

TOWARDS SUSTAINABLE DOWNY MILDEW RESISTANCE IN SUNFLOWER

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SUMMARY

Breeding for downy mildew resistance is one of the major goals in sunflower breeding programmes. In the last few decades new sources of resistance have been discovered and the position of 11 resistance genes was established on the genetic map of sunflower. For most of these resistance genes sequence specific markers have been developed, which facilitate their detection and make the selection process faster and more reliable. By combining the partial resistance provided by minor genes with specific resistance genes, durable resistance could be achieved.

Key words: sunflower, downy mildew, resistance, molecular markers, breeding

INTRODUCTION

Downy mildew is a sunflower disease caused by *Plasmopara halstedii* (Farl.) Berlese et de Toni, an oomycete with high virulence, aggressiveness and a great potential in developing new races.

Downy mildew infection can be primary (soilborne and seedborne) and secondary (airborne) (Aćimović, 1998). Primary infection causes more severe symptoms and a decay of a plant, while secondary infection usually results in small angular spots on leaves without limiting the sunflower yield (Jocić *et al.*, 2009). Under humid conditions, oospores germinate into zoosporangia, which liberate flagellate zoospores. Spores move towards the roots and penetrate into the tissue, thereby initiating primary infection (Delanoe, 1971, according to Viranyi and Spring, 2011). Infected plants tend to be insufficiently developed, dwarfed, with chlorotic leaves covered with white mycelium (Dussle *et al.*, 2004).

Extent of the damage that the pathogen causes is influenced by several factors:

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- 1) environmental conditions, primarily humidity and temperature (Wehtje and Zimmer, 1978; Aegerter, 2003; Tourvieille de Labrouhe *et al.*, 2008b);
- 2) potential of pathogen for host inoculation, *i.e.*, zoosporangia viability (Meliala *et al.*, 2000; Tourvieille de Labrouhe *et al.*, 2000) and,
- 3) genetic resistance or susceptibility of the crop.

Efficient control of downy mildew requires an understanding of the pathogen's fundamental biology, molecular mechanism of infection, development and reproduction (Sakr *et al.*, 2009; Sakr *et al.*, 2010), as well as identification and determination of position and function of the host resistance genes.

Population dynamics and molecular characterization of the pathogen

Until 1980, only two races of downy mildew were known. After the races from the USA came in contact with the races from Europe, a number of races emerged in different parts of the world. Until the present day, 14 races of downy mildew have been identified in Europe (Delmotte *et al.*, 2008) and 35 worldwide (Gulya *et al.*, 1991; Gulya, 2007). In France, race 100 was the solely present from 1965 until 1987.

Zimmer and Fick (1974) found that gene Pl_1 provides resistance against race 100 and Pl_2 against races 100 and 300. These two genes controlled the downy mildew population in Europe until 1998 when new races emerged (710 and 703) in France (Tourvieille de Labrouhe *et al.*, 1991; Tourvieille de Labrouhe *et al.*, 2000; Delmotte *et al.*, 2008). Later research showed that these races were introduced from the USA *via* infected seeds (Roeckel-Drevet *et al.*, 2003). Afterwards, a number of highly aggressive races, *e.g.*, 304, 307, 314, 334, 704 and 714, evolved (Tourvieille de Labrouhe *et al.*, 2000; Sakr, 2010). In Serbia, race 100 was the only race until 1990. In 1991, the presence of race 730 was confirmed (Maširević, 1998), and it soon became the prevailing race in the country (Lačok, 2008).

Division on races is based on the reaction of an internationally accepted set of differential lines to the pathogen (Tourvieille de Labrouhe *et al.*, 2000). In the last decade advanced tools of biotechnology have enabled discernment of intraspecific groups of *Plasmopara* on molecular level and led to the shift from a morphological to a phylogenetic species concept (Spring and Thines, 2004). With molecular markers based on partial sequence of the nuclear ITS regions, Spring *et al.* (2006) detected polymorphism between profiles of races 100, 310 and 330, as well as between groups of populations representing races 700, 701, 703, 710 and 730. Giress *et al.* (2007) found high genetic variability between the isolates from France and Russia using SNP markers, whereas Sakr (2010) utilized EST - derived markers to determine genetic relationship between the races. Interestingly, new insights from molecular phylogenetics often verify a narrow species concept that was proposed by Gäumann in 1918 (Voglmayr and Constantinescu, 2008).

Disease-control measures

The disease control measures could be divided in:

- agrotechnical,
- chemical and
- breeding for resistance.

