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Marker-assisted selection and use of molecular markers in sunflower breeding for resistance to diseases and parasites

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Recently, the problem of phytosanitary condition of sunflower crops has been exacerbated, which is associated with violation of crop rotations and, as a consequence, spread of common diseases. Selection for resistance to biotic factors requires comprehensive research into the crop biology and pathogens. The use of starting material, which is resistant to major pathogens and environmental stressors, in selection is a prerequisite for the breeding of highly productive hybrids. Significant progress in the breeding of heterosis sunflower hybrids has been achieved primarily due to stable inbred lines. However, their creation is time-consuming, taking 8-12 years. Selection of desirable genotypes and initial forms for crossing is complicated by the fact that it is driven by a set of polygenic traits that are prone to significant modification variability. The use of molecular genetic markers is a way to accelerate breeding. Marker-assisted selection breeding (MAS) has been theoretically justified in numerous publications and implemented in most breeding institutions around the world. However, in domestic breeding programs, MAS has not become widespread compared to traditional methods. Nevertheless, this breeding trend opens new opportunities for studying genetic diversity and determining kinship at the intraspecies and genus levels. The review provides information on the status and prospects of implementation of MAS in traditional plant breeding and highlights the achievements of modern biotechnology in sunflower breeding for resistance to biotic factors owing to molecular genetic markers. The MAS principles are outlined and the advantages of this method are described. Specific examples of application of the molecular approach during the development of starting material of sunflower for breeding for resistance to common diseases and parasites are given. The main stages and components of PCR analysis are also described. Inbred sunflower lines – carriers of the gene for resistance to the downy mildew pathogen are characterized and genetic passports using STS markers to the Pl_6 locus have been formalized for 13 sunflower lines.

Key words: *DNA markers, Marker-Assisted Selection, sunflower, downy mildew, sunflower rust, broomrape.*

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Introduction

There are two main approaches in genetic studies - "direct" and "reverse" genetics. Since Gregor Mendel's discoveries of (1865), classical or "direct" genetics has been studying the inheritance of traits (phenotypes) in living organisms for several generations. Taking the phenotype as a starting point, "direct" genetics identifies genetic factors that affect the expression of any trait. Thus, "direct" genetics is

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directed from phenotype to genotype. Production of bulky mutant populations for further search for phenotypic changes in them is a key stage in the experimental application of this approach (Sulima, Zhukov, 2015).

Since the 1980s, the knowledge gained over the past period (discovery of DNA as a carrier of genetic information, decoding of the genetic code, development of sequencing and genome-modifying methods) (Inge-Vechtomov, 2010) opened a new approach that involves analyses of DNA sequences and the effects that are exerted by changes in these sequences (mutations) rather than analyses of phenotypes and their genetic control. This concept is called "reverse" genetics (Struhl, 1983; Reski, 1998).

Modern genetics has entered the "post-genomic era", when information about the genome structure of a wide assortment of organisms has become available. Nowadays, it is especially important to identify biological functions of genes, the sequences of which are already known (Eisenberg et al., 2000; Griffiths, Stotz, 2006; Hsiao, Kuo, 2009).

The object of "reverse" genetics is usually a gene with an unknown function (which was detected by Expressed Sequence Tag (EST) sequencing), the entire genome, or a separate part of it, etc. The research strategy is in altering the gene structure or activity and subsequent analyzing associated changes in the phenotype. With the development of large-scale genomic sequencing technologies, "reverse" genetics has received significant support, taking a leading position both in fundamental science and in applied research (Alonso, Ecker, 2006; Barrangou et al., 2007; Small, 2007; Boutros, Ahringer, 2008; Hirochika, 2010; Bolle et al., 2011; Upadhyaya et al., 2011; Liu, Fan, 2014).

Traditional breeding focuses mainly on phenotypic selection and is unable to distinguish effects of the environment or other factors related to growing conditions from genetic characteristics. This problem can be solved by using molecular markers in direct selection (MAS). The trait of interest is marked by a molecular marker that is closely linked to the gene determining this trait or to the gene that affects the trait expression.

MAS allows selecting in accordance with the genotype, regardless of environmental effects. In addition, MAS is used as a tool that complements and significantly facilitates traditional breeding.

The European Technology Platform ‘Plants for the Future’ in its proposed strategic research agenda for plant genomics and biotechnology until 2025 regards a rise in the selection efficiency as a priority. The molecular approach, namely Marker-Assisted Breeding (MAB) or Marker-Assisted Selection (MAS) is a promising way to solve this problem (Kozhukhova, 2011).

Literature Review, Summarization of Basic Provisions. The term "Marker-Assisted Selection" was first used in the literature in 1986 to describe the possible use (Beckmann, Soller, 1983). The first ground-breaking article on MAS in plant breeding using DNA markers was devoted to resistance of soybean to nematodes (*Heterodera glycines* Ichinohe) (Concibido et al., 1996). According to the Glossary of Biotechnology for Food and Agriculture of the Food and Agriculture Organization of the United Nations (FAO), MAS is the use of DNA markers to increase the selection efficiency, basing on the detection of markers of breeding traits (Zaid et al., 2007).

