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Identification of Powdery Mildew *Blumeria graminis* f. sp. *tritici* Resistance Genes in Selected Wheat Varieties and Development of Multiplex PCR

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Abstract: The aim of the study was to identify the *Pm2*, *Pm3a*, *Pm4b* and *Pm6* genes and to develop multiplex PCR reaction conditions to reduce time and limit analysis costs. The following molecular markers were used for gene identification: *Xcfd81*, *Whs350* and *Xgwm205* (for *Pm2*), *Pm3a* (for *Pm3a*), *STS_241* and *Xgwm382* (for *Pm4b*), *NAU/BCDSTS 135-2* (for *Pm6*). Plant material consisted of 7 popular European wheat varieties from the wheat collection at the Department of Genetics and Plant Breeding of the Poznań University of Life Sciences. The field experiment was established in 2017 and 2018 on 10 m^2 plots in a randomized complete block design in three replicates in the Dłoń Agricultural Experimental Farm of the Poznań University of Life Sciences (51°41'23.835"N 017°4'1.414"E). The analyses demonstrated that the accumulation of all identified *Pm* genes was found in the Assosan variety. The accumulation of the *Pm2*, *Pm4b* and *Pm6* genes was found in Atomic, Bussard, Lear, Sparta, Tonacja and Ulka varieties. The work also involved developing multiplex PCR conditions for *Xcfd81* and *STS_241* and *Xcfd81* and *Xgwm382* primer pairs, allowing the simultaneous identification of the *Pm2* and *Pm4b* genes.

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1 Introduction

In the last century, a growing proportion of the population living in cities with a simultaneous shrinking of crop area per person has been observed. For this reason, increasing the fertility of varieties is the main purpose of current plant breeding. Obtaining plants with beneficial economic features, including high yielding potential, is closely related to their resistance to biotic and abiotic stresses [1].

Wheat (*Triticum aestivum* L.) is cultivated on all continents and is the most important cereal in the Northern Hemisphere, but also in Australia and New Zealand [2]. The yield of winter wheat may be limited by many factors, including weed infestation, occurrence of pests, nutrient deficiencies, and pathogen infections [3-5]. In addition, stress factors, both abiotic and biotic, can also lead to lower yields and quality [6].

Powdery mildew of cereals and grasses caused by *Blumeria graminis* f. sp. *tritici* belongs to one of the most dangerous fungal diseases in cereal crops in the world, where it is the cause of large yield losses every year [7-9]. Currently, over 70 powdery mildew resistance alleles, located in 41 loci, and approx. 20 temporarily designated genes have been identified [10-20]. Research aimed at identifying new genes for powdery mildew resistance is ongoing. The powdery mildew (*Pm*) resistance genes have been introduced into strains by hybridization, homologous recombination or backcrossing [21] from ancestor or wild wheat species, e.g., *Triticum monococcum*, *Triticum durum*, *Triticum uratu*, *Aegilops speltioides*, *Aegilops tauschii*, *Haynaldia villosa* [22, 23]. Only 24 genes are derived from the *Trtiticum aestivum* gene pool [21].

Breeding resistant varieties provides the possibility of effective, cheap and safe eradication of harmful

Table 1: The degree of resistance to *Puccinia recondita* f. sp. *tritici* infection under field conditions (Dłoń Agricultural Experimental Farm) and the identification of the *Pm2*, *Pm3a*, *Pm4b* and *Pm6* genes by means of molecular markers.

Genotype	Field conditions		Molecular analysis						
	2017	2018	*Pm2 Xcfd81*	*Pm2 Whs350*	*Pm2 Xgwm205*	*Pm3a Pm3a*	*Pm4b STS_241*	*Pm4b Xgwm382*	*Pm6 NAU/STSBCD 135-2*
Asosan	4	2	+	-	+	+	+	+	+
Atomic	4	3	+	-	+	-	+	+	+
Bussard	5	4	+	-	+	-	+	+	+
Lear	6	4	+	+	+	-	+	+	+
Sparta	5	3	+	+	+	-	+	+	+
Tonacja	5	4	+	-	+	-	+	+	+
Ulka	5	2	+	-	+	-	+	+	+