- 1) Agrotechnical measures include a proper crop rotation and use of healthy seed treated with fungicides. The recommended cultivation practices include: the use of healthy seeds for planting; coating of the seeds with fungicides against downy mildew; proper crop rotation, *i.e.*, maintaining intervals of 4-5 years between two sunflower cultivation in the same field; selecting fields that are at least 500 m away from a field on which sunflower was grown the previous year. Since the infected harvest remains in that field, volunteer plants should be removed from the field and deep plowing of the field should be done after the sunflower harvest. Additionally, sowing should be performed at optimum time, with the avoidance of late planting (Jocić *et al.*, 2010).
- 2) Today a wide range of commercial fungicides are available on the market (Gisi, 2002) with different modes of action (Gisi and Sierotzki, 2008). Coating of seeds with metalaxyle derivates is most frequently used, as it provides protection at the time of the primary infection, *i.e.*, at early stages of development of sunflower. Concerning issues related to the use of chemical control include emerging of races resistant to the fungicide in France, Germany, Turkey and Hungary (Viranyi and Spring, 2011), negative environmental effects of the fungicides as well as the economic feasibility of this disease control measure.
- 3) Resistance genes, denoted *Pl* genes, provide complete resistance to pathogen. From 1978 on, commercial varieties in France have always carried one or more *Pl* genes (Miller and Gulya, 1988; Vear *et al.*, 2008). As the *Pl* genes are race-specific, there is a great probability that new races of downy mildew will overcome them in a relatively short period of time. Even though monogenic resistance is not durable, introgression of *Pl* genes is still among the most efficient methods of controlling downy mildew.
- 4) Induced resistance (often called systemic or acquired resistance) implies the use of specific chemical compounds for triggering plants defence mechanisms (Mauch-Mani, 2002; Korosi *et al.*, 2011). This method, which was developed in the last few decades (Mauch-Mani, 2002), has been proven to be efficient in diminishing the severity of infection in genotypes without genetic resistance (Gisi, 2002).

The frequency of resistant pathogen individuals often starts increasing after the intensive use of an effective method. While controlling the sensitive pathogen subpopulations, disease-control measures enable progress of the resistant subpopulations, which soon become dominant. In order to prolong resistance duration, several control measures need to be applied simultaneously. As far as genetic resistance is concerned, durable resistance could be achieved by combining quantitative with monogenic resistance (Vear *et al.*, 2008), or by introducing genes from different clusters and with different origin in a single genotype (Jocić *et al.*, 2010).

Genetic resistance of sunflower to downy mildew

Genetic resistance can be classified into two categories:

- 1) partial resistance which is controlled by minor genes (QTLs), and tends to influence the extent of disease development (Tourvieille de Labrouhe *et al.*, 2008a) and,
- 2) qualitative or monogenic resistance, conferred by the *Pl* genes.

Partial resistance controlled by minor genes

Partial resistance, also called field, general, quantitative or horizontal resistance, is usually not race-specific and is assumed to be polygenic. The use of partial resistances does not appear to have as strong effect on pathogen populations as the monogenic resistance. By combining the partial resistance provided by minor genes with specific resistance genes, durable resistance could be achieved (Tourvieille de Labrouhe *et al.*, 2008a). It has been shown that QTLs provide tolerance to a number of pathogens such as *Sclerotinia sclerotiorum* (Vasić *et al.*, 2004), *Diaporthe helianthi* (Mihaljčević *et al.*, 1982; Škorić 1985), *Phoma macdonaldii* (Vear *et al.*, 2008), and *Orobanche cumana* (Pérez-Vich *et al.*, 2004). Rachid Al-Chaarani *et al.* (2002) found four putative QTLs. The three major QTLs were located on LG 1, 9 and 17, and explained 54.9% of the total phenotypic variance. Vear *et al.* (2008) reported two QTL located on LG 10 and LG 8, and suggested microsatellite markers ORS613 and ORS389 for their detection.

