POLYMORPHISM OF TEN NEW MINISATELLITE MARKERS IN SUBPOPULATIONS OF PHYTOPATHOGENIC FUNGUS LEPTOSPHAERIA MACULANS DIFFERING WITH METCONAZOLE TREATMENT

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Abstract: Stem canker of brassicas is one of the most damaging diseases of oilseed rape worldwide. The disease is caused by two related Leptosphaeria species, and L. maculans is regarded as the more damaging one. Being an ascomycete, the pathogen is able to quickly create new variants that can overcome new resistance genes introduced by researchers and breeding companies. The aim of this work was to study polymorphism of L. maculans populations using 10 recently developed minisatellite markers. The studied subpopulations differed with metconazole treatment.

Seven minisatellite markers showed polymorphisms and formed alleles varying from 2 to 10 different core motifs, with 5 alleles on average. In total 36 alleles were found. The majority of alleles (72%) were found in both studied subpopulations of L. maculans. There were 28 alleles in the group of L. maculans isolates originating from plants not treated with any fungicide and 32 in the subpopulation treated with metconazole. Ten unique alleles and imbalanced ratios between some alleles contributed to differences between L. maculans subpopulations. The minisatellites MinLm555, MinLm935-2, MinLm939, MinLm1139 and MinLm2451 showed 6 new variants as compared to the isolates described so far.

Key words: genetic polymorphism, Leptosphaeria maculans, minisatellite marker, VNTR, oilseed rape, PCR, stem canker of brassicas

INTRODUCTION

Stem canker of brassicas is one of the most damaging diseases of oilseed rape in Australia, Canada and Europe, including Poland (Zhou et al. 1999; Khangura and Barbetti 2001; Fitt et al. 2006; Jędryczka 2007).

The disease is caused by Leptosphaeria maculans and L. biglobosa, two related species differing in harmfulness to oilseed rape. The first species is regarded as more damaging to rapeseed plants. It forms disease symptoms at stem base and root neck, leading to blockage of vessels or even breakage of a whole stem. The disease causes great economic losses and it is a high concern to oilseed rape growers (West et al. 2001; Mróczczyński and Pruszyński 2008).

Being the ascomycete, Leptosphaeria sp. pathogen is able to quickly create new variants that can overcome new resistance genes introduced by researchers and breeding companies. Numerous techniques, first of all based on genetic and chemical methods, allow to describe polymorphism of living organisms, including fungi. Populations of L. maculans were up to date typed using PCR-based methods such as RFLP (Johnson and Lewis 1990; Hassan et al. 1991; Patterson and Kappoor 1995; Voigt et al. 2001), AFLP (Pongam et al. 1991; Purwantara et al. 2000), rep-PCR (Jędryczka et al. 1999), as well as molecular karyotyping (Taylor et al. 1991; Morales et al. 1993), sequencing of DNA fragments (Mendes-Pereira et al. 2003; Voigt et al. 2005) and chemotaxonomical methods (Pedras and Seguin Swartz 1992; Kachlicki and Jędryczka 1994; Pedras and Biesenthal 2000). However, these methods were mainly used to show differences between two subpopulations, subsequently separated by Shoemaker and Brun (2001) in two species: L. maculans and L. biglobosa.

Minisatellite markers allowing to fingerprint numerous living organisms were recently used to characterise population structures of different species (Jeffreys et al. 1985). Minisatellites are usually defined as tandemly repeated short (6 to 100 bp) motifs spanning 0.5 to several kilobases (Vergnaud and Denoeud 2000). Variation in the number of core fragments and the ability of these arrays to cross-hybridize with numerous similar loci throughout the genome made minisatellites a powerful tool in genetic analyses. Minisatellite markers were also implemented in genetic studies of plant pathogens and they are successfully used to describe differences within subpopulations or even individual isolates of fungi. The first tandemly repeated core motif was described in Saccharomyces cervisiae (Horowitz and Haber 1984). Since then minisatellite fragments were found in several fungal species, including human, animal and plant pathogens.