9° scale: 1°-0.1% of infected area, 9°-60% of infected area
"+" indicates the presence of a given DNA fragment characteristic of the marker locus
"–" indicates the absence of a given DNA fragment characteristic of the marker locus

organisms, at the same time reducing the amount of used plant protection products and preserving the quality and quantity of the yield. Selection using molecular MAS markers (marker assisted selection) allows the detection of specific genes or QTLs in plants at the early stages of their development in a relatively short time. Resistance to powdery mildew, which is conditioned by only one gene, is characterized by low efficiency and persistence, therefore, the pyramidization of *Pm* genes is sought [24-26]. The development of multiplex PCR will allow the simultaneous identification of two or three genes, which will reduce the time and cost of analysis.

The aim of the study was to identify the *Pm2*, *Pm3a*, *Pm4b* and *Pm6* powdery mildew resistance genes in wheat varieties with different origins from the collection of the Department of Genetics and Plant Breeding of the Poznań University of Life Sciences and to develop multiplex PCR reaction conditions.

2 Materials And Methods

Plant material consisted of 7 *Triticum aestivum* ssp. *vulgare* L. varieties from the wheat collection at the Department of Genetics and Plant Breeding of the Poznań University of Life Sciences (Table 1). The varieties show good resistance to powdery mildew of cereals and grasses, according to information from the National Small Grain Collection at the Agriculture Research Station in Aberdeen, USA. The field experiment was established at the Experimental Station KGiHR housed in the Dłoń Agricultural Experimental Farm of the Poznań University of Life Sciences (51º41'23.835"N 17º4'1.414"E) in 2017 and 2018. Genotypes were sown on 10 m^2 plots, in a randomized block system, in triplicate. The assessment of the degree of plant infestation by *Blumeria graminis* f. sp. *tritici* was carried out at the milk maturity stage (BBCH 71-77) on 40 flag and underflag leaves of randomly selected plants from each plot. The field assessment was carried out in accordance with the recommendations of the European and Mediterranean Plant Protection Organization (EPPO) according to a 9 degree scale (1° – 0.1% of infected area, 9° – 60% of infected area).

The material for the tests was collected from 10-day-old seedlings, which were obtained from seeds germinated in laboratory conditions. DNA isolation was performed using the Genomic Mini AX PLANT plants DNA isolation kit (A&A Biotechnology, Poland) according to the included procedure. DNA concentration was determined using a DeNovix spectrophotometer. The samples were diluted with Tris buffer to obtain a uniform concentration of 50 ng/µL. PCR was carried out in a TProfesional Basic gradient thermocycler (Polygen, Poland).

The identification of powdery mildew resistance genes (*Blumeria graminis* f. sp. *tritici*) was carried out using the following molecular markers: *Xcfd81* [27], *Whs350* [28] and *Xgwm205* [29] for *Pm2*, *Pm3a* [30] for *Pm3a*, *STS_241* [31] and *Xgwm382* [31] for *Pm4b* and *NAU/STS BCD 135-2* for *Pm6* [32]. The primer sequences are derived from the Grain Genes database [33] and are presented in Table 2 with their annealing temperature and the size of the amplified products. The 12.75 µL reaction volume

Table 2: Primer sequences and their annealing temperature in the identification of the *Lr11*, *Lr13*, *Lr16*, *Lr26* genes.

Gene – Marker	Primer sequence	Annealing temperature	Product amplified
Pm2 – Xcfd81	F:5'TATCCCCAATCCCCTCTT3' R:5'GTCAATTGTGGCTTGTCCCT3'	57°C	283 bp
Pm2 –Whs350	F:5'AGCTGTTTGGGTACAAGGTG3' R:5'TCCCCTGTGCTACTACTTCTC3'	56°C	598 bp
Pm2 – Xgwm205	F:5'CGACCCGGTTCACTTCAG3' R:5'AGTCGCCGTTGTATAGTGCC3'	56°C	143 bp
Pm3a – Pm3a	F:5'GGAGTCTCTTCGCATAGA3' R:5'CAGCTTCTAAGATCAAGGAT3'	51°C	642 bp
Pm4b – STS_241	F:5'CTCATTCTTGTTTTACTTCCTTCAGT3' R:5'GTCTCGTCTTCAGCATCCTATACA3'	56°C	241 bp
Pm4b – Xgwm382	F:5' GTCAGATAACGCCGTCCAAT3' R:5' CTACGTGCACCACCATTTTG3'	52°C	125 bp
Pm6 – NAU/ STSBCD 135-2	F:5'GCTCCGAAGCAAGAGAAGAA3' R:5'TCTGCTGGTCCTCTGATGTG3'	57°C	230 bp