The MAS principle is as follows: if the localization of a gene that affects the expression of an agronomically important trait is known, inheritance of the gene that controls it, or the presence of the required allele in breeding material (not the expression of this trait) is monitored (Kozhukhova, 2011).

Available molecular markers are a necessary prerequisite for any MAS project. A molecular marker can be any DNA fragment used to detect a polymorphism and having a close genetic association with the gene responsible for the trait under investigation (Kalendar, Glazko, 2002).

The rapid development of new methods of molecular biology, including automation and computerization of different processes, development of appropriate methods and software for statistical processing and creation of available databases needed to study DNA polymorphism contribute to the arsenal of molecular markers and their increasing use in various fields of fundamental and applied biology (Kozhukhova, 2011).

Methods of molecular genetics have been widely used in various biological branches: botany, entomology, phytopathology, genetics, etc. The idea of markers is not new, as it was formulated by AS Serebrovskiy as a method of signals in the 1930s. Genetic and breeding achievements in agricultural plants are often attributed to different marker systems. After all, to optimize and accelerate the breeding process, one has first to identify genes of the desired traits in staring and breeding materials. Traditional methods of their detection (hybridological analysis) are labor- and time-consuming.

MAS, a novel comprehensive combining traditional breeding and achievements of such new disciplines as genomics and bioinformatics, offers exciting possibilities for the creation of harmful organism-resistant plant varieties and hybrids (Sivolap et al., 2011).

Marker as a general concept in breeding is a trait that is easily recognizable and is closely associated with a gene of breeders' interest. That is, the presence of the marker is a signal that the important for breeding trait is present too. Markers make it possible to conduct the desired trait-oriented selection (for example, for resistance) and to add fundamentally new genes to the plant genome. Data on markers of resistance genes allow researchers to quickly find the desired resistance genes and their combinations in wild and domestic plants as well as to monitor the presence of this trait while creating resistant or tolerant varieties and hybrids. The method of biochemical markers was developed and investigated in the late 1970s. It was found that alleles of genes that determine the synthesis of storage proteins or isoenzymes are linked to genes of wheat resistance to fungal pathogens (Lisova, 1999). Due to these findings, individual proteins can be used as markers in breeding for resistance.

Markers for plant breeding began to gain popularity in the early 1980s, when isozyme markers were used to accelerate the introgression of monogenic traits from exotic germplasm into cultivated background (Tanksley, Rick, 1980; Tanksley, 1983). In the mid-1980s, the first use of restriction fragment length polymorphism (RFLP) markers in agricultural crop improvement was described, including theoretical issues related to marker-assisted backcrossing (MABC) to improve quality triats (Beckmann, Soller, 1983).

Marker systems can be categorized into three groups: morphological, biochemical (storage proteins and isoenzymes) and genetic (DNA markers). The first two groups of markers, morphological and biochemical ones (markers based on the polymorphism of storage proteins and some enzymes), were widely used in genetic and breeding studies of agricultural crops until the 1990s.

Lande and Thompson (1990) first conducted theoretical studies of MAS for quantitative traits, thus motivating other scientists to a number of modeling studies in the 1090s (Zhang, Smith, 1992, 1993; Gimelfarb, Lande, 1994a, 1994b, 1995; Hospital, Charcosset, 1997; Whittaker et al., 1997). In the early 2000s, additional theoretical discussions were held on the application of MAS and strategies for pyramiding the necessary alleles by recurrent crosses (Hospital et al., 2000; Frisch, Melchinger, 2001, 2005; Hospital, 2002; Servin et al., 2004; Bernardo et al., 2006). These studies have answered many key genetic questions about MAS systems, such as sample size, number and type of markers, population type, and genome size (Avise, 2004; Guimaraes et al., 2007).

In MAS, when one marker is used, the selection reliability increases from 95% to 99.5%, and when two flanking markers are used, it is 100% provided that this marker is located within the gene (Collard, Mackill, 2008). The markers to be used must be close to the target gene (<5 recombination units). This is mandatory to ensure that only a small percentage of specimens will be recombinant. Typically, two markers are used rather than one, to reduce the probability of an error caused by homologous combination. That is, the first step in MAS is to map the gene(s) or quantitative trait locus(loci) (QTL(s)) of interest by different techniques. The recombination frequency between the target locus and the first marker is approximately 5%. Thus, the recombination between the target locus and the marker can occur in approximately 5% of the offspring. The recombination probability between marker 1 and marker 2 (i.e. double crossingover) is much lower than for one marker (about 0.4%). Thus, the selection reliability is much higher when one use flanking markers (Kozhukhova, 2011).

MAS in breeding is used for almost all major crops in four broad directions, namely:

- Traits which are difficult to manage through traditional phenotypic selection because of significant resource costs or complex heredity;
- Traits, the expression of which depends on the specific environmental conditions or on stages of development, which affects the target phenotype;
- To accelerate backcross breeding and maintain recessive alleles in this breeding;
- To pyramide several QTLs for one target trait with complex heredity (drought resistance or other adaptive traits) or several monogenic traits (qualitative traits and resistance to diseases and pests) (Xu, Crouch, 2008).

