Biparental QTL and Association Mapping for *Sclerotinia* Head Rot Resistance in Cultivated Sunflower

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ABSTRACT

- Sclerotinia Head Rot (SHR) is considered one of the most damaging diseases of sunflower worldwide, causing average yield reductions of 10 to 20%. Sunflower genotypes show large variations in resistance to SHR. Due to the multigenic nature of this trait, breeding approaches face serious limitations. The aim of this work was to present advances for biparental and association mapping (AM) as complementary strategies to identify both QTL and genes conferring resistance to SHR.

- A total of 94 Recombinant Inbred Lines (RIL) encompassing the biparental population obtained from PAC2 (resistant genotype) X RHA266 (susceptible genotype) and an Association Mapping Population (AMP, 94 inbred lines) were evaluated for SHR during three seasons. Assisted inoculations with the fungal pathogen *Sclerotinia sclerotiorum* were applied. Severity, incidence and spread of the disease were assessed by visual scoring. The RIL population was genotyped with 671 DNA markers. A total of 52 candidate genes were selected and 34 resulted polymorphic in a Core Set of inbred lines selected for SNP and Indel development. Genotyping of AMP and statistical analysis is on going in the context of the AM approach.

- A total of 20 quantitative trait loci (QTL, LOD score >3) accounting for incidence (I), severity (S) and the area under the disease progress curve (AUDPC) were identified on specific regions of linkage group (LG) 10, 13, 14. A significant association was found between one of the evaluated candidate gene haplotypes and SHR incidence (P<0.01).

- The presence of several QTL at diverse genomic regions for traits measured at different times during the course of the disease suggests that different genes may be activated during the resistance response to SHR in sunflower. The genomic region associated with SHR resistance detected in the LG 10 is a strong candidate for the development of molecular assisted markers, since it was detected using data from field trials conducted over years, as well as independent studies using different biparental population.

- This work provides clear evidence about the power of the biparental and association population in the genetic study of disease resistance, and set an appropriate platform for the analysis of SHR disease and other complex traits through candidate gene approach.

Key words: Association Mapping – Molecular Marker – QTL Mapping – *Sclerotinia sclerotiorum* – Sunflower

INTRODUCTION

Cultivated sunflower (*Helianthus annuus* L.) is susceptible to several pathogens. One of the most important disease affecting sunflower growing regions around the world is Sclerotinia Head Rot (SHR) caused by *S. sclerotiorum* (Pereyra and Escande, 1994).

If the flowering period occurs with high humidity environmental conditions, SHR can provoke serious loss production because of capitulum destruction and declining seed quality. Disease control on *S. sclerotiorum* is difficult, since the fungus persists in soils for long periods and at high inoculum levels (Huang and Dueck, 1980; Troglia, 2003).

It has been shown that resistance determinants are associated with both pathogen entry into plant host cells and prevention of the spread of the mycelium (Bioley et al., 1987). Large variation in phenotypic behavior has been detected in sunflower probably due to different sources of resistance present in sunflower genotypes (Thuault and Tourvielle de Labrouhe, 1988). Indeed, SHR resistance has been described as quantitative and mostly additive (Vear and Tourvieille de Labrouhe, 1988), comprising many genes each one with small effect involving physiological and anatomical reactions (Castaño et al., 1993; Gulya et al., 1997). These features lead breeding programs to the identification of partial resistance to conduct marker-assisted gene pyramiding for experimental line development.

The genomic regions associated with SHR resistance in sunflower can be studied by Mendelian segregation from crossings of genotypes with contrasting behavior against *S. sclerotiorum*. Previous works have revealed quantitative trait loci (QTL) associated with resistance to SHR. These QTL explain only a low percentage of phenotypic variance, are distributed throughout the genome and their expression is highly dependent on the genotype and the environment (Gentzbittel et al., 1998; Mestries et al., 1998; Bert et al., 2003; Maringolo, 2007; Yue et al., 2008).