consisted of: water – 5 µL, DreamTaq™ Green PCR Master Mix – 6.25 µL, primers – 2 × 0.25 µL (final concentration was 20 µM), DNA template – 1 µL. PCR after optimizing was carried out under the same conditions regardless of the marker being identified. The profile differed only in the primer annealing temperature, determined based on their melting point: initial denaturation for 5 minutes at 94°C, 40 cycles (denaturation – 45 sec at 94°C, primer annealing – 1 min at 51°C, 52°C, 56°C, 57°C, elongation – 1 min at 72°C), final extension – 5 min at 72°C, storage – 4°C max. for 24 hours.

Simultaneous identification of the *Pm2* and *Pm4b* genes was carried out using *Xcfd81* (*Pm2*) and *STS_241* (*Pm4b*) and *Xcfd81* (*Pm2*) i *Xgwm382* (*Pm4b*) marker pairs. Primer sequences are shown in Table 2. The multiplex PCR reaction for *Xcfd81* and *STS_241* marker identification was carried out in a volume of 25 µL and the mixture composition was as follows: water – 10 µL, PCR Mix Plus (A&A Biotechnology, Poland) – 12.5 µL, *Xcfd81* primer – 2 × 0.25 µL, *STS_241* primer – 2 × 0.25 µL, DNA template – 1.5 µL. The reaction was based on the thermal profile specific for the identification of the *Pm2* gene – primer annealing temperature was 57°C. In order to simultaneously identify the *Xcfd81* and *Xgwm382* markers, the multiplex PCR reaction was carried out in a volume of 25 µL. Mixture composition: water – 10 µL, PCR Mix Plus (A&A Biotechnology, Poland) – 12.5 µL, *Xcfd81* primer – 2 × 0.25 µL, *Xgwm382* primer – 2 × 0.25 µL, DNA template – 1.5 µL. Primer annealing temperature was 52°C.

To visualize the results, PCR products were separated in a 2.5% agarose gel containing 0.01% µL ethidium bromide in TBE 1x buffer. The voltage was 100 V and the current was 200 mA. The duration of electrophoresis was 1 h. A Molecular Imager Gel Doc™ XR UV transilluminator was used with the Biorad Bio Image™ Software to visualize the PCR products.

3 Results

3.1 Field assessment

Assosan and Ulka were the most resistant varieties in field conditions in 2018. The degree of infection of these varieties by *Blumeria graminis* f. dp. *tritici* was only 2°. However, these varieties showed less resistance in 2017, which might be caused by very intensive precipitation during the growing season, which promoted pathogen development. The least resistant in field conditions were the varieties: Lear (5° – 2017 and 6° – 2018), Bussard (4° – 2017 and 5° – 2018) and Tonacja (4° – 2017 and 5° – 2018). The Atomic (4° – 2017 and 3° – 2018) and Sparta (5° – 2017 and 3° – 2018) varieties were characterized by moderate resistance in both 2017 and 2018 (Table 1).