If one combines simultaneous selections for large number of target traits in traditional breeding, it will lead to a general weakening of the resulting material and increase the number of selection cycles to obtain final accessions. On the other hand, MAS ensures fewer selections and fewer losses when breeders build up several target traits in the same genotype.

It should be noted that the improvement of complex traits, such as resistance to diseases and pests, is complicated by large numbers of additional genes with unpredictable epistatic effects, impacts of different environmental factors and weak heredity (Kozhukhova, 2011). Modern methods based on the achievements of DNA technologies enable searching for resistance genes in starting material more accurately and quickly. At present, in the breeding practice, it is DNA markers that are successfully used: short fragments of DNA that are closely linked with a gene that is responsible for a particular trait or directly characterizes the target gene. MAS, which is based on such markers, is suitable in the selection for different agronomic traits, including resistance to pathogens and pests. The main advantage of DNA markers is that it is possible to study almost any part of the DNA molecule, while there is no need for repeated reseeding of breeding material on infection backgrounds, which significantly shortens the time of creation of resistant varieties and hybrids.

Of molecular methods used in marker-assisted plant breeding, polymerase chain reaction (PCR), which is widely applied to identify most DNA markers, is the most effective tool of DNA analysis. PCR was invented by American biochemist Kary Mullis, and he won the 1993 Nobel Prize in chemistry for this invention (Korovaeva, Popova, 2015).

The PCR method is based on the identification of specific DNA (RNA) fragments in the test material, their selective synthesis to a concentration at which they are easy detected and subsequent determination of amplicons (amplification reaction products) (Fedorenko et al., 2007). Except for RNA viruses, DNA is a unique carrier of genetic information in all organisms on Earth (Glazko, 2003).

DNA has a unique property - the ability to double after unraveling the helix and the separation of DNA strands (replication). DNA replication is catalyzed by enzymes called DNA polymerases using the complementarity principle. To start replication, this enzyme needs an initial double-stranded DNA fragment. Such a fragment is formed when a short single-stranded DNA fragment (primer) interacts with a complementary region of the corresponding parental strand of DNA. Two strands of DNA are replicated, but they grow in opposite directions. As a result, two double-stranded molecules are synthesized from one double-stranded DNA molecule, each of which contains one strand from the parental molecule of DNA and the other daughter, newly synthesized strand (Oleshchuk et al., 2014).

Each DNA replication cycle includes three main stages:

- The DNA helix is unraveled and the double-stranded DNA template is separated into single strands (denaturation);
- Primers anneal, or bind, to the DNA template;
- A daughter strand of DNA is synthesized.

In the PCR machine, these processes are cycled *in vitro*. The transition from one stage of the reaction to another is achieved by changing the temperature of the incubated mixture (Lopukhov, Eldeinshtein, 2000; Fedorenko et al., 2007; Kutyrev et al., 2010). To perform PCR analysis, it is necessary to prepare a sample of biomaterial (DNA or RNA extraction), complete PCR (amplification) and to detect the PCR products (amplified nucleic acid) (Romanenko et al., 1998). The requirements for PCR laboratories are formulated and summarized in corresponding regulations and guidelines (Edwards et al., 2004; Kutyrev et al., 2010; Stehnii et al., 2010; Kalachniuk et al., 2012).

Polymerase chain reaction allows one to selectively synthesize certain (target) DNA sites of several hundred to thousands of nucleotide pairs (usually not longer than 2 kilobases (kb)), using any DNA sample as a template, including a sample of degraded DNA. Today, the PCR principles remain unchanged despite numerous modifications. They consist in DNA amplification via synthesis of complementary strands catalyzed by DNA polymerase. To start replication, this enzyme needs two artificially synthesized single-stranded oligonucleotide primers. Primers are normally between 18 and 30 nucleotides in length. The primers are oriented in opposite directions with their 3' ends pointing towards each other. so that the elongation reaction proceeds from 5′ to 3′ across the region between the two primers, i.e. the distance between the primers determines the length of DNA fragments amplified during PCR. As a result of amplification, new DNA fragments of a certain size are synthesized (they appear after cycle 2).

Polymerase chain reaction is a cyclic process and usually consists of 30-40 cycles. Starting from cycle 2, the newly synthesized DNA molecules serve as templates for further synthesis of the target DNA region. Therefore, PCR will lead to an exponential increase in the number of copies of the DNA region of interest, which was flanked on both sides by primers. The number of amplicons can be approximately

estimated by formula 2^n, where n is the number of cycles. Correspondingly, the target DNA region can be 2^{20}-fold amplified in 20 cycles (Patrushev, 2004).

Polymerase chain reaction has been widely used in medicine, veterinary science, biology, criminology, history, archeology and other branches of human activities. This method is supersensitive and specific in diagnostics of infections. Thanks to PCR, a lot of modern scientific challenges are successfully solved; organisms are genotyped; genetic diseases are diagnosed and liability to them is evaluated. PCR can accurately test family relations, identify individuals, analyze ancient remains and expose GMOs (Rybicki, 2005; Mahony, 2008).

