Genomics, mapping and marker assisted selection strategies for disease resistance

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ABSTRACT

- Biotic stress is considered one of the most important factors affecting yield all over the world. In sunflower downy mildew, black rust, Sclerotinia rot, Verticillum wilt and Alternaria leaf spot are considered the most important fungal diseases. In the last ten years, several attempts have been made through conventional breeding and molecular biological studies to dissect the bases for fungal resistance to allow molecular assisted selection. The detection and genetic location of resistance gene candidates and QTL for complex diseases, together with the development of molecular markers, linkage maps and genetic association studies have largely contributed to that purpose. There exists a vast array of gene/gene complexes for disease resistance, tracing back to cultivated or wild sunflowers species. Linkage drag around disease resistance genes (DRG), especially when the resistance comes from wild species, is one of the most limiting issues which may decrease yield potential. However, molecular tools as marker assisted selection (MAS) have greatly increased the efficiency of resistance selection to different diseases.
- The detection of new downy mildew and black rust R genes, their close genomic location, their arrangement in clusters and its implication in breeding strategies is discussed. Main results of association mapping employed as a complementary tool of family mapping strategies for head rot resistance QTL location are presented. Stacking genes controlling the resistance to main Argentinean races of *Verticillium dahliae* have been obtained for the first time. Pyramiding major quantitative trait loci (QTL) to head rot and *Alternaria* is presented as a relative simple way to increase the resistance level for these diseases.
- The state of the art of the genomics of resistance and MAS in breeding processes for some of the main sunflower pathogens are reviewed, The collaborative work between public and private institutions has largely contributed to the elucidation of the architecture of disease resistance in sunflower which will provide many tools to build up durable and sustainable disease resistances in modern sunflower hybrids.

Key Words: Alternaria leaf blight - black rust - downy mildew – *Helianthus* - Sclerotinia head rot - Verticillium wilt

INTRODUCTION

Sunflower (*Helianthus annuus*) is grown as an oilseed crop in temperate and subtropical climates on every continent, being Argentina, Russia, Eastern Europe, the European Union, and the United States the largest producers (Gulya, 2004).

Considering that sunflower is cultivated in vast marginal areas and therefore, the application of fungicides is not always feasible, breeding for generation of inbred lines with genetic tolerance to most common pathogens represents a main goal (Sackston, 1962). Downy mildew (*Plasmopara halstedii)*, Sclerotinia stalk and head rot (*Sclerotinia sclerotiorum*), black rust (*Puccinia helianthi)*, Verticillium wilt (*Verticillium dahliae)*, Alternaria leaf blight (*Alternaria helianthi*) among others, appear as higher impact diseases since seriously reduced yield in many sunflower crop regions (see Viranyi, 2008, for a review).

Breeding for genetic disease resistance has been, and will continue to be, essential to strength this crop. There exist several sources of resistance genes in sunflower, including numerous wild sunflower species, landraces and old open pollinated varieties that have been introgressed into cultivated germplasm (Seiler, 2010; Gulya et al., 2010) but several of them remains to be discovered, genetically characterized and incorporated in the high yielding cultivated genetic pool.

The advances in genomics in the last decade allowed the discovery and functional characterization of many genes simultaneously on a genome-wide scale. In the case of crop species, genomic tools can improve the rate of genetic gain in breeding programs by either extending the amount or nature of variation available for selection, or by accelerating the selection process to produce varieties more rapidly (Langridge and Fleury, 2011).

In the case of sunflower, genomic approaches have already started to impact fundamental and applied sunflower pathology, both in the public and private sectors, which resulted in the generation of several tools that are likely to change some paradigms of sunflower breeding (Jan and Seiler, 2007; Paniego et al., 2007; Seiler and Jan, 2010, Kane et al., 2011, Fernandez et al., 2012). One of the relevant results obtained from a fundamental perspective is that resistance genes are clustered together in certain regions of the genome. These clusters, which were described in many species, are mainly distributed in the sunflower genome at the upper and lower end of most linkage groups (Radwan et al., (2008) A fine elucidation of this particular architecture will be of great impact over molecular breeding, not only by the design of molecular markers targeting these regions, but also for the design of completely new regions combining useful disease resistant genes (DRG) from different sources by recombination and selection.