3.2 Pm2 gene identification

The analyses with the *Xcfd81* marker linked to the *Pm2* gene resulted in the identification of a 283-bp specific product in all tested varieties. The occurrence of non-specific products was also observed. The result was reproducible (Table 1., Figure 1). The *Whs350* marker was the second one tested for the *Pm2* gene. A specific product with a length of 598 bp was identified only in two tested

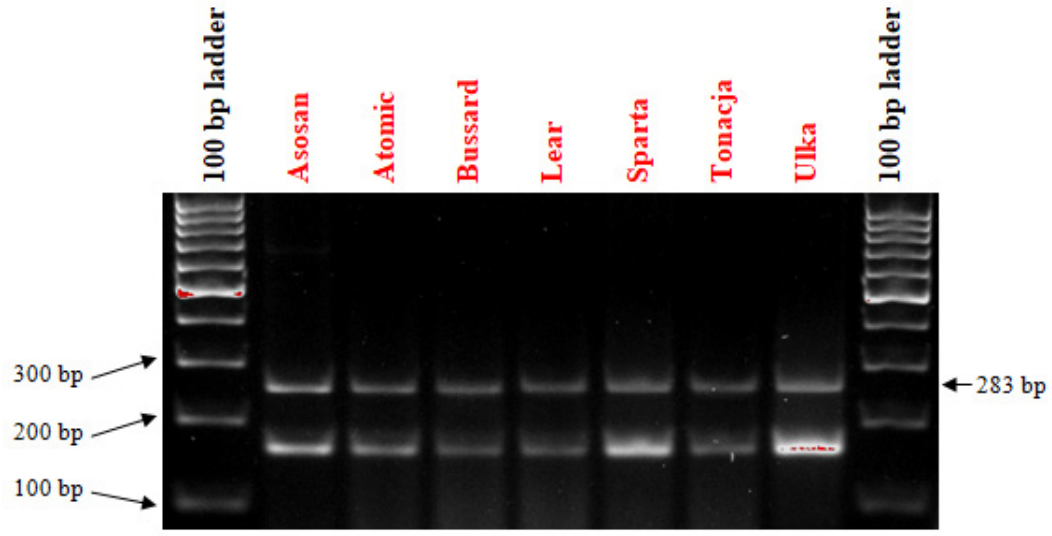

Figure 1: Electropherogram showing the presence of the *Xcfd81* marker of the *Pm2* gene in wheat varieties.

varieties: Lear and Sparta. The marker was not present in the Assosan, Atomic, Bussard, Tonacja and Ulka varieties (Table 1). *Xgwm205* was the following marker linked to *Pm2*. A specific product of 143 bp was identified in all tested varieties. Moreover, the occurrence of non-specific products was also observed. The result was reproducible (Table 1)

3.3 Pm3a gene identification

The analyses using the *Pm3a* marker demonstrated a specific amplification product of 642 bp only in the Assosan variety. The marker did not appear in the Atomic, Bussard, Lear, Sparta, Tonacja and Ulka varieties. The result was reproducible (Table 1).

3.4 Pm4b gene identification

The experiments concerning the identification of the *Pm4b* gene using the STS_241 marker, which gave a specific product of 241 bp, identified the marker in three varieties: Assosan, Atomic and Sparta. No distinct product was obtained in the Bussard, Lear, Tonacja and Ulka varieties (Table 1., Figure 2). *Xgwm382* was another marker tested for the *Pm4b* gene. A specific product of 125 bp was identified in all tested varieties. The result was reproducible (Table 1., Figure 3).

3.5 Pm6 gene identification

NAU/STSBCD 135-2 coupled to the *Pm6* gene was the last tested marker. The presence of a specific product of 230 bp was identified in all tested varieties. The result was reproducible (Table 1).

3.6 Multiplex PCR DNA amplification for the Pm2 and Pm4b genes

After PCR with *Xcfd81* and *STS_241* primer pairs, 283-bp and 241-bp products were obtained, indicating the presence of the *Pm2* and *Pm4b* genes, respectively. Two strong products were obtained in Atomic, Bussard, Lear and Sparta varieties. In the case of the Assosan, Tonacja and Ulka varieties, only a product of 241 bp was observed, indicating the presence of the *Pm4b* gene (Figure 4).

After PCR reactions with *Xcfd81* and *Xgwm382* primer pairs, 283-bp and 125-bp products were obtained, indicating the presence of the *Pm2* and *Pm4b* genes, respectively. Two strong products were obtained in the Assosan, Bussard, Lear and Sparta varieties. As regards the Assosan, Tonacja and Ulka varieties, only a product of 283 bp was observed, indicating the presence of the *Pm2* gene. No specific products were observed in the Ulka variety. The occurrence of non-specific products was also observed (Figure 5).

