IDENTIFICATION OF SOME MOLECULAR MARKERS ASSOCIATED WITH RESISTANCE OF SOME OILSEED RAPE CULTIVARS (*BRASSICA NAPUS* L.) AT THE PATHOGEN V*ERTICILLIUM LONGISPORUM*

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Abstract

Oilseed rape (*Brassica napus* L) is a relative young species which appeared a few hundred years ago through a spontaneous interspecific hybridization between cabbage (*Brassica oleracea* L.) and turnip rape (*Brassica rapa* L.) (Rygulla et.al., 2007). Among the main diseases that can affect the oilseed rape culture we can find also verticillium wilt caused by the pathogen *Verticillium longisporum.* The mycelium is colonizing the vascular system of the plant and cause his obstruction, so due to the water stress the plant can die. Because, until now there are no available approved chemicals to prevent this disease, the phytosanitary control of this disease can be realized by cultivating some resistant varieties. The aim of this study was utilization of the SSR markers to identify some resistance sources of oilseed rape to *Verticillium longisporum* pathogen attack. For this purpose we used a number of 130 oilseed rape cultivars which were artificially infected with the pathogen *V. longisporum*. For the molecular studies, we used 51 SSR markers which amplified a number of 139 specific fragments. Correlating the molecular data obtained after the SSR analysis and the phenotypic data obtained after the artificial infection, we identified 18 SSR markers associated with resistance to *V. longisporum.* This results are very important for the next studies regarding the breeding of oilseed rape, for the identification of the resistance genes to *V. longisporum*.

Key words: molecular markers, association, rezistance

Oilseed rape (*Brassica napus* L. *oleifera*) is considered at this moment to be one of the most important oilseed from Europe. Due to the increased of the cultivated area with this crop, disease problems had become a major issue in current breeding effort (Enyck, 2007). The pathogen *Verticillium longisporum* is causing one of the most principal diseases in the cultivation of oilseed rape besides blackleg and stem canker caused by *Phoma lingam* and stem rot caused by *Sclerotinia sclerotiorum* (Zeise & Steinbach, 2004). The control of this disease is difficult to make, because the microsclerotia produced by *V. longisporum* can survive in the soil for several years and until now there are no chemical produced for this disease (Schnathorst, 1981). In present, long term control of these disease can be made by using cultivars with resistance to this disease (Obermeier et al., 2012). The aim of this study was to evaluate the resistance of 130 oilseed rape cultivars to *V. longisporum* using SSR markers. For this purpose, the 130 oilseed rape cultivars were artificially infected with *V. longisporum* spore suspension in the green house. For the molecular studies we used 51 SSR markers. After the studies which were made, we identified 18 SSR markers associated with the level of resistance at *V. longisporum*.

MATERIAL AND METHODS

The plant material for this study comprised 130 genotypes of rapeseed cultivars proceeded from the Centre for Genetic Resources Netherlands. The plants were sown in the field in order to collect the fresh tissue material for DNA extraction.

SSR analysis

For the SSR analysis we used a number of 51 markers which amplified 139 polymorphic fragments *(tab.1*). Leaf samples were taken from all plants of each cultivar, bulked and immediately frozen in liquid nitrogen. The DNA extraction was made using CTAB method modified according to Doyle and Doyle (1990). DNA content was measured using a Nano Drop 2100 spectrophotometer. Based on these data DNA was diluted to a concentration 20 ng/ µl for the SSR

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analysis. Polymerase chain reaction (PCR) mixture (25 µL) for SSRs contained 20 ng of DNA template, 0.75 pmol of each primer, 0.2 mM dNTP mix, 1 mM MgCl2, 10XPCR reaction buffer and 5 unit of Taq DNA polymerase (Qiagen). Amplifications were performed using a standard amplification cycle in a GeneAmp PCR System 9700 thermal cycler, and SSR