In this paper will be reviewed the inheritance of several resistance traits, the genetic mapping of resistance genes or QTL, and the genomic and bioinformatic approaches currently used to understand the inheritance and physiology of resistance to many important sunflower diseases, with special emphasis in the generation of new breeding tools and strategies.

CATEGORIES OF DISEASE RESISTANCE

Two general categories of disease resistance have been long recognized in plants: (i) complete resistance conditioned by a single gene, and (ii) incomplete resistance conditioned by multiple genes of partial effect. In their extreme forms, these types of resistance are clear and easily distinguished (Poland et al., 2009). A variety of terms have been used to refer to this perceived dichotomy, including horizontal versus vertical, complete versus incomplete, major-gene versus minor-gene and narrow-spectrum versus broad spectrum. Here, we use the terms ‘qualitative disease resistance’ and ‘quantitative disease resistance’ (QDR) to refer to the respective phenomena. We use the term ‘DRG’ to refer to genes that confer qualitative effects and ‘QTL’ (quantitative trait loci) to refer to loci or genes that confer QDR. Although the phenomena of qualitative and quantitative resistance can be considered different, there is a great deal of gray area between the extremes, suggesting that it might be useful to reexamine the concepts in light of emerging evidence on mechanisms of resistance. The mechanisms involved in conditioning QDR are likely to have implications for resistance spectra and durability associated with specific QTLs. Mechanisms of specific recognition might eventually be overcome as a result of pathogen evolution, whereas non-specific defense mechanisms could provide resistance that is relatively broad in spectrum and robust in the face of pathogen evolution (Poland et al., 2009). For example, downy mildew resistance in sunflower has been recognized as a qualitative disease resistance with several major genes involved although QDR for this disease have recently been discovered (Vear et al., 2008a) which allow breeders to combine both of them to obtain a durable mechanism of resistance.

DOWNY MILDEW

Several major resistance genes to downy mildew have been identified in cultivated and wild *Helianthus* species (Korell et al., 1996; Miller, 1992). These dominant resistance genes have been designated as *Pl*

genes, and approximately 20 of them have been described up today. Some of them provide resistance to a single race of *P. halstedii*, whereas others conferred resistance to two or more races (Miller, 1992).As soon as the resistance provided by the *Pl2* was overcome, new resistance genes, mainly derived from public USDA lines were deployed in sunflower cultivars. The *Pl5* gene from *H. tuberosus*, a perennial hexaploid sunflower, protects against race 3 (virulence phenotype 700) (Leclercq et al., 1970; Pustovoit, 1966). Resistance to races 1, 2, 3 and 4 (virulence phenotype 730) was introgressed into cultivated sunflower from three sunflower species: *H. annuus ssp. annuus* (*Pl6*), *H. praecox ssp. runyonii* (*Pl7*) and *H. argophyllus* (*Pl8*) (Miller and Gulya, 1988; 1991). Also from *H. argophyllus* was isolated *PlArg*, a gene which conferred resistance to at least four tested races (virulence phenotypes 300, 700, 730, 770). Recently, two new sources of resistance were described, one derived from the inbred line HAR5 which was designated as *Pl13* (Mulpuri et al., 2009), while the other was designated as *Pl14* (Bachlava et al., 2011). The effectiveness of the major resistance genes *Pl6* and *Pl7* has been overcome by new races in France (Delmotte et al., 2008) and in USA (Gulya et al., 2010); however, there are no records of races overcoming other broad spectrum (*Pl8*, and *PlArg*) genes (Gulya, 2007a; Gulya et al., 2010).

A new gene designated as *Pl15* was found in RNID, a proprietary restorer inbred line which traces back to an Argentine sunflower open pollinated population. This gene shows resistance not only to the four predominant races of downy mildew in all sunflower producing countries (300, 700, 730, and 770; Gulya 2007a), but also to the less prevalent races, including those recently described in USA (714 and 734) and in France (304) which overcome *Pl6* (Gulya et al., 2010; Vear, 2004). This resistance gene was located on LG8 and, based on its map position, it was reported as a new member of the *Pl1-Pl2-Pl6-Pl7* gene cluster (Romano et al., 2010). The molecular characterization of the *Pl1-Pl2-Pl6-Pl7-Pl15* cluster on LG8 using multilocus intron fragment length polymorphism (IFLP) permit to distinguish each of the members of the gene cluster (Slabaugh et al., 2003; Romano et al., 2010) and could be useful as molecular markers for marker assisted selection (MAS) and for the molecular prospection of genetic resources.