To date, a lot of modifications of classical PCR analysis have been developed, among which the following types of PCR can be distinguished:

Real-time PCR. This approach is able to determine the number of DNA copies or mRNA in the sample under study. Today, real-time PCR is the most common method used in different sectors. This method is based on the quantitative determination of the PCR product content in the reaction mixture in each cycle of the reaction. Fluorescent-labeled oligonucleotides are used to quantify the PCR products. When performing real-time PCR, one should compare the obtained graphs of fluorescence accumulation between several samples, for example, between a standard sample and a test sample.

Real-time PCR is now widely used in medicine and plant production to determine viral load in living organisms, to elucidate transcript levels, to assess mononucleotide polymorphism and to quantitatively determine the content of a foreign DNA molecule in organisms and food (the presence of genetically modified sources).

Multiplex PCR. Several primers specific for different genetic loci are added to the reaction mixture simultaneously.

Polymerase chain reaction with reverse transcription (RT-PCR). cDNA is synthesized on the RNA template by reverse transcriptase, and the resulting DNA sequence is used for classical PCR. RT-PCR is used for the following purposes:

- To study the differential expression of genes at the transcription level;
- In DNA diagnostics of infectios.

PCR *in situ*. This technique is designed to amplify DNA or RNA sequences directly on fixed slides of tissues, cells or chromosomes.

Allele-specific PCR. It is used to detect mutations in genomic DNA with allele-specific primers, which are complementary to the mutant sequences, while the wild type (norm) is not amplified (Patrushev, 2004).

Different molecular methods are used to detect DNA markers of the trait under investigation. Gupta et al. (2008) grouped DNA markers according to the detection technique as follows:

1) DNA markers based on restriction fragment length polymorphism (RFLP). RFLP markers are detected after treatment of genomic DNA with restriction endonucleases.

2) DNA markers, which are detected by various types of PCR analysis. These include the following types of markers:

- RAPD (random amplified polymorphic DNA) - randomly amplified polymorphic DNA; RAPD markers include DNA sequences obtained through amplification with arbitrary primers (9-11 bp);
- ISSR (inter simple sequence repeats) – intermicrosatellite sequences; during amplification, a DNA fragment located between intermicrosatellite loci is replicated;
- AFLP (amplified fragment length polymorphism) – DNA regions are identified by treating the DNA with two restriction enzymes, ligating adapters to the ends of the target nucleic acid and further amplifying with primers that are complementary to the adapter nucleotides;
- SSR (simple sequence repeat) - simple repeating sequences or microsatellites; these are tandem repeats of 2 - 6 bp in length, for example, (A) n, (AT) n, (GA) n, where n varies between 10 and 80 bp;
- EST (expressed sequences tags) - expressed DNA sequences, anonymous sequences, or sequences of unknown function, obtained from sequencing cDNA libraries;
- SCAR (sequence characterized amplified region) - a sequence that characterizes the amplification region; first, the RAPD fragment is excised from the gel, cloned and sequenced, and then specific primers are designed for this site with a length of 14–20 bp;
- CAPS (cleaved amplified polymorphic sequence) - amplification polymorphic sequences that are cleaved; amplification products are treated with endonucleases;

- IRAP (inter-retransposon amplified polymorphism) - amplification polymorphism of interretrotransposon sequences; amplification occurs between primers that are complementary to the sequence of two adjacent LTR regions of the retrotransposon;

- STS (sequence tagged site) - a sequence that characterizes the locus. A fragment of genomic DNA obtained by amplification with primers that are specific to the primary structure of a known locus.

3) DNA markers, which are detected by sequencing and using DNA chips. Single nucleotide polymorphism (SNP). SNPs are sites in the genome at which more than one nucleotide is found in a population, and the frequency of the rare allele should be at least 1% (Malyshev, Kartel, 1997; Lesk, 2009).

As it has been mentioned above, MAS is applied to almost all major crops. Below, we describe some molecular markers of resistance genes to biotic factors exemplified by one of the most common oil crops in the world - sunflower.

Downy mildew (*Plasmopara helianthi* Novot.). Breeding for resistance to downy mildew is a difficult challenge due to the large number of pathogen races and their significant variability. To date, 20 genes (Pl_1 – Pl_{20}) are known to determine race-specific resistance to downy mildew. These genes were found in different accessions, and *Pl* alleles are dominant. Pl_1 and Pl_2 are the most common genes, which are present in almost all breeding specimens of sunflower. There are published data on marking some of them, which helps to significantly accelerate the selection of valuable genotypes. In search for markers of these genes, scientists extensively use 13 lines - differentiators, which are included in the international standard for the identification of the downy mildew pathogen (Table 1).