Table 1

The sequence of SSR markers

Nr. crt	Denumire primer	Secvenţa Forward	Secvenţa reverse
1.	CB-10065	CGGCAATAATGGACCACTGG	CGGCTTTCACGCAGACTTCG
2.	Na10-G08	TTTCTTTTAACCTGATGTTTTGG	TCACTGTGTTTACTTGCGCC
3.	Ol10-B02	CACGAACGCGAGAGAGAGAG	TGCATAAGCTCGAAGAGACG
4.	Na12-C01	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC
5.	BRMS-30	TCAGCCTACCAACGAGTCATAA	AAGGTCTCATACGATGGGAGTG
6.	Na10-D11	GAGACATAGATGAGTGAATCTGGC	CATTAGTTGTGGACGGTCGG
7.	CB 10536	-	-
8.	Ol10-E12	TGCTCTGCAAGATATTCCCC	AACCGTCACAGATCCTGTCC
9.	MD-60	-	-
10.	CB 10028	CTGCACATTTGAAATTGGTC	AAATCAACGCTTACCCACT
11.	CB 10206	TACAACGCAAACGTTCCT	TTGATGTTCTTGGTGCCT
12.	CB 10437	-	-
13.	CB 10097	ACTTCGGTGGTTCTATTTCT	CGACGGTTAATCAAGTTTCT
14.	CB 10347	-	-
15.	CB 10536	-	-
16.	Na12-H06	AAATCCCAAAACCCAACCTC	CAAAGAAAAACGTGTCTAGAGAGAG
17.	CB 10611	GTATCTGCGACAGTGGGA	AGCTTGGCTGTAATGACG
18.	BRMS 20	AACAAGAGAAGGAGAGCCACCG	CGCTTATAAAATGGCAGTCGCA
19.	Ol10-D08	TCCGAACACTCTAAGTTAGCTCC	GAGCTGTATGTCTCCCGTGC
20.	BRMS 309	-	-
21.	Na10-B11	TTTAACAACAACCGTCACGC	CTCCTCCTCCATCAATCTGC
22.	Na12-D08	ACGACGATTCAACTCATCTTC	TTAACCAACTTCGCTTTTTG
23.	OL10-C10	AAGAAGGCGTAGAGATTGCC	GCAGATAAGATTCGAGTCCCC
24.	Ra2-F04	CCTACAAACACATAAATAAAGAGAGAG	AACAACATAAAAGATTCATTTCG
25.	Na14-H12	CACATTGGCACGTATCCATC	GGCTGATCGAACACAAATAAG
26.	Ol10-D01	TCTCTGCCAAAAGCAAATAGC	CTTGGCTCTCTCTCACCACC
27.	CB10600	CGCTTCTCTTCATCTGCT	AATTTGTTTCTTCAGCCG
28.	Na12-A01	GCATGCTCTTGATGAACGAA	GCTTCAACCTCTCAATCGCT
29.	Ol10-D03	GCCAAAGACCTCAAAGATGG	AAGCCACGTGAAGAAAGTCC
30.	Na14-G06	AAACGGCTTGCATTGTTCTC	GGCTTGCTTGATCCAGTCTC
31.	Ol10-F12	TCCATGTTTCATGTTGGAGG	CTCTCCGGCTTCACTTTCC
32.	Ol11-B05	TCGCGACGTTGTTTTGTTC	ACCATCTTCCTCGACCCTG
33.	Ni2-C12	ACATTCTTGGATCTTGATTCG	AAAGGTCAAGTCCTTCCTTCG
34.	Na12-B11	AAGCTTCCTCGTTCTCCTCC	TTGTCTTCACTCGTTTTGCG
35.	Ol13-E08	TTCGCAACTCCTCCTAGAATC	AAGGTCTCACCACCGGAGTC
36.	Ol10-G06	GACAAGTTCCCTTGTAATGGC	TGTAATCATCACACATTTTGGG
37.	OL10-E05	GCCAGAAACAGGAGAAATGG	GAAGCCGAAGAAAATAAGCG
38.	OL13-F08	GTGGACGTTCATGTCCCTTC	CCTGAATCGATTTTCGTCTTG
39.	Na12-B07	TTGAGATCGAAAGTGATTAGGG	GATCCCGATCAGCTCAACC
40.	Ra12-E12	-	-
41.	Na12-A02	AGCCTTGTTGCTTTTCAACG	AGTGAATCGATGATCTCGCC
42.	Na12-B05	CAAATATCCGTCATCGGAGC	CCTGCGGGATATTGAAGACC
43.	HMR416	-	-
44.	Ra2-F11	-	-
45.	Ol11-H02	TCTTCAGGGTTTCCAACGAC	AGGCTCCTTCATTTGATCCC
46.	Na10-C01	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC
47.	HMR354	-	-
48.	Na14-G10	ACGAAGTGGGTTAGTAGGCG	GAAGCCTTTCTCCACCATTG
49.	HMR562	-	-
50.	HMR585	-	-
51.	Na12-G05	CCGATCATACCTTTTACTCTAGCC	GATGTTCCTCTCGGTGATGC