A second linkage group, LG13, was found to contain two clustered resistance genes: *Pl5* and *Pl8* (Bert et al., 2002). The *PlArg* (Dußle et al., 2004), *Pl13* (Mulpuri et al., 2009) and *Pl14* (Bachlava et al., 2009) loci, on the other hand, were localized on linkage group LG1.

Although these dominant genes confer functional complete resistance to one or more races of *P. halstedii*, new races continually appear. This threat, combined with the development of resistance to chemical control (Gulya et al., 1999), has spurred a vigorous search for determinants specific to new races. In addition, pyramidization of different DRG might be another strategy to cope with this threat (see below).

BLACK RUST

The rapid changes that occur in the virulence of *P. helianthi* represent a continuous threat to the effectiveness of existing rust-resistance inbred lines and hybrids. Several sources conferring resistance to rust have been identified in sunflower including *R1, R2, R3, R4, R5, Pu6,* and *Radv* (Putt and Sackston 1957; Miah and Sackston 1970; Miller et al., 1988; Yang et al., 1989; Goulter 1990; Lawson et al., 1998), but only a few of them have been genetically characterized, mapped and linked to molecular markers.

Molecular markers linked to rust resistance genes were first described for *R1*, and were localized on LG8. This gene was found in the restorer inbred line Rha279 (Lawson et al., 1996). Many genes were later associated with markers from LG13, like *Radv* from a propietary hybrid from Australia (Lawson et al., 1998), *Radv* from Rha340 (Bachlava et al., 2009) and *R4* from HAR3 (Qi et al., 2011). *R1*, both *Radv* and *R4* are close to the largest clusters of NBS-LRR resistance gene candidate currently described in sunflower genetic maps (Lawson et al., 1998, Yu et al., 2003, Rawdan et al., 2008, Lawson et al., 2010; Qi et al., 2011b). *R2* from MC29, a rust resistance line from Canada, was located using SSR markers in LG9 (Lawson et al., 2010).

Inheritance and mapping of rust resistance genes from the sources P386, HA-R4, HA-R6, "Caburé Precoz", B648, and PNR1 were recently reported (Bulos et al., 2012). Using a classical genetic approach and bulk segregant analysis mapping strategy it was demonstrated that rust resistance is controlled by a single factor in each source and that all of them are located on LG 13. Currently, research is in progress to elucidate the allelic relationships among all these sources and to generate allele specific markers for all of them. A pre-breeding strategy to eliminate linkage drag from the wild donor species are also in progress.

SCLEROTINIA HEAD ROT

S. sclerotiorum is a highly polyphagous and necrotrophic fungus that causes head rot, one of the major diseases of sunflower. Different aspects of the disease were investigated, pathological issues (Ekins et al., 2007; Li et al., 2009), the resistance sources (Gulya et al., 2010; Vear and Grezes-Besset, 2010, Zubryzcki et al 2012), the anatomical and biochemical resistance mechanisms present in different floral

pieces (Tourvieille et al., 1997; Rodriguez et al., 2004; Prats et al., 2003; 2006; 2007, Perchepied et al., 2010), the transcriptomic and metabolic profiles of sunflower genotypes with contrasting response to the disease (Peluffo et al., 2010; Fernandez et al., 2012), and traditional breeding strategies (Vear et al., 2007).

The nature of resistance has been described as partial, quantitative and mostly additive (Vear et al., 1988). Recurrent selection after mycelial and ascospore infections for over 16 sunflower generations, resulted in a reduction of 50% of natural attack on hybrids and doubled the delay period before symptom appearance of inbred lines (Vear et al., 2007). QTL for head rot resistance were identified in every linkage group of sunflower by several researchers (Gentzbittel et al., 1998; Mestries et al., 1998; Bert et al., 2004, Vear et al., 2008b and 2010; Yue et al., 2008; Zubryzcki et al., 2012).