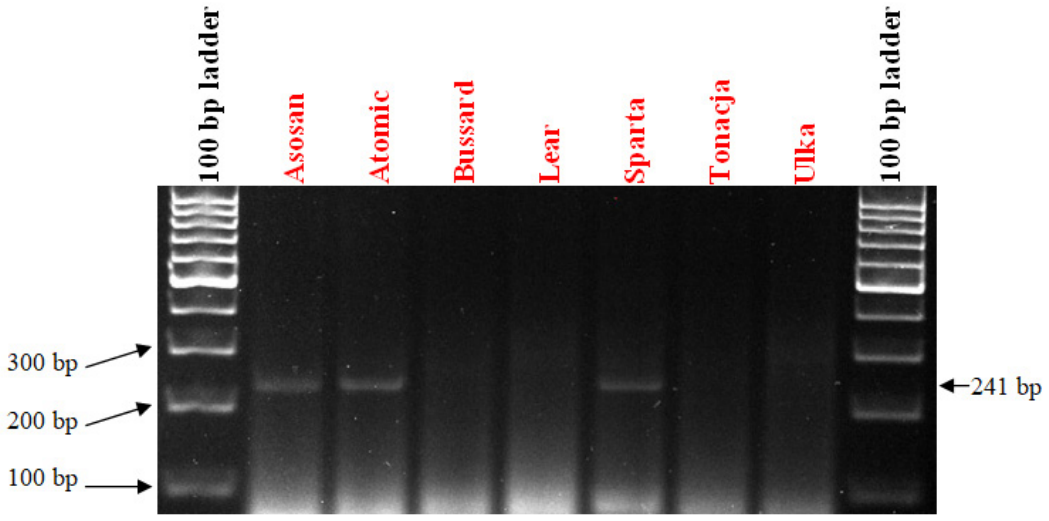

Figure 2: Electropherogram showing the presence of the *STS_241* marker of the *Pm4b* gene in wheat varieties.

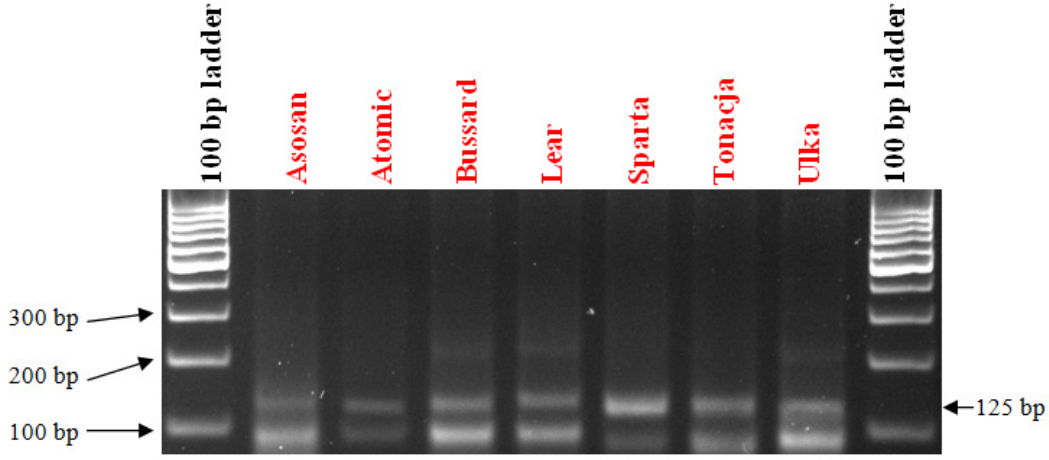

Figure 3: Electrophorogram showing the presence of the *Xgwm382* marker of the *Pm4b* gene in wheat varieties.