Table 1. Characteristics of lines – differentiators (Jocić et al., 2012)

Line	Genes of resistance	Downy mildew races, to which resistance is ensured
HA-288	–	susceptible
RHA-265	Pl_1	100
RHA-274	Pl_1, Pl_2	100, 300, 304, 310, 330, 334
DM-2	Pl_2, Pl_5	100, 300, 304, 700, 703
PM-I3	Pl_2, Pl_5	100, 300, 700, 703
PM-17	Pl_5	100, 300, 304, 310, 700, 710, 703, 714
803-I	Pl_8	100, 300, 304, 330, 334, 700, 710, 714, 730, 733, 734
HA-R4	Pl_2, Pl_{13}	100, 300, 304, 330, 334, 700, 710, 714, 730, 734, 770
HA-R5	Pl_2, Pl_{13}	100, 300, 304, 330, 334, 700, 710, 714, 730, 734, 770
QHP-1	Pl_8 , Pl_{13}	100, 300, 304, 330, 334, 700, 710, 714, 730, 734, 770
FT-226 (analogue of QHP-1)	Pl_8 , Pl_{13}	100, 300, 304, 330, 334, 700, 710, 714, 730, 734, 770
HA-335	Pl_1, Pl_2, Pl_6	100, 300, 330, 700, 710, 730, 733, 770
RHA-419	Pl_{ARG}	Universally resistant

To date, the following genes of resistance to downy mildew are marked: Pl_1, Pl_2, Pl_{5-8}, Pl_{13-14}, Pl_{16-20}, and Pl_{ARG}. Carriers of the Pl_{ARG} gene, which determines the universal resistance against all known races of *Plasmopara helianthi*, are the most valuable sources of resistance in further sunflower breeding for resistance to downy mildew (Jocić et al., 2010). The Pl_{ARG} gene was mapped with SSR markers in linkage group (LG) 1 of the sunflower genetic map (Duble et al., 2004), and it was shown to be closely linked with microsatellites ORS716, ORS509, ORS1128, and ORS543 (Wieckhorst et al., 2010).

Scientists from the Plant breeding and genetics institute - National center of seed and Cultivar Investigation (Solodenko et al., 2014) studied 16 microsatellites in LG 1 in order to identify markers of the Pl_{ARG} gene. Amplicons loci ORS543, ORS606, ORS710, ORS716, and ORS959 were obtained. Collection accessions of the wild species *Helianthus argophyllus* L. were compared with line - differentiator RHA-419 (the Pl_{ARG} gene carrier) and other lines - differentiators (carriers of the *Pl1*, Pl_2, Pl_6, Pl_8, Pl_{13} genes). As a result, marker alleles of loci ORS509, ORS605, ORS610, ORS675, ORS1039, and ORS1182 that allow distinguishing the Pl_{ARG} carriers from the other studied genotypes, were identified. Thus, *H. argophyllus* carries the following marker alleles: 190 bp (ORS605), 220 bp (ORS1039), and 315 bp (OR716), while line RHA 419 carries 197 bp (ORS605), 130 bp (ORS610), and 190 bp (ORS1039).

Alleles 207 bp (locus ORS509), 165 bp. (ORS1182) and 220 bp (OR 675) can identify a fragment of LG1, which originates from *H. argophyllus* L. or line RHA-419.

Table 2. Characteristics of the *Pl6* locus (Bouzidi et al., 2002)

STS-marker	R/S*	Primer	Amplicon length,
Ha-NBS 1	S	HaP1	1,901
Ha-NBS 2	R	HaP1	1,484
Ha-NBS 3	S	HaP2	1,694
Ha-NBS 4	S	HaP2	1,979
Ha-NBS 5	R	HaP2	1,763
Ha-NBS 6	S	HaP2	1,700
Ha-NBS 7	R	HaP2	1,589
Ha-NBS 8	R	HaP2	1,414
Ha-NBS 9	R	HaP2	1,260
Ha-NBS 11	R	HaP3	1,811
Ha-NBS 12	S	HaP3	1,406
Ha-NBS 13	R	HaP3	1,119
Ha-NBS 14	R	HaP3	988

Note: R = resistance; S = susceptibility.

Table 3. Nucleotide sequences of the primers used to identify the *Pl6* locus

Primer	5′–3′ sequence
HaP1	F: GGTAATGGCTGTTGAATTTATGGAGC R: AGCATGATCCGGCTAGAGCCTTCTA
HaP2	F: GTCTACTACATGGTTTCCGTTTTC R: TGCTTCTTCCTTCTATCTCACTC
HaP3	F: GTTTGTGGATCATCTCTATGCG R: TGCTTCTTCCTTCTATCTCACTC

Table 4. Characteristics of the *Pl5* and *Pl8* loci (Bouzidi et al., 2002; Ramazanova, Antonova, 2018)

STS-marker	Locus	R/S*	Primer	Amplicon length, bp
Ha-NT8R 1	*Pl8*	R	Ha-P1	1,569
Ha-NT8R 2	*Pl8*	R	Ha-P1	2,119
Ha-NT8R 7	*Pl8*	R	Ha-P1	2,237
Ha-NT8S 1	*Pl8*	S	Ha-P1	1,153
Ha-NT8S 2	*Pl8*	S	Ha-P1	1,610
Ha-NT5R 2	*Pl5*	R	Ha-P1	2,021
Ha-NT5S 1	*Pl5*	S	Ha-P1	1,303
Ha-NT5S 2	*Pl5*	S	Ha-P1	1,424
Ha-NT5S 3	*Pl5*	S	Ha-P2	387
Ha-NT8R 3	*Pl8*	R	Ha-P3	1,584
Ha-NT5R 1	*Pl5*	R	Ha-P3	1,584
Ha-NT8R 4	*Pl8*	R	Ha-P4	1,840
Ha-NT8R 5	*Pl8*	R	Ha-P5	2,419
Ha-NT8R 6	*Pl8*	R	Ha-P6	2,437

Note: R = resistance; S = susceptibility.