Qualitative or monogenic resistance

The outcome of the infection is predetermined by genetic constitution of the host. Sunflower and downy mildew have a typical "gene-for-gene relationship" - for each virulence gene presented in the pathogen exists a corresponding resistance gene in the host plant. If the plant has an effective resistance gene that will counteract the virulence gene in the pathogen, the infection will be stopped near the penetration site. Cotyledon limited infection (CLI, Gulya *et al.*, 1991) is a phenomenon observed in resistant genotypes. It is caused by hypersensitive reaction (HR) that is manifested as an extensive cell death in the tissue of infected hypocotyls (Mouzeyar *et al.*, 1993; 1994). Molecular mechanisms of HR include activation of glutation-peroxidase gene (Herbette *et al.*, 2003) and hsr230J-like gene (Radwan *et al.*, 2005). When the cascade is activated, a number of linked processes occur in the apoptotic cells - respiration rates increase, production of nitric oxide (NO) and reactive oxygen species (ROS) elevates, leading to an oxidative burst. As a result, invading pathogen cells are neutralized.

Genes that confer resistance to downy mildew are dominant and often form clusters. Until the present day a number of *Pl* genes have been reported (Pl_{1-15} , Pl_r , Pl_w , Pl_{x-z} , M_w , M_x , Pl_{arg} , Pl_{HA-R4}) (Table 1), and position of 11 genes has been determined on the SSR genetic map (Figures 1 and 2). It is usually the case that new resistance genes provide resistance to earlier races of the pathogen.

Table 1: Review of the downy mildew races, resistance sources and resistance genes in sunflower

	Races of <i>Plasmopara halstedii</i>													Donor Line	Gene	Location of the gen		Literature source				
																RFLP map	SSR map					
	100	300	304	310	314	330 USA	330 ESP	334	700	703	704	710	714			717	730		733	734	770	
R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	RHA-266, RHA-265	Pl ₁	LG 1	LG 8	Zimmer and Fick, 1974; Gedil et al., 2001; Bertero de Romano et al., 2010
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	RHA-274	Pl ₂	LG 1	LG 8	Zimmer and Fick, 1974; Bertero de Romano et al., 2010
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	HIR-34	Pl ₄	?	?	Sackston, 1990
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	XRQ YSQ	Pl ₅	LG 6	LG 13	Bert et al., 2001; Radwan et al., 2004
R	R	S	?	S	R	R	S	R	S	R	S	R	S	R	R	S	R	HA-335, HA-336	Pl ₆	LG 1	LG 8	Miller and Gulya, 1991; Bouzidi et al., 2002; Year et al., 2003; Pankovic et al., 2007
R	R	S	?	S	R	R	S	R	S	R	S	R	S	R	R	S	R	HA-337, HA-338	Pl ₇	LG 1	LG 8	Miller and Gulya, 1991; Bert et al., 2001;
R	R	R	?	R	R	R	?	R	R	?	?	?	?	?	?	?	?	83RM	Pl ₇₊	?	?	Sackston, 1990
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	RHA-340	Pl ₈	LG 6	LG 13	Miller and Gulya, 1991; Radwan et al., 2004; Bachalava et al., 2011
R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	RHA-274	Pl ₉	?	?	Sackston, 1990; Molinero-Ruiz et al., 2003
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	HA-R5	Pl ₁₃	?	?	Mulpuri et al., 2009; Bertero de Romano et al., 2010
R	R	?	R	?	R	?	R	?	R	?	R	?	R	?	R	?	R	29004	Pl ₁₄	?	LG 1	Bachalava et al., 2011
R	R	R	?	R	R	?	R	R	?	R	R	?	R	R	?	R	R	RNID	Pl ₁₅	?	LG 8	Bertero de Romano et al., 2010
R	R	R	?	R	R	S	?	R	R	R	R	R	R	R	?	S	DM4	Pl ₁₆	?	?	?	Molinero-Ruiz et al., 2003
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	RHA-419	Pl _{arg}	?	LG 1	Dussle et al., 2004; Wieckhorst et al., 2010; Imerovski et al., 2011
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	HA-R4	Pl _{HA-R4}	?	LG 1	Liu et al., 2010
R	R	R	?	R	S	S	R	R	R	R	R	R	R	R	S	S	S	PM-17	Pl?	?	?	Year, 2010
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	803-1	Pl?	?	?	Year, 2010
R	R	R	?	R	R	R	?	R	R	R	R	R	R	R	?	R	R	QPR1	Pl?	?	?	Year, 2010
R	R	R	?	R	R	R	?	R	R	R	R	R	R	R	?	?	R	QHP-1	Pl?	?	?	Year, 2010
R	R	?	S	S	S	R	R	R	R	S	S	?	?	?	?	?	?	PMI-3	Pl?	?	?	Year, 2010

R - resistant; S - susceptible; LG - linkage group on the genetic map; ? - unknown;

Resistance genes are often found in wild sunflower species. Inbred lines have been developed from these wild species, which are then used as donors of resistance genes. Thus, Pl_6 was introduced from wild ecotype of *H. annuus* (Miller and Gulya, 1991), Pl_5 from *H. tuberosus* (Vranceanu *et al.*, 1981), Pl_7 from *H. praecox* (Miller and Gulya, 1991), and Pl_8 and Pl_{arg} from *H. argophyllus* (Miller and Gulya, 1991; Seiler *et al.*, 1991). Along with resistance genes, lines developed from wild sunflower species also inherit a number of undesirable characteristics. When the donor lines are used in commercial breeding programs they have to be crossed with inbred lines which have desirable agronomical traits (Jocić *et al.*, 2010).

