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DNA Marker-Based High-Throughput Identification of Downy Mildew Infected and Non-Infected Sunflower Plants

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Abstract: Developing hybrids, resistant to causal pathogen of Downy mildew (*Plasmopara halstedii* (Farl.) Berl. & de Toni), is one of the critical tasks in sunflower breeding. Molecular markers have advanced breeding practice in the past decades, however there are still unmet needs for reliable high-throughput (HT) selection of the pathogen resistant starting material and differentiation of the plants infected by different pathogens. In this study, we tested the known DNA marker (308 bp fragment from ribosomal DNA of *P. halstedii*) for detection of pathogen in different tissues of sunflower plant and at different stages of plant development. Specified DNA marker was re-validated in the total DNA, isolated from sporangium as well as from seedlings of infected pathogen resistant/susceptible inbred lines of Ukrainian breeding and 60 F_2 crosses. An independent set of field grown plants with unknown resistance to *P. halstedii*, having symptoms of the bacterial/fungal/viral pathology were used for HT screening and genotypes, infected with downy mildew, were successfully identified. Pathogen appeared to be concentrated in the vessels of sunflower leaves, in contrast to parenchymal tissue. Our study demonstrates an addition to whole seedling inoculation technique of *P. halstedii* detection, which allows HT identification of the pathogen infected and non-infected sunflower plants.

Keywords: sunflower, *Plasmopara halstedii*, DNA marker, high-throughput test, pathogen detection

Introduction

Ukraine is one of the world's leaders in production of the sunflower seeds. Breeding and seed production of sunflower facing constant problems, caused by susceptibility of sunflower crop to pathogens and pests. Specifically, increasing the cultivated areas with sunflower in Ukraine led to spreading of the multiple

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pathogens infection, including Downy mildew. The causal agent *Plasmopara halstedii* is an obligate fungus-like (Oomycete) pathogen. Primary route of infection is through the root system by zoospores, growing up in worm and wet conditions from environmentally stable oospores. The pathogen slows down growth of the plant, shortens the leaves petiole, causes mosaicism and corrugation of leaves, on the lower surface of which spore-producing colonies appear. The manifestation of disease can be seen already at the stage of 3–4 leaves. Usually, plants are dying quickly. There are currently more than 30 pathotypes of Downy mildew discovered in the cultivation areas of sunflower (Jocic *et al.*, 2010) and new pathotypes of *Plasmopara halstedii* often appear.

Breeding of varieties and hybrids, carrying genetically determined resistance to pathogen is considered to be the most environmentally and economically beneficial approach to overcome Downy mildew. Breeding process includes field- (Tihonov *et al.*, 1981) and lab-tests (Dolgova *et al.*, 1990) of the initial material for resistance. However, field-tests are not always reliable due to dramatic changes in the field resistance upon environmental conditions. Lack of rain can make the pathogen somewhat dormant and invisible to the eye in susceptible forms of sunflower. Lab-test, developed for evaluation of pathogen resistance in early stages of plant development (12–14 days seedlings), requires climatic growth chambers and constant keeping of pathogen cultures.

Molecular genetics and genomics methods, extended knowledges about the structure and functions of different genomes, provided us with new technologies for development of molecular markers. PCR-based tests were developed for detection of quarantine pathogen in tissue and seed of different crops (Berg *et al.*, 2005; Bonants *et al.*, 2003). In order to develop molecular marker, the specific DNA sequence, characteristic for the target gene and allowing to distinguish the genotype or group of genotypes from other ones, needs to be identified. Sequence analysis of 28S ribosomal RNA (GenBank) in 9 isolates of *P. halstedii* and 60 isolates of other oomycetes allowed to identify two polymorphic sequences, one of which was specific only for *Plasmopara halstedii* (Ioos *et al.*, 2007).

The aim of this study was to demonstrate the possibility of usage of specific sequence of *Plasmopara halstedii* genome as DNA marker for detection of pathogen in seedlings and tissues of sunflower plant.

Materials and methods

The lines-differentiators of sunflower resistance to particular races of Downy mildew 803-I, RHA 419 were investigated as well as susceptible inbred line Od

9A, created at Plant Breeding and Genetics Institute (PBGI, Odessa, Ukraine), the seedlings of F_2 (Od 9A x 803-I), the blooming plants of the same F_2 population and different breeding lines, grown on the testing field. Seed and plant material for study was kindly provided by Dr Boris Varenyuk, PBGI.

