozygous with respect to allele 1 (A1: 0.8733, A2:0.1784 frequency), as shown in Figure 3b. While 18 of the 30 $F_2$ individuals were heterozygous (A1: 0.4279–0.5923; A2: 0.1126–0.1788 frequency ranges), 7 $F_2$ individuals were homozygous with respect to allele 2 and resistant (A2: 0.2513–0.3023 frequency ranges) after crossing with HA-R5, and 5 $F_2$ individuals were homozygous with respect to allele 1 (A1: 0.9199-0.9790 frequency ranges) and susceptible.

According to greenhouse and field observations, P64LC53 and HA-89 are resistant and susceptible regarding the $Pl_{8}$ gene, respectively. Analysis with NSA002220 containing a T/C base pair substitution showed that P64LC53 was heterozygous, with allele 1 and 2 showing luminescence (0.8132 and 0.3056 frequency), and that HA-89 was homozygous with respect to allele 2 (A1: 0.0079, A2: 0.4118 frequency), as shown in Figure 3c. While 11 of the 23 $F_2$ individuals were heterozygous and resistant (A1: 0.5988–0.8315; A2: 0.2233–0.3214 frequency ranges) after crossing with P64LC53, 12 $F_2$ individuals were homozygous and susceptible (A2: 0.3948-0.4310 frequency ranges) after crossing with HA-89. Analysis of P64LC53 × HA-89 and 23 $F_2$ individuals with NSA002251 marker yielded similar results to the analysis with

**Table 5.** Discrimination results of SNP markers by KASP assay (R: resistant, S: susceptible).

| Crosses | | Discrimination of SNP markers | |
|---|---|---|---|
| $Pl_{arg}$ | | NSA002867 | NSA006138 |
| RHA-419 [R] | P64LC53 [S] | Discriminative | Discriminative |
| RHA-419 [R] | Colombi [S] | Discriminative | Discriminative |
| RHA-419 [R] | Oliva [S] | Discriminative | Discriminative |
| $Pl_{13}$ | | NSA000052 | NSA000354 |
| HA-R5 [R] | P64LC53 [S] | Indiscriminate | Discriminative |
| $Pl_{8}$ | | NSA002220 | NSA002251 |
| P64LC53 [R] | HA-89 [S] | Discriminative | Discriminative |

NSA002220 marker. P64LC53 was heterozygous, with allele 1 and 2 showing luminescence (0.5799 and 0.2465 frequency) and HA-89 was homozygous with respect to allele 2 (A1: 0.0203, A2: 0.3789 frequency). According to the allelic discrimination plot of the results of the analysis with NSA002251 marker in Figure 3d, while 13 of the 23 $F_2$ individuals were heterozygous and resistant (A1: 0.3829–0.5763; A2: 0.1611–0.2493 frequency ranges) after crossing with P64LC53, 10 $F_2$ individuals were homozygous and susceptible (A2: 0.3682–0.3996 frequency ranges) after crossing with HA-89.

All SNPs except NSA000052 had compatible results in terms of downy mildew phenotype and genotype, as shown in Table 5. NSA002867 and NSA006138 could give discriminative results in segregation of all parental crosses for $Pl_{arg}$. NSA000354 could separate the cross of HA-R5 × P64LC53 as allele 2 homozygous (C) for HA-R5 and allele 1 homozygous (T) for P64LC53; however, NSA000052 could not classify these parents for $Pl_{13}$. P64LC53 and HA-89 genotypes showed heterozygous and homozygous patterns, respectively, for $Pl_{8}$ with NSA002220 and NSA002251 markers in the allelic discrimination plot.

The first genetic maps of sunflower for downy mildew were based on random amplification of polymorphic DNA (Mouzeyar et al., 1995), restriction fragment length polymorphism (Roeckel-Drevet et al., 1996; Vear et al., 1997; Gentzbittel et al., 1999), nucleotide binding site (Gedil et al., 2001), and non-Toll/interleukin-1 receptor–nucleotide binding site–leucine-rich repeats) (Radwan et al., 2003). Microsatellite markers (SSRs) that provide higher density genetic maps than other markers were then developed. The first study using maps based on SSRs was performed by Tang et al. (2002). Most *Pl* genes were identified with microsatellites in sunflowers (Yu et al., 2003; Kursterer et al., 2004; Mulpuri et al., 2009; Gascuel et al., 2015). The study into *Pl* genes in sunflower provided two SSR markers (ORS1008 and expressed sequence tag) linked with $Pl_{16}$ and $Pl_{13}$ (Liu et al., 2012). There is another study on the use of STS markers $Pl_{5}$, $Pl_{6}$, and $Pl_{8}$ associated with *Plasmopara halstedii* resistance in sunflower, according to an article published by Usatov et al. (2014). Currently, molecular marker technology is developing with SNP genotyping. SNP markers closely linked with rust-resistance gene $R_{12}$ were studied in $F_2$ sunflower mapping populations (HA-89 × RHA464) by Talukder et al. (2014). In a study carried out by Long et al. (2016), both SNPs and SSRs were used to identify molecular markers linked with the $Pl_{17}$ gene in sunflower. The latest study on SNP markers for downy mildew resistance was carried out by Qui et al. (2017). In that study, SNP markers linked with $Pl_{arg}$ and $Pl_{8}$ were developed and the SNPs were tested in sunflower breeding material. According to the PCR gel pictures of the study, RHA-419 carries $Pl_{arg}$ with NSA002867 and NSA006138. NSA002867 and RHA-419 markers carry a single G nucleotide, and NSA006138 and RHA-419 have a deletion. Moreover, HA-89 does not carry $Pl_{8}$ with NSA002220 and NSA002251. These results were confirmed by our KASP assay study. This study is the first small-scale molecular breeding study in terms of using SNP markers in combination with KASP for MAS studies about downy mildew resistance in sunflowers. Using KASP to screen for SNP markers related to trait loci provides an opportunity for the selection of genotypes as resistant/susceptible or homozygotes/heterozygotes.

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