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Search for carriers of the Pl_6 resistance gene, which determines resistance to 11 races, including races 710, 730 and 330, is no less important in sunflower breeding for resistance to downy mildew (Ramazanova, Antonova, 2018). Table 2 summarizes the characteristics of 13 STS-markers within this locus and three primers that were developed by Bouzidi et al. (2002) and are used by researchers from different countries to identify the Pl_6 locus in sunflower genotypes. The nucleotide sequences of these primers are presented in Table 3.

The introgression of the Pl_8 locus into breeding accessions is also promising, since this locus is in the same LG with the Pl_5 locus, so one can use them in the crop breeding to create universally resistant sunflower lines and hybrids, i.e. with resistance to the most common races. To flank the Pl_5 and Pl_8 loci, researchers used six primers (Bouzidi et al., 2002), the characteristics of which are summarized in Table 4, and their nucleotide sequences are presented in Table 5.

To successfully breed sunflower for resistance to downy mildew, one should analyze not only plants, but also the pathogen. Hence nowadays, PCR methods are used to determine the molecular genetic variability of *P. helianthi*. This approach allows elucidating the structure of the pathogen population and the rate of its variability, which significantly accelerates the breeding work to create resistant starting material (Radwan et al., 2008).

Table 5. Nucleotide sequences of the primers used to identify the Pl_5 and Pl_8 loci

Primer	5′–3′ sequence	Locus
Ha-P1	F: GCCCAAAATTGAAAGAAAGGTGTG R: GGCGAAATTGGTTCCCGTGAGTCG	Pl_5, Pl_8
Ha-P2	F: AATCTTGAGTCATTACCCGAGC R: CAGCGTCTCTGGTAGATCGTTCACC	Pl_5, Pl_8
Ha-P3	F: GCTGTTACTGCCCTCTTCAAAGTC R: TTTGAAAGATAAGTTCGCCTCTCG	Pl_5, Pl_8
Ha-P4	F: GCTGTTACTGCCCTCTTCAAAGTC R: CCCAACTCGACATATCTTCAAACC	Pl_8
Ha-P5	F: TAGTTAACATGGCTGAAACCGCTG R: CCCCATATTGACAAAGAGTTGAGG	Pl_8
Ha-P6	F: TAGTTAACCATGGCTGAAACCGCTG R: CGTCTCTGGTAGATCGTTCACCTT	Pl_8

Sunflower rust (*Puccinia helianthi* Schw.). The introgression of resistance to rust from wild species, mainly from *H. argophyllus*, in modern sunflower varieties and hybrids has led to the discovery of 13 resistance genes (R_{1-5}, R_{10-12}, R_{13a}, R_{13b}, *R14*, R_{adv} and P_{u6}) (Bachlava et al., 2011). Molecular mapping of 11 genes showed they were located in several LG of the genetic map of sunflower. Thus, five *R* genes are located in LG 2 (R_5), LG 8 (R_1), LG 11 (R_{12} and R_{14}) and LG 14 (R_2), while the remaining six genes (R_4, R_{11}, R_{13a}, R_{13b}, R_{adv} and P_{u6}) are located in LG 13, which is divided into two subclusters, subcluster 1 (R_{11}, R_{adv} and P_{u6}) and subcluster 2 (R_4, R_{13a} and R_{13b}) (Lawson et al., 1996; Yu et al., 2003; Qi et al., 2011, 2012a, 2012b, 2015b; Gong et al., 2013a, 2013b; Bulos et al., 2013).

The fact that sunflower rust resistance genes quickly lose their effectiveness due to the emergence of new virulent races in a short period of time after the introduction of resistant varieties and hybrids into production poses a serious problem. Therefore, it is important to search for new genes of rust resistance and for molecular markers that can identify these genes as well as to pyramide several resistance genes in one genotype.

The first molecular studies were conducted to detect markers of the R_1 and R_{adv} genes using RAPD- and SCAR-markers (Bulos et al., 2013; Lawson et al., 1998). Subsequent molecular studies of the *R* genes were designed to identify molecular markers linked to the R_1, R_{adv}, P_{u6}, R_{11}, R_{13a}, and R_{13b} genes, which are located in LG 13. Qi et al. (2015b) identified two markers flanking the R_4 gene (ORS581 and ZVG61), which were later also used to mark the rust resistance genes R_{13a} and R_{13b} (Bulos et al., 2013; Qi et al., 2015a, 2015b; Talukder et al., 2014, Solodenko, Fait, 2016) (Table 6). Bulos et al. (2014) mapped the P_{u6} gene and identified closely linked SSR-markers (ORS316, ORS224) in LG 13.