60 seedlings of F_2 (Od 9A x 803-I) were lab-tested for resistance (Dolgova *et al*., 1990) as following: seeds were germinated in during 4–5 days, after which 5–9 cm seedlings were kept at temperature 13–15 °C in a suspension of Downy mildew zoosporangia (population of pathogen collected from infected plants grown on PBGI fields). After inoculation the seedlings were placed on filter paper, which was rolled and kept in the growth chamber for further seeds growing at 20 °C and 90–95 % humidity. In 6–7 days the seedlings with the first pair of true leaves were put in the humidity chamber for 20 hours in the dark to induce sporulation. Emergence of visible signs of sporulation on the leaves, hypocotyls and cotyledons of some plants made it possible todefine them as susceptible to Downy mildew.

CTAB protocol (Doyle and Doyle, 1987) was used for DNA isolation from the sunflower seedlings, the leaves of flowering plants with chlorosis and *P. halstedii* sporangium removed from infected samples. In order to isolate DNA from different parts of the leaves a fragments of leaf parenchyma and fragments of veins of the leaves were cut out separately. PCR was performed with primers PHAL-F and PHAL-R, developed by Ioos *et al*. (2007) for analysis of polymorphic sequence of ribosomal DNA in *Plasmopara* genome, as well as primers ORS1039-F and ORS1039-R, detecting corresponding microsatellite loci of sunflower. PCR mix in 20 µl consisted of 1 x reaction buffer (Fermentas), 0.2 mM of each dNTP, 0.2 µM of each primers, 20 ng DNA and 1 unit Tag polymerase (Fermentas). PCR reactions were performed using "Tercik" thermocycler under following protocol: 1 min at 92 °C, 30 cycles of 1 min at 92 °C, 30 sec at 60 °C, 30 sec at 72 °C, with final extension for 3 min at 72 °C. The amplification products were visualized by electrophoresis on polyacrylamide gels followed by silver staining.

Results and discussion

According to the standard practice, sunflower lines and hybrids are evaluated for resistance to Downy mildew every year during winter using the lab-test. To accomplish molecular testing, we collected 60 seedlings of F_2 (Od 9A x 803-I) when they were lab-tested and visible sign of *P. halstedii* infection started to occur. Upon presence or absence of fungus spores, seedlings were categorized as susceptible and resistant. Seedlings tissue was used for DNA isolation. Prior

to isolation, susceptible samples were thoroughly cleaned off from sporangium. Collected sporangium were used for DNA isolation as a positive control. PCR with primers, specific to *P. halstedii* genome, generated product of 308 bp only with DNA templates, isolated from infected seedlings and sporangium (Figure 1).

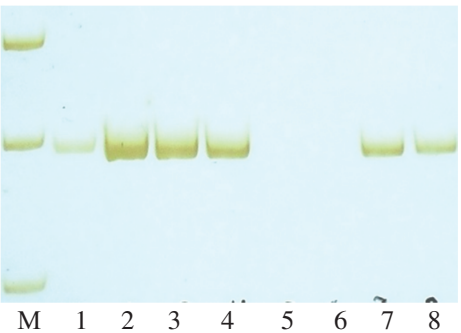

Figure 1: PAGE gel with PCR samples after amplification DNA with *P. halstedii* specific primers: 1–4 – Downy mildew infected F_2 seedlings; 5, 6 – non-infected F_2 seedlings; 7, 8 – *P. halstedii* sporangium. M – molecular weight marker pUC 19/MspI (bands 404, 331, 242 bp).

Since our isolation method delivers total DNA mix from both fungus and plant, it was critical to evaluate the specificity of the used DNA marker as well as probability of getting false positive and false negative PCR products. Figure 2 shows PCR products obtained with primers specific either to *P. halstedii* genome (lanes 1, 3, 4, 5) or to microsatellite locus (*ORS1039)* in *H. annuus* genome (lanes 2, 6, 7). Note, PCR template, containing fungal and plant DNA, was isolated only at the final stage of the lab-test. The lines-differentiators RHA 419 and 803-I, carrying genes Pl_{Arg} and *Pl8*, correspondingly, and posing resistance to all known races of *P. halstedii* (Jocic *et al.*, 2012), were not infected with Downy mildew in our lab-test. Furthermore, pathogen was not present in the tissues of these lines, as confirmed by the absence of *P. halstedii* DNA marker (308 bp) in PCR test (Figure 2, lane 4). This result demonstrates the actual inability of pathogen to grow in the tissue of RHA 419 and 803-I lines. The same DNA templates allow clear amplification of microsatellite locus (*ORS1039)*, fragment 190 bp for RHA 419 and 803-I (Figure 2, lanes 6, 7).