4 Discussion

The *Xcfd81, Whs350* and *Xgwm205* markers were analyzed for their usefulness in the identification of the *Pm2* gene conferring resistance to powdery mildew – *Blumeria graminis* f. sp. *tritici*. The analyses demonstrated that the most effective markers were: *Xcfd81*, which gave a 283-bp product and *Xgwm205* with a 143-bp amplification product, which confirmed the presence of the *Pm2* gene in all 7 analyzed *Triticum aestivum* ssp. *vulgare* varieties. The *Whs350* marker turned out to be the least suitable. Its 598-bp amplification product occurred only in 2 out of 7 analyzed varieties. All three tested markers were identified in the Lear and Sparta varieties.

Ma et al. [34, 35, 36] also demonstrated the efficacy of the *Xcfd81* and *Xgwm205* markers in the identification of the *Pm2* gene. Moreover, Gao et al. [37] showed that these markers were also linked to the *Pm46* gene, which was previously considered to be an allelic form of the *Pm2* gene. They showed that the Tabasco variety had the *Pm46* gene, and not *Pm2* as previously thought.

Tomkowiak et al. [38] also evaluated the usefulness of the Xgwm205, Xcfd81 and Whs350 molecular markers to identify the *Pm2* resistance gene against powdery mildew of cereals and grasses in 27 wheat varieties. As a result of SSR analyses, the *Xgwm205* marker was considered the most effective in *Pm2* gene identification; its product appeared in 25 out of 27 analyzed varieties. The *Xcfd81*

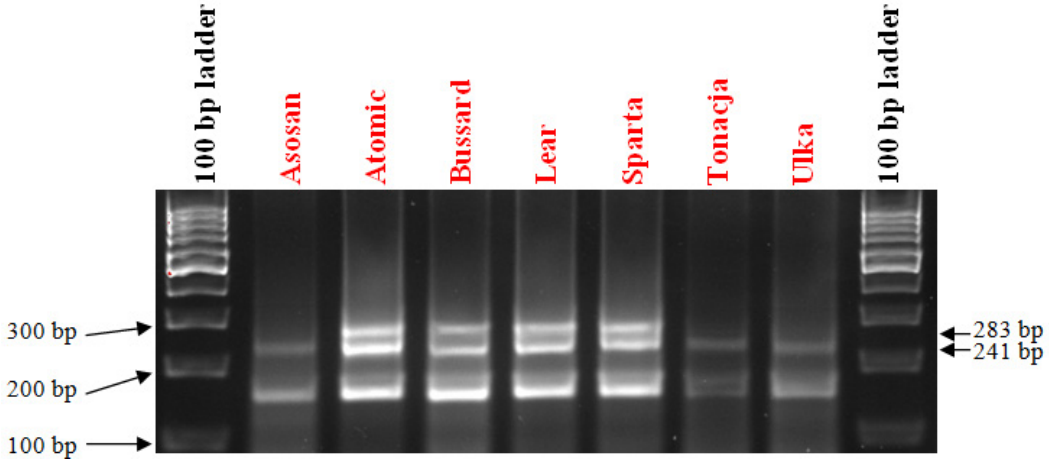

Figure 4: Electrophorogram showing the presence of the following markers: *Xcfd81* of the *Pm2* gene and *STS_241* of the *Pm4b* gene in wheat varieties.