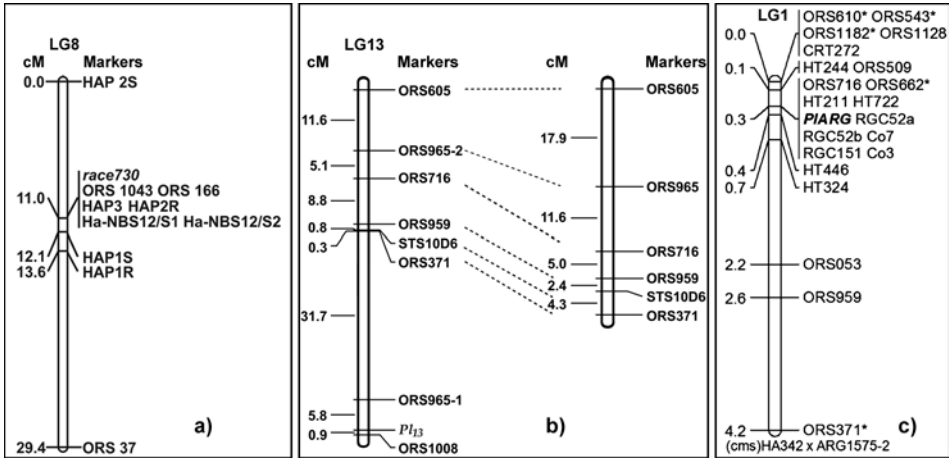


Figure 1: Location of some of the Pl genes on genetic maps: a) position of the Pl_6 on LG8 (Panković *et al.*, 2007), b) position of the Pl_{13} on LG 13 (Mulpuri *et al.*, 2009), c) position of the Pl_{arg} on LG1 (Dussle *et al.*, 2004)

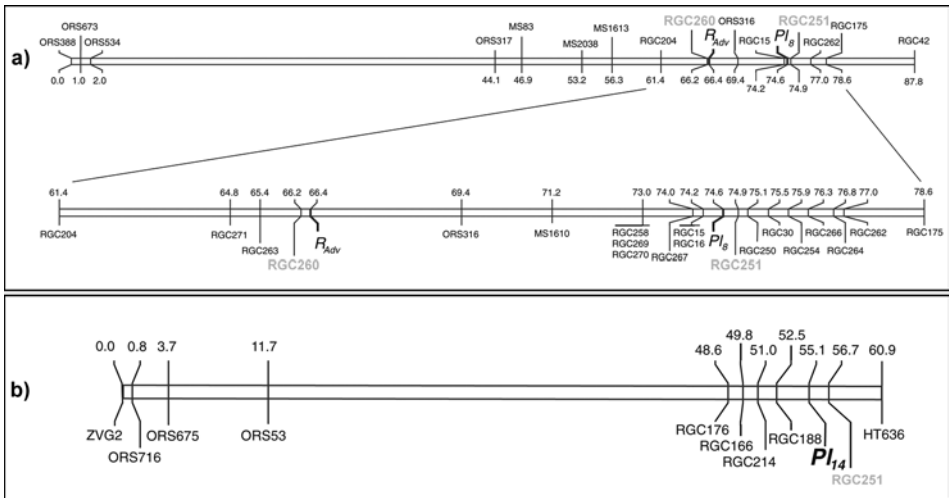


Figure 2: Position of the genes Pl_{14} (a) and Pl_8 (b) (Bachalava *et al.*, 2010).

Molecular markers have become a valuable tool in breeding programs (Kaya *et al.*, 2012). Molecular techniques, such as RFLP, SSR, INDEL, TRAP and SNP markers, enabled construction of genetic maps (Gentzbittel *et al.*, 1995; Yu *et al.*, 2002; Tang *et al.*, 2002; Yu *et al.*, 2003; Lai *et al.*, 2005) that greatly facilitated mapping of *Pl* genes. The position of most of the *Pl* genes is known.