P. halstedii specific fragment of 308 bp (Figure 2, lane 1) and *H. annuus* specific fragment 205 bp (Figure 2, lane 2) were detected in total DNA mix, isolated from the same infected seedling from F_2 (Od 9A x 803-I). PCR-test results coincided with standart lab-test results for all F_2 seedlings and parent lines Od

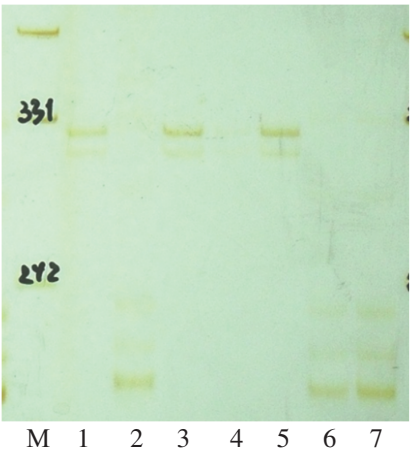

Figure 2: PAGE gel with PCR products after amplification of DNA, isolated from: Downy mildew infected seedling with *P. halstedii* specific primers (1) and *H. annuus* specific primers (2); *P. halstedii* sporangium with *P. halstedii* specific primers (3, 5); resistant line RHA 419 with *H. annuus* specific primers (6); resistant line 803-I with *P. halstedii* specific primers (4) and with *H. annuus* specific primers (7). M – molecular weight marker pUC 19/MspI (bands 404, 331, 242 bp).

9A and 803-I. All resistant samples did not have the marker fragment of *P. halstedii* DNA in PCR-test. This result shows high specificity and efficacy of used DNA marker for the differentiation of resistant and susceptible samples. Since marker was re-validated and performed reliably in our sample set with known resistance/susceptibility, at the next step we evaluated test performance on the blinded samples, with unknown response to *P. halstedii* infection, but with visible lesions, which could be caused by fungus, bacteria and/or virus.

For molecular detection of *P. halstedii* fungus we used tissues of sunflower plants with unknown sensitivity, grown in the field and developed signs of lesions, similar to Downy mildew pathogen. We demonstrated that DNA of P. *halstedii* is detected in the veins of sunflower leaves of plants with typical symptoms of Downy mildew infection like stunting, leaf corrugation and chlorosis, and withering flower heads. However, DNA isolated from leaf parenchyma of the same samples did not contain the specified marker of 308 bp or it was appearing slightly. This fungus develops inside the infected plants in all plant parts and fungus mycelium can take place in the root, the stem and especially the leaves including the parenchima as well as the vessels. In our study detection of DNA of the pathogen was less effective in the leaf parenchima than in the

leaf vessels. Perhaps under insufficiently favorable conditions of development, the pathogen is predominantly localized in the leaf vessels in order to have better access to assimilates and water.

Sunflower breeding for resistance to diseases is getting more and more importance every year. This is triggered by the constant appearance of new races and pathotypes of causative agents, which keep overcoming the resistance of hybrids and varieties. Oospores of Downy mildew are preserved in the seeds of infected plants as well as in the soil. Due to the harmfulness of this disease, all breeding materials must undergo the lab-test for resistance to the pathogen. PCR-based molecular identification of pathogenic DNA could provide more objective estimation of the pathogen resistance. Moreover, DNA-test might be useful to control the degree of contamination of sunflower plants with Downy mildew spores in the field, especially for diagnosis of the hidden disease.

Conclusion

DNA marker of *P. halstedii* genome allows detection of pathogen in the seedlings and the leaves of flowering plants of sunflower. Total mixed DNA of the host plant and fungus can serve for DNA-test of Downy mildew infection in sunflower plants. To increase the quality and reliability of the test on the samples of mature plants, it is necessary to use the vessels tissue of leaves for DNA isolation. Test allows high-throughput identification of infected and non-infected by the pathogen sunflower plants. PCR-test could be an addition to the lab-test of seedlings or an independent test of mature plants, grown in the field.

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