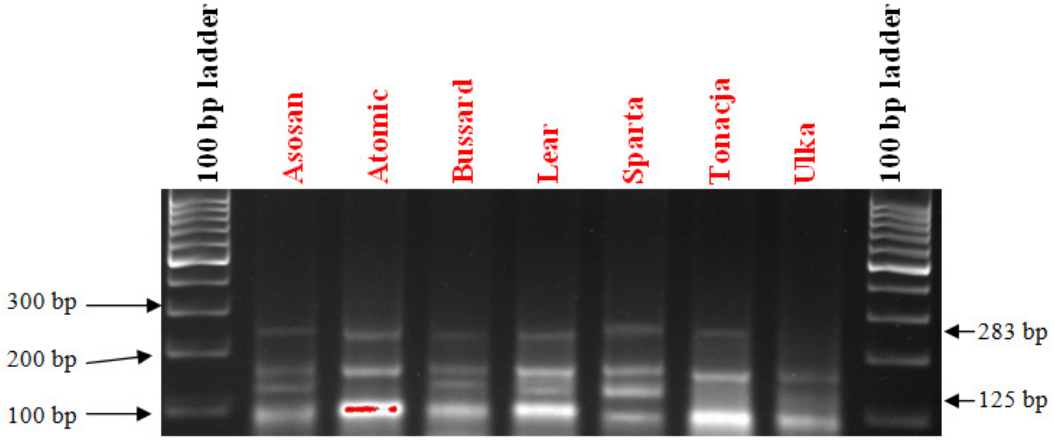

Figure 5: Electrophorogram showing the presence of the following markers: *Xcfd81* of the *Pm2* gene and *STS_241* of the *Pm4b* gene in wheat varieties.

marker was found in 21 varieties and *Whs350* marker product in only 9 varieties. All three tested markers linked to the gene were successfully identified in the Atomic, Bussard, Sparta, Lear Tonacja and Ulka varieties.

Huang and Roder [11] conducted research on 32 genotypes of wheat for the presence of different *Pm* genes and confirmed the presence of the *Pm2* gene in the genotype of the Ulka variety.

The *Pm3a* gene marker was analyzed as the next one. The analyses showed the presence of a 642-bp product only in the Assosan variety. Huang and Roder [11] and Tommasini et al. [30] also identified the *Pm3a* gene in the Assosan variety. The latter variety may be a good source of powdery mildew resistance, because, the analyses showed that it contained all the analyzed genes, i.e., *Pm2, Pm3, Pm4b* and *Pm6*. According to literature data, Kredo might be an equally useful variety, in which Huang and Roder [11] identified the *Pm3a* gene, and Tomkowiak et al. [39] showed the presence of the *Pm2, Pm4b* and *Pm6* genes.

Subsequently, the usefulness of the *STS_241* and *Xgwm382* markers for the identification of the *Pm4b* gene was tested. The *Xgwm382* marker turned out to be significantly more effective, as its product of 125 bp was identified in all the analyzed varieties. The *STS_241* marker was characterized by a lower suitability. A 241-bp marker product appeared only in 3 out of 7 tested varieties: Assosan, Atomic and Sparta. Tomkowiak et al. [39] also

analyzed, among others, Assosan and Atomic varieties for the presence of the *Pm4b* gene using the *STS_241* marker and did not show the presence of the gene in these varieties. Yi et al. [31], Hao et al. [20] and Tomkowiak et al. [39] considered the non-analyzed VPM line as a reliable source of the *Pm4b* gene.

The STS *NAU/STS BCD 135-2* marker, specific for the *Pm6* gene, was tested as the last one. The analyses showed the presence of a 230-bp product in all varieties. Literature data provide different lengths of the product, which indicate the presence of the *NAU/STS BCD 135-2* marker. Ji et al. [32] studied four markers specific for the *Pm6* gene: *NAU/STS BCD 135-1*, *NAU/STS BCD 135-2*, *STS003* and *STS004*. The authors showed that both *NAU/STS BCD 135-1* and *NAU/STS BCD 135-2* markers were strongly linked to the *Pm6* gene, however, the *NAU/STS BCD 135-2* marker turned out to be more effective. According to the methodology included in these authors' study, a product of 230 bp would indicate the presence of the marker. Kowalczyk et al. [24] confirmed the obtained results by using the marker to detect the *Pm6* gene in triticale varieties. Kęska et al. [40] identified the *Pm6* gene in new wheat breeding lines using the *NAU/STS BCD 135-2* marker and applying primers designed by Ji et al. [32]. The authors identified the *Pm6* gene in 8 genotypes obtained from breeding lines as a result of conducted analyses. However, the results are doubtful, because the authors indicated that the presence of the *Pm6* gene was indicated by a product of 135 bp, which is contrary to the source methodology. Similar studies were also conducted by Tomkowiak et al. [39], who identified a product of 230 bp in all analyzed varieties and lines.

All *Pm* genes were found in the Assosan variety. The accumulation of the *Pm2*, *Pm4b* and *Pm6* genes was demonstrated in the Atomic, Bussard, Lear, Sparta, Tonacja and Ulka varieties. These varieties can be an effective source of genes in breeding programs and may serve as reference materials.