Molecular markers facilitate detection of *Pl* genes, make breeding faster and more reliable, and are becoming an essential part of selection process (Imerovski *et al.*, 2011b; Miladinović *et al.*, 2011).

The link between the resistance genes and molecular markers

Cloning of resistance genes gave a better understanding of their structure, and provided a key for their classification (Gentzbittel *et al.*, 1998). Nearly three-fourths of the *R*-genes identified in plants encode nucleotide binding site (NBS) leucine-rich repeat (LRR) proteins (Radwan *et al.*, 2008). NBS-LRR encoding genes are highly duplicated in genomes of all known plants and are arranged as large multi-gene clusters. Hundreds of NBS-LRR encoding genes have been identified in sequenced genomes. Assuming that similar genes exist in sunflower genome as well, Gentzbittel *et al.* (1998) designed degenerate primers and found *R*-genes in two tightly linked families. These resistance gene analogues (RGA) were then cloned and sequenced (Bouzidi *et al.*, 2002). As a result, full-length RGA sequence was obtained and specific PCR primers that amplify 13 markers were created. These markers covered a genetic distance of about 3 cM around the *Pl*₆ locus, and gave a better insight into complexity of this locus. The utility of the markers created by Bouzidi *et al.* (2002) was verified by Panković *et al.* (2004). Subsequently, a co-dominant marker that completely co-segregates with *Pl*₆ gene and can be used for MAS was developed (Panković *et al.*, 2007). Two SSR markers, ORS 166 and ORS 1043, were also located within the HaRGC1 cluster (Slabaugh *et al.*, 2003). These markers co-segregated with *Pl*₆ (Panković *et al.* 2007), however later research suggested that the CAPS marker is probably in closer vicinity to *Pl*₆ than the microsatellite markers (Imerovski *et al.*, 2011c).

Several resistance gene candidates were also mapped on LG 8 and 13, and a smaller NBS-LRR-encoding clusters were mapped on linkage groups 1, 4, 9, and 15. Based on the sequences of two RGAs, 14 STS markers were developed within the *Pl*₅/*Pl*₈ locus (Radwan *et al.*, 2003; 2004). Opposed to TIR-NBS-LRR that characterize *Pl*₆ gene, RGA mostly linked to *Pl*₅/*Pl*₈ cluster are non-TIR-NBS-LRR (Radwan *et al.*, 2003; 2004; Dimitrijević *et al.*, 2011).

Bachlava *et al.* (2011) identified RGC markers tightly linked to *Pl*₈ and *Pl*₁₄. They 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 and *Pl*₁₄. Dussle *et al.* (2004) mapped *Pl*_{arg} in the telomeric region of LG 1, and found closely linked SSR markers that are useful for molecular assisted selection (Imerovski *et al.* 2011a). Wieckhorst *et al.* (2010)

identified three NBS-LRR class resistance gene candidates (RGC151, RGC52a and RGC52b), and provided a groundwork for map-based cloning of this resistance locus.

Conclusions and future prospects

Even though a big step has been made towards understanding the complex interaction of the pathogen and sunflower, as well as the mechanisms of sunflower resistance, a number of questions have still remained unanswered. One of the problems that need to be addressed is the classification of pathogen races. It would be of a great significance if a unique molecular method for pathotype identification would be proposed. With the use of molecular markers, race composition in different sunflower cultivating regions could be determined with a higher accuracy. Additionally, tracking of the pathogen population dynamics, *i.e.*, new race occurrence, would become possible.

A great number of authors have contributed to better understanding of the mechanisms involved in downy mildew resistance. The development of biotechnology resulted in molecular markers for detecting *Pl* genes, and provided means for marker assisted selection (MAS).

Candidate resistance genes that have been proposed until now need to be confirmed in the future. Genes that were denoted differently and were introduced from the same resistance source need to be compared to exclude the possibility that they are the same one. To avoid confusion, a unique nomenclature should be used for denoting gens.

In order to keep up with the occurrence of new pathogen races, novel sources of resistance genes and corresponding sequence specific molecular markers need to be found. Mechanisms of quantitative resistance should be examined in more detail, and genes that are involved in the unspecific defence mechanism identified. Finally, a set of molecular markers that could be used for tracking QTL should be proposed. Marker assisted selection could in this way be used not only for detecting major but also for minor genes, and would bring us a step closer to achieving sustainable resistance to *Plasmopara halstedii*.

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