Today, R_5 is the only sunflower rust resistance gene in LG 2. Qi et al. (2012b, 2015a) identified two SSR- and two SNP-markers that flank the gene (SFW03654, $ORS653_a$, NSA_000267, ORS1197-2).

Table 6. Markers of the sunflower genes of resistance to *Puccinia helianthi*

Linkage group	Resistance gene	Accession used to map the gene	Marker	Reference
LG 2	R_5	Ha-R_2	SFW03654 ORS653a NSA_000267 ORS1197-2	Qi et all., 2012a Qi et all., 2015a
LG 8	R_1	RHA 279	$SCT06_{950}$	Lawson et all., 1998
LG 11	R_{12}	RHA 464	NSA_003426/NSA_00455/ NSA_000064/NSA_008884/ NSA_003320. CRT275 ORS1227 ZVG53	Talukder et al., 2014
	R_{14}	PH3	NSA_000064 ORS1227 ORS542 ZVG53	Zhang et al., 2016
LG 13	R_4	HA-R3	SFW05240/SFW05630/SFW06095/ SFW08283 SFW01497/SFW05630/SFW08875 ORS581 ZVG61	Qi et al., 2011 Qi et al., 2015a
	R_{HAR6}	HA-R6	ZVG61 ORS581	Bulos et al., 2013
	R_{13a}	HA-R6	SFW05743 RGC15/16 ORS316/ZVG61 ZVG61 SUN14	Gong et al., 2013a Qi et al., 2015a
	R_{13b}	RHA 397	SUN14 SFW00757 RGC15/16 ZVG61, ORS316 SFW04275/SFW04317/SFW05743 ZVG61, ORS316	Gong et al., 2013a Qi et al., 2015a
	R_{adv}	Advance RHA 340	SCX20600 RGC260 ORS316	Lawson et al., 1998 Bachlava et al., 2011
	P_{u6}	P386	ORS316 ORS224	Bulos et al., 2014
	R_{11}	Rf ANN-1742	ORS728 ORS728 ORS45	Qi et al., 2012b
LG14	R_2	MC 29 (USDA)	SFW01272 NSA_002316 SFW00211	Qi et al., 2015b

LG 11 contains two rust resistance genes: R_{12} and R_{14}. The two genes were aligned with markers ORS1227 and ZVG53. Talukder et al. (2014) used five SNP-markers (NSA_000064, NSA_004155, NSA_003426, NSA_008884, NSA_003320) to identify the R_{12} gene, but only two of these markers (NSA_003426 and NSA004155) were effective in identifying the R_{12} gene.

Qi et al. (2012b) also used previously developed SSR- and SNP-markers to identify homozygous multi-race-resistant genotypes in a population of F_2 hybrids derived from crossing BC3F2 accession - carrier of the R_5 gene and HA-R6 accession - carrier of the R_{13a} gene. The offspring obtained from plants selected from this hybrid population were more resistant to races 336 and 777 compared to lines with only one resistance gene. The researchers also pyramidized the R_5 and R_{13a} genes in confectionery sunflower using SSR- and SNP-markers (Qi et al. 2015a). They revealed that pyramidation of the *R* genes could ensure long-term resistance to the causative agent of sunflower rust. Thus, the creation of sunflower genotypes combining several rust resistance genes is a very important objective in breeding, and the breeding process can be significantly facilitated and accelerated by using the molecular markers described above.

Broomrape (*Orobanche cumana* Wallr.). Broomrape (a plant - parasite)-caused damage significantly reduces sunflower yields. The most reliable way to control this parasite is to create resistant varieties and hybrids with prior studies of the inheritance of resistance to broomrape. The genetics of this trait is studied by Ukrainian and foreign scientists. Sunflower has been bred for resistance to broomrape for almost a century (Shindrova, 2006). Currently, 8 broomrape races with different virulence are known. They are denoted by the Latin letters as A, L, B, C, D, E, F, G, H (Melero-Vara et al., 1989; Alonso *et al.,* 1996; Akhtouch *et al*., 2002; Shindrova, 2006; Pacureanu et al., 2009; Antonova *et al*., 2011). Until recently, the first five physiological races of broomrape were spread in all regions of sunflower cultivation; resistance to them is determined by individual *Or* genes (Sukno, 1999). Studies have confirmed that resistance to races A to E is determined by the genes Or_1 to Or_5, which are allelic or strongly linked, and resistance to race E is controlled by a single dominant gene, Or_5 (Lu et al., 2000; Fernández-Martínez et al., 2000; Fernández-Martínez et al., 2008).

Most molecular analyses were performed to study and create different types of molecular markers to identify the Or_5 gene, which determines resistance to races E and below (Tang et al., 2003; Fernández-Martínez et al., 2004; Guchetl et al., 2012). Foreign scientists (Tang et al., 2003) identified some DNA markers in the same linkage group with Or_5. The nearest SCAR-marker is mapped at a distance of 5.6 cM from the distal end of Or_5 (Table 7).