In spite of these advances, practical results to overcome this complex disease are still insufficient. Bazzalo et al. (2010) evaluated an ample base of germplasm by ascospore seminatural infection over many years and environments and some resistance sources from very different genetic origins were detected. Resistance QTL were mapped in bi-parental crosses of the two best resistance sources by different susceptible lines. Through a meta-analysis of all obtained results, two major QTL (2 & 3) were detected in different linkage groups. By backcross program with MAS a NIL combining both QTL was derived. Susceptible line L1 and its NIL L1 (QTL2&3) were artificially inoculated under three different environments. Highly significant differences were detected for both incidence and percentage of affected head at 35 days after inoculation. Susceptible L1 exhibited 87 % of diseased plants and L1 (QTL2 &3) 34% of diseased plant. The mean proportion of head covered by the lesion was 44 % for the susceptible line and 11 % for the NIL with 2 QTL. No GxE interactions were detected. None significant differences were observed between NIL for yield, oil content and some morphological parameters like head diameter, leaves size and plant height.

The possibility of increasing the level of resistance of elite lines by introgression of only two QTL with the help of MAS and embryo culture tools, without affecting yield components, constitutes a relative simple way to reduce the impact of this complex disease.

Association mapping based on candidate genes were carried out to study Sclerotinia head rot resistance using a population of 94 public and proprietary inbred lines preserved in the local germplasm bank (Fusari et al., 2008; Fusari et al., 2010; Zubryzcki et al, 2012). Disease incidence was measure using assisted inoculation with the fungal pathogen during two campaigns in replicated field trials. Given that either no biological mechanisms or biochemical pathways have been clearly identified for Sclerotinia head rot, 43 candidate genes were selected based on previous transcript profiling studies in sunflower (Pelufo et al., 2010) and *Brassica napus* (Zhao et al., 2007) infected with *S. sclerotiorum*. A total of 30 candidate genes were amplified in 10 sunflower inbred lines selected as Core Set (CS) for polymorphism discovery, 16 candidate genes were genotyped in the association mapping population. Finally, associations among 69 haplotypes and Sclerotinia head rot incidence were tested using a Mixed Linear Models that account for the population structure and kinship relationships. A significant association was found between one of the evaluated candidate gene haplotype and disease incidence (Fusari et al., 2010; Zubryzcki et al., 2012).

These results demonstrate that association genetics via candidate gene in sunflower is a valuable approach for elucidating the molecular basis of complex traits and for developing DNA-based markers for "precision breeding" of improved varieties. Future efforts will be devoted to expand the association mapping population and to incorporate large-scale gene sampling as well as high-throughput genotyping methods.

ALTERNARIA LEAF BLIGHT

A. helianthi is a worldwide distributed pathogen attacking leaves, stems petioles, bracts and head of sunflower (Kong, 1995). Premature foliar senescence and defoliation due to spots coalescence or petiole necrosis are associated to important yield reduction. Non-availability of good sources for resistance to *A. helianthi* was a major limiting issue in sunflower breeding (Reddy et al., 2006). The nature of resistance is polygenic and limited information about resistance QTL location has been reported (Virányi, 2008).

In order to detect resistance sources, 370 lines were evaluated under natural infection in the Northern crop region of Argentina. The lesion density and the percentage of totally necrotic leaves were recorded 15 days after the beginning of flowering. F2 plants, from the cross of most contrasting lines, were artificially inoculated under controlled conditions. Phenotypic and molecular data of individual plant were analyzed by simple and composite interval mapping association. Three major QTLs in LG 4, 10 and 17 showing additive effect were detected (Galella et al., 2010). The QTL were introgressed by backcross from the resistance source to L97 and L03 susceptible lines using MAS. The converted lines and their respective

counterparts were evaluated under very severe natural infection conditions. The percentage of total necrotic foliage was reduced from 100 to 50 % in L03 and from 70 to 40 % in L97 backgrounds, respectively.

VERTICILLIUM WILT

Genetic resistance, based on a single, dominant gene from wild *H. annuus*, was previously identified and incorporated into released inbreds (Gulya, 2007b). Using this gene, resistance oilseed hybrids were produced. A race of *V. dahliae* not controlled by the *V1* gene was identified in Argentina (Bertero and Vazquez, 1982).