polymorphisms were separated and visualised using a LI-COR GeneReadir 4200 (MWG Biotech, Ebersberg). For the SSR analysis it was used M13-tailing technique. In this method the fluorescently labeled universal M13 primer 5'-AGGGTTTTCCCAGTCACGACGTT- 3' is added to the PCR reaction, and the forward primer of each SSR is appended with the sequence 5'-TTTCCCAGTCACGACGTT-3'. The amplification

was performed after a touch-down PCR cycle as follows: an initial denaturation was performed at 95°C for 2 min, followed by five cycles of denaturation for 45 s at 95°C, annealing for 5 min beginning at 68°C and decreasing by 2°C in each subsequent cycle, and extension for 1 min at 72°C. Then five cycles were performed with 45 s denaturation at 95°C, 1 min annealing beginning at 58°C and decreasing 2°C in each subsequent cycle, and 1 min of extension at 72°C. The PCR was then completed with an additional 27 cycles of 45 s denaturation at 94°C, 2 min of annealing at 47°C, and 30 s of extension at 72°C, with a final extension at 72°C for 10 min. The visualization of the amplified fragments was made using Saga generation software version1. Each primer was scored manual, for the presence of the band using ‘1“and 0 were the band was absent.

Resistant tests for V. longisporum

The resistance tests were performed with *V. longisporum* isolates VL 43 which was provided by the division of Plant Pathology and Plant Protection, Gottingen, Germany. The isolate was chose on the basis were chosen on the basis of results of preliminary virulence tests of several Verticillium strains from *B. napus* described by Zeise & von Tiedemann (2002a). Long-term storage of fungi was performed as conidial suspensions in a concentration of 1-3 x 106 conidia mL-1 in Czapek-Dox medium supplemented with 25% glycerol. The inoculums for the infection were produced by 500 µL of spore stock solutions to 250 mL potato dextrose broth. The cultures were subsequently incubated for 7 days at 23°C on a rotary shaker (100 RPM). After the incubation period, the resulting suspension was filtered through sterile gauze and using a haemocytometer was diluted to 1 x 106 spores mL-1. In our experiments, we used as reference controls the winter oilseed rape cultivars varieties Express (less susceptible) and Falcon (highly susceptible). The seeds of the control an test plants, surface sterilized by immersion in 70% ethanol for 2 minutes. After the sterilization, the seeds were washed with tap water and then sowed in silica sand. After 10 days, the roots of the plans were carefully washed from the sand. Inoculations were performed by cutting 2 cm from the roots and and submerging the capped roots for 30 min in the spore suspension. For each cultivar we used 10 plants inoculated and 10 controls. After that, the plantlets were transferred in pots containing a mixture of sand, peat and compost (1:1:2) and grown in the green house with a light/dark cycle of 14/10h. Disease scores were taken weekly from each plant for a four-week period using an assessment key with nine classes, slightly modified from ZEISE (1992), as described by EYNCK et al. (2007). For each accession it was calculated area under disease progress curve (AUDPC) from the disease severity values.