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Phytopathogenic *L. maculans* is currently one of the fungal species well recognized with minisatellite markers. Sequencing of its genome allowed to find numerous tandemly repeated fragments, of which 39 showed polymorphisms what led to study them in detail (Attard et al. 2001; Eckert et al. 2005; Jędryczka 2006; Stachowiak 2008). Some of these minisatellites, such as *MinLm2*, contained numerous core motifs. Recent experiments showed that *L. maculans* populations in Europe have at least 20 alleles of this minisatellite, of which 14 were found in Poland (Stachowiak 2008). High pathogen variation was demonstrated, showing that as many as 12 alleles could be detected within one oilseed rape field containing plants infected with *L. maculans* (Jędryczka et al. 2009).

The aim of this work was to characterize genetic polymorphism of *L. maculans* using 10 new minisatellite markers, which have never been used for population studies of this pathogen in Poland. Variability of minisatellite alleles concerned plants not treated with any fungicides and the sub-population that received a single autumn spray with metconazole.

**MATERIALS AND METHODS**

Fungal isolates of *L. maculans* originated from winter oilseed rape cultivar Bosman (Plant Breeding Strzelce, Poland). The experiment was done in the vegetative season 2006/2007 at the Agricultural Experimental Station of IPP-NRI in Winna Góra (central-west Poland). Field experiment was designed in standard randomized blocks with individual plots of 21 m² in 4 replicates. Fungicide treatment was done in autumn 2006, according to SPEC recommendation (www.spec.edu.pl). Metconazole (triazole fungicide) was applied using the solution of 60 g of the active ingredient per 1 litre (Caramba 60 SL).

The studied population contained 40 hyphal tip isolates of *L. maculans* originating from control and sprayed plots. DNA isolation procedure was done according to Irzykowski et al. (2005). PCR protocol followed the methods described by Stachowiak (2008) with further modifications. The primers ranged from 18 to 23 bp, with annealing temperatures from 54°C to 60°C (Table 1). Product amplification was done using the thermal cycler MJ Research PTC-200. PCR reaction profiles consisted of initial DNA denaturation for 2 min at 94°C, followed by 45 cycles: 30 s – 94°C, 30 s – 58–60°C, 60 s – 72°C. For the convenience of experiment performance the annealing temperature for *MinLm1139* was raised to 58°C. The final elongation step lasted 5 min at 72°C.

Separation of PCR amplicons was done at 180 V for 1.5 to 4 hours. The gels contained 2% agarose. Sample DNA was separated in the presence of Gene Ruler 100 bp DNA Ladder (Fermentas) and internal markers varying from 68 bp to 471 bp, which helped to precisely evaluate the size of the studied products. Gel documentation was done using Scion IMAGE for Windows system (Scion Corporation).

At first, 8 randomly selected isolates – 4 per each sub-population of *L. maculans* – were used. The further study of the whole isolate collection was done only in case of polymorphisms found in the preliminary experiment.