Association Mapping (AM) was suggested as a promising alternative to QTL biparental mapping to elucidate the genetic basis of complex trait (Yu et al., 2006). The AM approach is based on the extent of Linkage Disequilibrium (LD) observed in a set of accessions that are not closely related. In contrast to classical biparental population mapping, AM is a method that detects relationships between phenotypic variation and gene polymorphisms in existing germplasm, without development of mapping populations. This method incorporates the effects of recombination occurring in many past generations into a single analysis and thus, it is complementary to the classical biparental approach (Jorde, 2000). The main drawback of AM is the possibility of false-positive results due to unrecognized population structure. In order to avoid population stratification effects, information on the relatedness among genotypes is commonly included trough the recognition of the population structure and/or as a kinship matrix between genotypes (Yu et al., 2006). Two possible strategies have been proposed for AM: (1) Genome Wide Association (GWA) and (2) candidate gene approaches, being the latter most widely used in plants, probably due to the lack of complete genome data for many plant species. Great efforts have been done in sunflower for detecting QTL by biparental mapping. However, even though LD and nucleotide diversity have been assessed in germplasm from different origins (Liu and Burke, 2006; Kolkman et al., 2007; Fusari et al., 2008), no AM studies have been published to date either for SHR or any other trait in sunflower.

This work presents advances of biparental mapping and association mapping (AM) as complementary strategies to identify QTL and genes conferring resistance to SHR in a local germplasm collection. This strategy should allow us to identified both, several QTL and a putative causal allele associated with SHR resistance as a starting point to establish a marker assisted SHR breeding program in Argentina.

MATERIALS AND METHODS
Plant Material and Field trials
A set of 94 Sunflower Recombinant Inbred Lines (RIL) from PAC2 (R) X RHA266 (S) crossing and the AMP, encompassing 94 inbred lines, were evaluated for SHR during three seasons.

Sunflower capitula at the R5.2 phenological stage (Schneiter and Miller, 1981) were inoculated with the fungal pathogen *S. sclerotiorum* (2500 ascospores/mL). Flowers were immediately covered with paper bags since the inoculation time up to 15 days post inoculation and then they were uncovered. Severity, Incidence and Spread of the disease were assessed by visual scoring every three days till R9 developmental stage (Schneiter and Miller, 1981).

DNA extraction from lyophilized young leaves of 3-weeks-old plants was performed using the NucleoSpin Plant II system (Macherey-Nagel).

QTL biparental mapping

A set of 30 SSR markers was selected by aligning public genetic maps delivered by Tang et al. (2003) and Kiani et al. (2007) with that constructed by (Maringolo, 2007), using the Cmap tool available in the Compositae Genome Project database (www.sunflower.uga.edu/cgi-bin/cmap/viewer) and were added to the genetic map produced by Talia et al. (2010). On the other hand, 7 candidate genes characterized by Fusari et al. (2008) as informative for this population, were genotype in the RIL and added to the PAC2xRHA266 map.

Marker segregation was assessed using the Chi-squared Test. To add a genotyped marker, we specified a recombination frequency of 0.35, LOD= 4 and Kosambi mapping function (Zubrzycki et al., 2011). The estimated recombination frequencies were corrected by the factor that consider the multiple generation of meiosis under self-breeding (Haldane and Waddington, 1931; Tang et al., 2002). QTLs were identified using the Composite Interval Mapping procedure within the QTL Cartographer (version 1.16).

Candidate gene selection and Association Mapping

Candidate genes for SHR resistance were selected from three different data sources: (1) loci at least twofold over-expressed in a *Brassica napus* resistant genotype after 24 hours post-infection (hpi), were selected from the study of Zhao et al. (2007); (2) candidate genes selected according to their annotated function (e.g. biotic and abiotic stress related proteins, cell wall and cell membrane proteins) from an EST library obtained through suppressive subtractive hybridization from capitula of the genotype RHA801 infected with *S. sclerotiorum* at 48 hpi (Peluffo, 2010); (3) WRKY transcription factors and germin-like proteins, previously identified in sunflower and reported to participate in the defense response against *S. sclerotiorum* infections in sunflower and other species (Fernandez et al., 2003; Giacomelli et al., 2010).

Since the knowledge of the sunflower genome and transcriptome is still partial, a protocol for identifying sunflower genes orthologous to those detected by Zhao et al. (2007) was developed (Fusari et al., 2009; Filippi et al., 2011).