Table 7. Markers of the sunflower broomrape resistance genes

Linkage group	Gene of resistance	Accession used to map the gene	Marker	Genetic distance, cM	Reference
LG 3	Or_5	RPG01 PHD	CRT214 RTS05 CRT392 ORS1036	1.1 5.6 6.2 7.5	Lu et all., 2000 Tang et all., 2003
LG 13	$Or_{ab\text{-}vl\text{-}8}$	AB-VL-8	ORS683 ORS657	1.5 4.7	Imerovski et all., 2016

Tang et al. (2003) identified SSR-markers closely linked to Or_5 and mapped this locus in the upper part of LG 3 in the genetic map of SSR loci. The nearest SSR-markers are at distances of 6.2 cM (CRT392) and 7.5 cM (ORS1036) from the Or_5 locus.

Search for donors of resistance to highly virulent broomrape race F is urgent today. This race originated at the end of the last century in Spain. Imerovski I. et al. (Imerovski et al., 2016) used line AB-VL-8, resistant to this broomrape race, to map markers of a resistance gene, which was named $Or_{ab\text{-}vl\text{-}8}$ by the researchers. $Or_{ab\text{-}vl\text{-}8}$ was established to be in LG 13. In their molecular studies, the researchers found the nearest SSR-markers, which are at distances of 1.5 cM (ORS683) and 4.7 cM (ORS652) from the locus.

For traits with weak heredity, typical breeding programs involve the cultivation of millions of individual plants in thousands of populations to achieve greater homozygosity in lines, which occurs in approximately F_5-F_6 generations. This process requires significant resource costs and a significant time of 5-12 years. Due to the rapid development of agriculture and life in general, breeding programs with their scale, complexity of selection, numbers and sizes of populations, etc., require the latest approaches, which certainly include MAS.

Conclusions

Thus, MAS has been theoretically justified in numerous scientific publications and is implemented in most breeding institutions around the world. This trend in breeding opens new opportunities for studying genetic diversity and relations at the species and genus levels. Molecular marker-based selection is necessary in modern plant breeding, including in sunflower breeding for resistance to biotic factors.

The achievements of scientists in marking genes of resistance to the pathogen of downy mildew and sunflower rust as well as to the plant - parasite, broomrape, opens opportunities of identifying reliable sources of resistance and involving them in breeding programs in order to create valuable starting material. The emergence of new virulent races of these diseases and broomrape forces researchers to carry out deeper studies in finding new resistance genes and identifying molecular markers for these genes.

The effectiveness of molecular markers in sunflower breeding for resistance to diseases and parasites is confirmed by the results of scientific studies. Today, the search for donors of resistance to highly virulent races, which will significantly accelerate the selection of valuable genotypes in breeding for resistance, is urgent.

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Маркери генів стійкості соняшнику до основних хвороб та паразитів

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Останніми роками відмічається загострення проблеми фітосанітарного стану посівів соняшнику, що пов'язано із порушенням сівозмін і як наслідок – поширенням основних хвороб. Селекція на стійкість до біотичних чинників потребує всебічного вивчення біології культури та патогенів. Необхідною умовою для створення високопродуктивних гібридів є використання в селекційному процесі вихідного матеріалу, стійкого до основних патогенів та стресових умов середовища. Значних успіхів в селекції гетерозисних гібридів соняшнику досягнуто, насамперед, шляхом використання стійких інбредних ліній. Проте нині процесс їх створення є досить тривалим та займає 8–12 років. Процес добору потрібних генотипів та вихідних форм для схрещування ускладнюється тим, що іде за комплексом полігенних ознак, які зазнають значної модифікаційної мінливості. Одним з шляхів прискорення селекційного процесу є використання молекулярно-генетичних маркерів. Маркер-асоційована селекція (MAS) отримала теоретичне обґрунтування в численних публікаціях та впроваджена у більшості селекційних установ різних країн світу. Але у вітчизняних селекційних програмах маркер-асоційована селекція порівняно з традиційними методами не набула широкого розповсюдження. Проте даний напрям в селекції відкриває нові можливості вивчення генетичного різноманіття, визначення спорідненості на внутрішньовидовому і родовому рівнях. В огляді наведено інформацію щодо стану та перспектив впровадження в традиційну селекцію рослин так званого добору за допомогою генетичних маркерів Marker-Assisted Selection (MAS), висвітлено досягнення сучасної біотехнології в селекції соняшнику на стійкість до біотичних чинників з використанням молекулярних маркерів. Представлено принципи MAS, охарактеризовано переваги даного методу. Наведено приклади конкретного використання молекулярного підходу при створенні вихідного матеріалу соняшнику для селекції на стійкість до основних хвороб та паразитів. Також описано основні етапи та компоненти для проведення ПЛР-аналізу. Надано характеристику

інбредних ліній соняшнику з геном стійкості до збудника несправжньої борошнистої роси та сформовано генетичні паспорти 13 ліній соняшнику за STS-маркерами до локусу Pl_6.

Ключові слова: *ДНК-маркери, маркер-асоційована селекція, соняшник, несправжня борошниста роса, іржа соняшнику, вовчок соняшниковий.*

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