### Table 1. Characterization of new minisatellite markers used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer symbol</th>
<th>Primer sequence 5' – 3'</th>
<th>Annealing temperature [°C]</th>
<th>Core sequence [bp]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>MinLm555F</em></td>
<td>CACTGTCATTCCTCCTCCTTTTTT</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td><em>MinLm555R</em></td>
<td>TGCAGCCGTTTTAGTTCCTCATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>MinLm585F</em></td>
<td>GTCCAGACGGGCTCTAAATG</td>
<td>60</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td><em>MinLm585R</em></td>
<td>TGCAATACCTATCACTATGCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>MinLm935-2F</em></td>
<td>AGTAGGCAACACACAGCACACACA</td>
<td>58</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td><em>MinLm935-2R</em></td>
<td>CCCCTCCTGCAATTCTTCAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>MinLm939F</em></td>
<td>ACCCTCTTCTTGCATGCAAACC</td>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td><em>MinLm939R</em></td>
<td>CGAGAGTGCCGAGTTGAGGTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>MinLm1139F</em></td>
<td>AGCCACCGGAGAGGTTT</td>
<td>54</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td><em>MinLm1139R</em></td>
<td>ACCATCTACTGCTAGCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>MinLm1188F</em></td>
<td>CCGTCTTCTCTGTACTTCAC</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td><em>MinLm1188R</em></td>
<td>CAATTCTCCAGACTGCAAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>MinLm2448F</em></td>
<td>TTAGGATCTCATTGGGAGA</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td><em>MinLm2448R</em></td>
<td>AAAGTTGCTCAGGATTGGAAGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>MinLm2448-1F</em></td>
<td>CGCAACATGCTCTTGAGCCTCTACT</td>
<td>58</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td><em>MinLm2448-1R</em></td>
<td>CAAGTGGCTAGTGAGGATTGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td><em>MinLm2451F</em></td>
<td>GGCGCGAGTGGTATGGTGTTGTATG</td>
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<td>24</td>
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<tr>
<td></td>
<td><em>MinLm2451R</em></td>
<td>CGAACACAAATTCCTACCAACACACACTC</td>
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<tr>
<td>10</td>
<td><em>MinLm2452F</em></td>
<td>GTACATGGCCGAGCACAGGC</td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td><em>MinLm2452R</em></td>
<td>CATTIACACTGCAACACCTAGCTCA</td>
<td></td>
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</tbody>
</table>
RESULTS

In the preliminary experiment performed to check whether the new markers generate polymorphic PCR products, 7 minisatellites showed differences among randomly chosen 8 isolates of *L. maculans*. These markers were selected for further experiments. The markers MinLm585, MinLm1188 and MinLm2448 did not generate polymorphic products. The remaining 7 minisatellite markers generated from 2 to 10 alleles (Fig. 1). On average there were 5 alleles. In total 36 alleles were found. The majority of alleles (72%) were found in both studied subpopulations of *L. maculans*. There were 28 variants in the group of *L. maculans* isolates originating from plants not treated with any fungicides and 32 in the subpopulation treated with metconazole.

Ten alleles were unique; they were found in 5 out of 7 polymorphic minisatellites, excluding MinLm2448-1 and MinLm2452 (Fig. 1d, g). In most cases the percent of isolates with the same variant of the core motif was comparable. The exception, with the difference exceeding 30% were the following 3 alleles: MinLm939 5x (51.7% difference), MinLm939 8x (39.1%) and Min935-2 2x (30.2%) (Fig. 1b, c). Considerable imbalance between the isolates of *L. maculans* originating from untreated oilseed rape plants and from plants sprayed with metconazole was also found for MinLm935-2 3x and MinLm1139 3x; in both cases the difference was 24.8% (Fig. 1e).

Out of 10 alleles unique for one of the isolate subgroups, 8 alleles were unique for the cultures of *L. maculans* obtained from plants treated with metconazole; these were the following core motifs: MinLm555 7x (Fig. 1a), MinLm939 11x (Fig. 1b), MinLm935-2 5x and 9x (Fig. 1c), MinLm1139 12x and 14x (Fig. 1e), MinLm2451 2x and 6x (Fig. 1f). Only two alleles: MinLm935-2 7x (Fig. 1c) and MinLm2451 10x (Fig. 1f) represented the opposite situation, when unique variants were found in *L. maculans* isolates originating from untreated plants.

The obtained PCR products greatly differed in size. The shortest was 85 bp allele of MinLm2452 consisting of 2 core motifs of 21 bp each supplemented with 43 bp flanking sites. The longest product (478 bp) was generated by MinLm555 minisatellite, it consisted of 7 replicates having 63 bp core motifs and 37 bp flanking sites. Apart from a size marker, internal markers were also used and selected in the way allowing to evaluate products’ size with maximal precision. In most cases sizes of obtained products did not differ from the expected sizes by more than a few base pairs.
Fig. 1. Frequency of minisatellite alleles in populations of the phytopathogenic fungus *L. maculans* isolated from oilseed rape plants untreated (grey bars) and treated with metconazole (black striped bars): e) MinLm1139; f) MinLm2451; g) MinLm2452

The selection of internal markers was based on knowledge concerning sizes of PCR products obtained in previous experiments (Stachowiak 2008). However, in a few cases the PCR products obtained in this study were different than expected, what sometimes resulted in products shorter or longer than the internal markers (Fig. 2, 3). In this case the evaluation of the exact size of the amplicon could be done with a relatively bigger error.