Candidate genes selected were amplified with primers designed with Primer 3 (Rozen and Skaletsky, 2000). First, DNA polymorphisms were screened in a group of 10 to 15 inbred lines named Core Set (CS). Characterization of nucleotide diversity and polymorphism was done as reported previously (Fusari et al., 2008). Polymorphic genes were selected to be genotype using different methods according to the complexity and number of haplotypes detected in each one (Fusari et al., 2010). The statistical analysis for AM needs to include Population Structure analysis. Therefore, 8 out of 35 SSR markers were selected to perform Principal Coordinates Analysis (PCO) and the detection of population substructuring by the Bayesian method implemented in STRUCTURE software (Falusch et al., 2000). The association mapping tests were carried out using Mixed Linear Models (MLM) that account for the population structure and kinship relationships.

RESULTS AND DISCUSSION

Identification of QTL involved in SHR resistance

QTL discovery through CIM allowed identification of 20 quantitative resistance loci (QRL, LOD scores >3), accounting for incidence (I), severity (S) and the area under the disease progress curve (AUDPC), located mostly in linkage groups (LG) 10, 13 and 14 (Figure 1).

In the upper end of GL 10, two reproducible QRL associated with lower incidence, one at 43.8 cM and the second at 56 cM, were found on this population and on the population derived from the cross of HA89 X RHA801 analysed by (Maringolo, 2007).

In the GL 13, three QTL associated with resistance were found in a 27 cM region located at the bottom end of the LG, these QTL were detected at different times after inoculation for the three measured parameters.

In the GL 14, a QTL associated with S was found at different times after inoculation in a region spanning from 108 to 114 cM.

The presence of several QRL at diverse genomic regions for I, S and AUDPC measured at different times during the course of the disease, suggest that different genes may be activated during the resistance response to SHR in sunflower.

The genomic region associated with SHR resistance in the LG 10, may be an excellent candidate for the development of molecular assisted markers, because it was detected during the different evaluations performed on this population, as well as independent studies on other crosses.

Setting-up the platform to conduct AM studies for SHR resistance in sunflower

SHR incidence was evaluated during 2008-2009, 2009-2010 and 2010-2011 in replicated field trials. Adjusted means were calculated for each inbred line according to the MLM and 43% of AMP showed an

intermediate behaviour against the disease, i.e. between 40% and 60%. In addition, 37% of inbred lines showed an intermediate-to-bad behavior against SHR (60-80 % SHR incidence).