RESULTS AND DISCUSSIONS

For the molecular analysis based on SSR markers, we used 51 markers which amplified a number of 139 polymorphic fragments which ranged between 80 an 340 bp (base pairs). The obtained data after the SSR amplification were centralized in Excel, concretized in a binary matrix. For identification of the molecular markers which are associated with the resistance of oilseed, we correlated the AUDPC values obtained after the artificial infection with *V. longisporum* with the genotypic data obtained after the SSR amplification. For the realization of the correlation between the 51 SSR markers and the AUDPC values the simple linear regression method it was used and the statistic calculation was performed with SPSS v13 program.

After the correlation that it was made, we identified 18 markers which were significant associated with the resistance of oilseed rape to *V. longisporum* (*tab. 2*). The smallest p value (p=0) was obtained at the marker Na12-C01. In this case, the value of R^2 was of 0.093 which demonstrate that 9 % of the phenotypical variation of the AUDPC values is explained by this marker. Another marker associated with the resistance to *V. longisporum* was Na12-B07, which recorded a p value of 0, but only 1% of the phenotypical variation is explained by this marker. For the SSR analysis we used the SSR markers which were used in previous studies for realization the genetic maps at oilseed rape and for QTL identification. In this study, we identified 18 SSR markers which were associated with the resistance of the studied cultivars at *V. longisporum*. From these markers Na12-C01_40, Na12-B07_145, Na10-D11_220, Ol10-E12_280, Na12-B07_136 and Ra12-E12_150 are special because had obtained a phenotypic variation grater than 5% for the AUDPC values of the studied cultivars.The marker Na12-C01_40, which in this experiment proved to be associated with the resistance to *V. longisporum*, was localized on the C5 chromosome where was also identified the first QTL for resistance to this diseases in a segregate population of oilseed obtained by the cross of two different *Brassica* species (Rygulla *el al*., 2008).The marker Ol10-E12 which also proved to be associated with the level of resistance to *V. longisporum* was used in other studies for identification of a QTL for resistance to this disease (Obermeier *et al*., 2012).

CONCLUSIONS

The fact that this markers which were used in identification of some QTLs for resistance to *V. longisporum* in different studies and in this study proved to be associated with the resistance in a genetically different population of oilseed rape demonstrates that this markers may be correlated with the genes that are related with the resistance.

Table 2

The SSRs with significance for the resistance of the cultivares to V. longisporum

Nr. Crt	Primer	R	R Square	Adjusted R Square	p-value	Semnificaţie
1	Na10-G08_310	0.176	0.031	0.023	0.045	***
2	Na10-G08_320	0.175	0.03	0.023	0.047	***
3	Na12-C01_40	0.317	0.1	0.093	0	***
4	Na10-D11_220	0.268	0.072	0.065	0.002	***
5	Ol10-E12_280	0.256	0.066	0.058	0.003	***
6	MD 60_185	0.185	0.034	0.027	0.035	***
7	Na12-D08_140	0.188	0.035	0.028	0.033	***
8	Na12-D08_145	0.194	0.038	0.03	0.027	***
9	OL10-C10_190	0.217	0.047	0.04	0.013	***
10	Ol10-D01_270	0.188	0.035	0.028	0.032	***
11	Ol13-E08_170	0.176	0.031	0.024	0.045	***
12	OL10-E05_160	0.22	0.048	0.041	0.012	***
13	OL13-F08_145	0.18	0.032	0.025	0.04	***
14	Na12-B07_136	0.235	0.055	0.048	0.007	***
15	Na12-B07_146	0.342	0.117	0.11	0	***
16	Na12-B07_147	0.191	0.036	0.029	0.03	***
17	Ra12-E12_150	0.234	0.055	0.047	0.007	***
18	Ra12-E12_240	0.173	0.03	0.022	0.049	***

ACKNOWLEGMENTS

The financial support for our work is from the project POSCCE ID714/1268 funded by EU.

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