Frequency of alleles depends on their number, selection pressure exerted on particular isolate variants as well as the length of time when the allele was created. The most frequent variants are usually detected when the number of alleles is small, although the situations with imbalanced ratios among numerous alleles can also be found. The most popular allele among the studied ones was MinLm2452 3x with frequency exceeding 90%, both in *L. maculans* isolates obtained from treated and untreated plants (Fig. 1g). The other usually found variant was 5x core motif of MinLm555 minisatellite, found in 78.6% and 73.1% fungal strains isolated from plants that were respectively untreated and sprayed with metconazole (Fig. 1a). Minisatellite MinLm2248-1 2x core motif was also very popular; it was found in 78.6% and 61.5% of the respective isolates (Fig. 1d). Minisatellite fragment MinLm939 repeated 5 times (5x) was also frequent (73.1%), but only in the subpopulation of *L. maculans* obtained from metconazole-treated plots (Fig. 1b). Core fragment of MinLm2451 replicated 3 times (3x) was found in more than half of the studied isolates, but its frequency was equal in both subpopulations of *L. maculans* (Fig. 1f).
In contrast, some alleles were rare and did not reach 10% frequency. Such phenomenon concerned several alleles of minisatellites with high DNA polymorphism, but it was also true for 2x core motif of the minisatellite MinLm2452, which produced only two variants among the studied isolates of L. maculans (Fig. 1g).

Six alleles, including MinLm555 7x, MinLm935-2 9x, MinLm939 11x, MinLm1139 13x, MinLm1139 14x and MinLm2451 10x, were found for the first time. Their identity must be confirmed by sequencing.

**DISCUSSION**

High demand for plant oil used for human consumption, high energy cake and meal for animal feed and technical oil for biofuel led to the great success of oilseed rape cultivation worldwide. However, intensification of its production, connected with greater areas of oilseed rape cultivation increased damage caused by insect pests and fungal pathogens. Stem canker of brassicas, regarded as one of the most important factors decreasing yields of this crop plant is currently one of the biggest concerns and key topic for research and breeding activities. One of the most advanced scientific programmes is the L. maculans genome initiative launched in 2004 by INRA (France) and the University of Melbourne (Australia). By now, the pathogen’s genome has been sequenced and assembled. At present the structural and functional annotations are in progress (http://urgi.versailles.inra.fr/projects/lmaculans). In such way, L. maculans entered the genomic era (Rouxel and Balesdent 2005) and the first genetic linkage map and genome organization data were recognized (Kühn et al. 2006).

The access to sequences of L. maculans genome allowed to find out numerous tandemly repeated DNA fragments, known as VNTRs (Variable Number of Tandem Repeats) or minisatellites. It was proved that minisatellites could be associated with many interesting features of human genome biology and evolution, usually revealed by pathologies of genetic origin (Vergnaud and
denoed 2000). Hence, these fragments were also found interesting for plant pathologists and geneticists researching on *L. maculans*.

The first studies on polymorphisms in *L. maculans* isolates from Poland using 4 minisatellite markers from *MinLm1* to *MinLm4* showed 6, 9, 3 and 2 alleles respectively, with *MinLm1* 2x, *MinLm2* 7x, *MinLm3* 3x and *MinLm4* 3x as the most frequent variants in particular minisatellites (Jędryczka 2007). The studied population composed of 103 *L. maculans* isolates gathered from infected leaves of oilseed rape collected in 3 subsequent autumn seasons, starting in 2001. Further evaluation of DNA polymorphisms in Polish isolates of *L. maculans* using *MinLm1*-*MinLm3* and *MinLm5* minisatellite markers, using twice as big number of isolates as compared to the former study, showed 6, 14, 17 and 6 alleles respectively (Stachowiak 2008).