In 2004 Galella et al., reported two new races isolated from Argentina which were called Varg1 and Varg2. A new strain of *V. dahliae* has been identified in the U.S. which as all Argentinean races, is able to overcome *V1* resistance gene (from HA89 inbred line) (Gulya, 2007b; Galella et al., 2004 *op.cit*.). A major resistance Varg1 QTL, mapped and incorporated by MAS to hybrids of different background, was effective under natural infection at four locations independently of the background of the parental lines (Creus et al., 2007). A second resistance Varg2 QTL was mapped in a different linkage group. Both QTLs were transferred to the same susceptible line conferring resistance to the two most abundant physiological races present in Argentina under controlled independent inoculations. Nevertheless, few individual plants presented typical symptoms under natural infection conditions indicating the presence of minor *Verticillium* races (Galella et al., 2012). These results indicate that it is possible to combine resistance QTL to different races in order to obtain a more durable resistance to this disease.

CURRENT STATUS AND THE WAY AHEAD

Many DRG trace back to populations of wild species or landraces, linkage drag around these DRG may decrease yield potential or its stability when incorporated into high yielding backgrounds, as it was demonstrated for the herbicide resistance gene *Ahasl1-1* from wild *H. annuus* (Trucillo et al., 2010). For example, Pl_6 from wild *H. annuus* shows a linkage drag of 42.9 cM in the inbred line HA335, meanwhile the linkage drag around Pl_8 from *H. argophyllus* is 37.3 cM in the inbred line RHA340 (Slabaugh et al., 2003). The importance of mapping disease resistance genes is not only to initiate MAS programs, but also to ameliorate the original resistance sources since the linkage drag can only be eliminated by recombination and selection by molecular markers. By this way, high yielding resistance sources can be obtained. For example, a rust resistance gene discovered in "Caburé Precoz" maps in LG 13 (Bulos et al, 2012), close to the *Rf1* locus. However, this source does not restore PET1, which prevents its use as a source for the restorer germplasm. By using molecular markers, it was possible to link in coupling phase the *Rf1* allele with the DRG, permitting its incorporation as a new rust resistance source for the restorer germplasm (Bulos et al., unpublished).

From the breeding point of view, it is highly desirable to combine in a new cultivar as many DRG as possible in order to increase the durability and sustainability of the resistance. Tracking the accumulation of resistance genes in a new breeding line based on phenotypic evaluation data alone is hardly feasible. This strategy of pyramidization of DRG in sunflower can be achieved by three different ways. The first procedure involves the incorporation of different DRG in each parental line, so that these genes will be stacked in the final hybrid variety. The second is to include DRG belonging to different LG in the same parental line. The third strategy involves the pyramidization of closely linkage DRG or DRG belonging to the same DRG cluster. In the second and third strategy, if the genes to be stacked show complete resistance to all races of a pathogen, the only way to pyramidize them is by a molecular marker based approach. The outcome of these briefly outlined strategies is rather different. By the first one, a stacked hybrid is obtained; by the second one a stacked line is produced. However, by the third strategy a new cluster of DRG is generated, that behaves as a new DRG with monogenic inheritance in the practice. In fact, "running to stand still" is not a desirable strategy to cope with fast shifting fungal diseases. Sunflower breeders have to conduct anticipatory breeding in order to be prepared for racial shifts. Piramidization of useful genes in new DRG clusters, parental breeding lines and/or thoughtful combinations in F1 hybrids is a critical approach to underpin a durable genetic resistance.

CONCLUSIONS

The collaborative work between public and private institutions had contributed to the elucidation of the architecture of disease resistance in sunflower. This knowledge will provide the tools and strategies for the construction of durable resistance in modern sunflower hybrids. An integrated disease resistance breeding based on the clear understanding of the genetic organization of resistance, together with the permanent monitoring of pathogenic populations/races, the staking of both horizontal and different

monogenic resistances, the use of converted elite lines as resistance donors as a way to avoid linkage drag, the construction of "super donor lines" including resistance genes/clusters that could be transferred together as monogenic traits, are some of the strategies that will permit rising sunflower productivity worldwide in the near future.

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