A total of 37 genes were suitable for amplification in the CS out of the 52 candidate genes selected from different sources and 34 candidate genes resulted polymorphic. The gene panel includes transcription factors, enzymes involved in cell wall degradation and transport, germin-like proteins, proteins involved in defense mechanisms and oxidative burst. A total of 22,628 of aligned base pairs (bp) were analysed per individual. Gene length ranged from 166 to 1713 bp including indels, with an average of 501 bp per region. Polymorphism index, nucleotide diversity and number of haplotypes were explored to select the genotyping method that best fitted each gene under study. Polymorphisms were abundant, finding 280 SNPs and 50 Indels. Number of SNPs ranged from 0 for *HaPRP1*, *HaROPGEF3*, *HaHP1*, to 18 for *HaGDPDI*. On average, 1/66 SNP and 1/371 indels were detected.
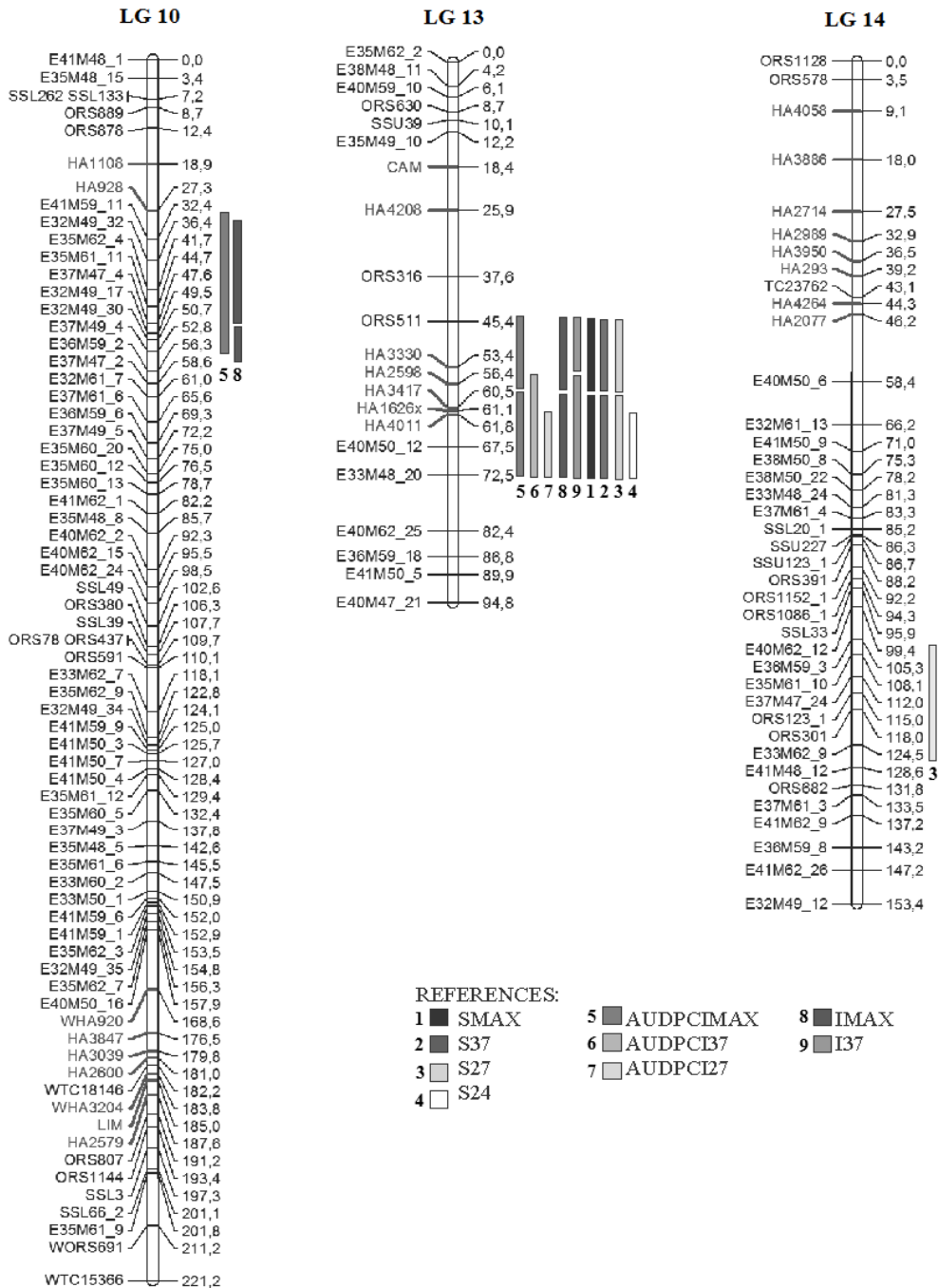

Fig. 1. LG 10, 13, 14

Summarizing, moderate levels of DNA polymorphism were found. Genetic variation at the nucleotide level was estimated from mean nucleotide diversity (π_T=0.0074) and from the number of segregating sites (θ_W= 0.0069).

The number of haplotypes per locus ranged from 1 to 6 among the CS and average haplotype diversity was 0.5022. Different genotyping methods were optimized according to the numbers of SNPs and haplotypes found in the CS. Those genes which showed only two haplotypes in the CS were selected to be genotyped with denaturing High Performance Liquid Chromatography (dHPLC), those which had indels were selected to be genotyped by Fluorescent Capillary Electrophoresis (FCE) and those with more than two haplotypes determined by both SNPs and indels were planned to be genotyped by direct sequencing. A total of 16 candidate genes have been already genotyped in the AMP and 7 additional genes are being optimized for the appropriate genotyping method.

Association Mapping tests detected an association between a SHR incidence and one candidate gene. Correction for multiple levels of relatedness by different approaches did not affect the association found. This gene might be a strong candidate, and since its molecular function is barely known in sunflower, functional genomic experiments are being performed to validate both, the gene function and the association. Taking into account these first approaches, new candidates are being genotyped in the AMP and a new field trial is being performed to include not only SHR incidence but also Severity in the AM test.

This work provides clear evidence about the power of the biparental population mapping studies in the genetic dissection of complex traits. The inclusion of candidate gene AM approach will complement QTL mapping by providing new delimited QTL or validating the QTL found by the former approach. Finally this work also provides with molecular markers to assist in sunflower breeding.

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