Following the detection of 20 variants of core motif numbers in *MinLm2*, this highly polymorphic marker was used to study variation in field populations of *L. maculans* isolates originating from plants differing by fungicide treatments (Jędryczka et al. 2009). Unexpectedly, 11 out of 14 alleles previously detected in Polish isolates of *L. maculans* could be found within one field of oilseed rape. Moreover, a novel 28x allele of *MinLm2* was additionally found. Only 3 alleles (6x, 9x and 12x) of this marker were found in both fungal subpopulations, whereas 5 and 4 alleles respectively were unique for fungal strains originating from control plants and the plants treated with metconazole. The most frequent product of 296 bp (MinLm2 9x) was present in 30.8% of *L. maculans* isolates from untreated plants and it was the second top product (23.1%) in the subgroup of isolates obtained from plants treated with metconazole. In this case the number of unique alleles was comparable for both subpopulations of *L. maculans*.

In the present study using 10 new minisatellite markers, 7 were found polymorphic. Eight out of 10 unique alleles were found in *L. maculans* subpopulation originating from plants treated with the pesticide. It suggests that the fungus population of *L. maculans* surviving on metconazole treated plants is very diverse and polymorphic. The hypothesized decrease of the number of *L. maculans* genetic variants that could survive in unfavourable conditions was not observed. In contrast, the number of alleles unique for this subpopulation was high. Additionally, 4 out of 6 newly found minisatellite variants were also detected uniquely in *L. maculans* isolates from plants sprayed with the fungicide. Conversely, only one allele was characteristic for unsprayed plants, and one was found in both subgroups of the pathogen. The results obtained in this study do not support any proof of the reduction of genetic diversity of *L. maculans* subpopulation originating from pesticide treated oilseed rape plants. However, it must be emphasized that the comparisons presented in this study concern the subpopulations that are equal in size, whereas in field conditions populations originating from untreated plants always greatly outnumber populations from plants subjected to a fungicide treatment.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Polymorphism of ten new minisatellite markers in subpopulations of phytopathogenic fungus... 109


**POLISH SUMMARY**

**POLIMORFIZM DZIESIĘCIU NOWYCH MARKERÓW MINISATELITARNYCH CHOROBOTWÓRCZEGO GRZYBA **

**LEPTOSPHAERIA MACULANS**

**W SUBPOPLACJACH ZRÓŻNICOWANYCH POD WZGLĘDEM TRAKTOWANIA METKONAZOLEM**


Celem pracy było oznaczenie polimorfizmu w obrębie populacji *L. maculans*, przy zastosowaniu dziesięciu nowych markerów minisatelitarnych. Badane subpopulacje patogena różniły się pod względem ich traktowania preparatem grzybobójczym zawierającym metkonazoł.

W zestawie badanych markerów minisatelitarnych 7 wykazało polimorfizm i tworzyło od 2 do 10 aleli – średnio 5 aleli. Sumaryczna liczba wariantów wynosiła 36, przy czym 28 aleli znaleziono w grupie izolatów *L. maculans* wyodrębnionych z roślin nie traktowanych preparatem grzybobójczym, natomiast w subpopulacji z rzepaku traktowanego metkonazołem stwierdzono występowanie 32 aleli. Do zróżnicowania badanych subpopulacji przyczyniło się 10 unikalnych aleli obecnych wyłącznie w jednej grupie izolatów oraz zróżnicowanie procentowego udziału niektórych aleli. Wśród produktów PCR otrzymanych dla markerów minisatelitarnych *MinLm555, MinLm935-2, MinLm939, MinLm1139* oraz *MinLm2451* stwierdzono występowanie sześciu nowych wariantów, nie znalezionych w dotychczas badanych izolatach grzyba *L. maculans*.