The effectiveness of breeding programs can be increased by the accumulation of different combinations of genes conditioning resistance not only to powdery mildew, but also to other agricultural diseases that are dangerous from the agricultural point of view. Pyramidization of genes is a commonly used method in breeding varieties throughout the world and allows minimizing the use of plant protection products that are not neutral to the environment as well as human and animal health.

The use of the multiplex PCR technique may be one of the methods allowing for acceleration of breeding processes. It is a technique based on the use of molecular markers, consisting of the simultaneous use of several primer pairs in the reaction mixture. This allows the identification of several genes simultaneously. The markers used should be selected to allow their amplification at the same primer annealing temperature to the DNA template. The method allows the reduction of research financial expenditures, but also saves time and work effort [41,42].

The literature provides many examples of attempts at simultaneous identification of multiple resistance genes. Leśniowska et al. [43] made a successful attempt to develop multiplex PCR conditions for the *Lr9* and *Lr19* resistance genes against leaf rust. Gogół et al. [44] identified the resistance genes to leaf rust – *Lr21* and powdery mildew – *Pm4b* in Polish wheat varieties and developed multiplex PCR conditions for simultaneous identification of these genes. The subject of their research was 30 Polish wheat varieties. Sumikova and Hanzalova [45] developed and optimized multiplex PCR conditions for the *Lr29* and *Lr37* resistance genes to leaf rust. De Froidmont [46] attempted simultaneous identification of the *1BL/1RS* wheat-rye translocation carrying the *Yr9* resistance gene to yellow rust, *Sr31* resistance gene to stem rust, *Lr26* resistance gene to leaf rust and *Pm8* conferring resistance to powdery mildew. Fraaije et al. [47] used the multiplex PCR method to simultaneously identify resistance genes to four wheat pathogens: *Septoria tritici*, *Stagonospora nodorum*, *Puccinia Striiformis* and *Puccinia recondita*. It has been shown that this method can be used to identify the presence of genes for fungal diseases in wheat.

The work also attempted to develop multiplex PCR conditions. Many combinations were tried within the identified markers; however, positive results were obtained only for the *Pm2* and *Pm4b* genes. Other combinations, e.g, *Xcfd81* with *Pm3a*, or *Whs350* with *NAU/STS BCD 135-2* may have failed, among others, due to the large differences in amplicon lengths, which made it difficult to separate them on a single electrophoretic gel.

Two distinct products with sizes of 238 bp and 241 bp were obtained in 4 out of 7 tested variants as a result of simultaneous DNA amplification by the multiplex PCR method for the pair of *Xcfd81* and *STS_241* markers. In addition, only one product was identified in the Assosan, Tonacja and Ulka varieties, indicating the presence of the *Pm4b* gene

Xcfd81 and *Xgwm382* were the second pair of identified markers. Both markers were characterized by high suitability for the identification of the *Pm2* and *Pm4b* genes, respectively. The products of both markers were obtained in 4 out of 7 analyzed variants as a result of the multiplex PCR reaction.

A 283-bp product, indicating the presence of the *Pm2* gene was also identified in the Atomic and Tonacja varieties.

5 Conclusions

Assosan and Ulka were the most resistant varieties in the field assessment in 2018. All four analyzed genes for powdery mildew of cereals and grasses were identified in the Assosan variety, while the *Pm2*, *Pm4b* and *Pm6* genes were identified in the Ulka variety. The Lear, Bussard and Tonacja varieties were characterized by the lowest resistance in the field assessment in 2017 and 2018. Molecular assessment, however, showed that they contained three pathogen resistance genes. The Atomic and Sparta varieties were characterized by medium resistance in the field evaluation, and molecular analyses revealed the presence of three resistance genes. The Assosan and Ulka varieties can be a good source of resistance to powdery mildew. In addition, the developed multiplex PCR conditions for simultaneous amplification of the *Pm2* and *Pm4b* genes can be used in breeding programs to shorten the time of molecular analysis.

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