BIULETYN INSTYTUTU HODOWLI I AKLIMATYZACJI ROŚLIN Nr 291 / 2020 : 41–46
E-ISSN: 2657–8913

Oryginalny artykuł naukowy
Original research paper

Analiza regionów genomu sprzężonych z odpornością porzeczki czarnej (*Ribes nigrum*) na wielkopąkowca porzeczkowego (*Cecidophyopsis ribis*)

Analysis of genome regions linked to the resistance of blackcurrant (*Ribes nigrum*) to blackcurrant gall mite (*Cecidophyopsis ribis*)

Anita Kuras✉, Bogumiła Badek

Zakład Hodowli Roślin Ogrodniczych,
Instytut Ogrodnictwa, ul. Konstytucji 3 Maja 1/3, 96–100 Skierniewice
✉ e-mail: anita.kuras@inhort.pl

Wielkopąkowiec porzeczkowy (*Cecidophyopsis ribis*), jest jednym z najgroźniejszych szkodników porzeczki czarnej. Powoduje on deformację pąków porzeczki czarnej i redukuje plon owoców. Wybranie najkorzystniejszych markerów molekularnych sprzężonych z odpornością na wielkopąkowca porzeczkowego może przyspieszyć proces hodowlany i poprawić skuteczność przy wyborze genotypów odpornych na tę chorobę. Celem pracy była analiza regionów genomu sprzężonych z odpornością porzeczki czarnej (*Ribes nigrum*) na wielkopąkowca porzeczkowego poprzez ocenę stopnia polimorfizmu w grupach sprzężeń obejmujących regiony Ce i P i poszukiwanie nowych fragmentów genomu regulujących odporność na *C. ribis.* W wyniku analizy elektroforegramów z produktami reakcji CAPS, zidentyfikowano 119 polimorficznych fragmentów DNA, z których wytypowano 100 do analizy sekwencji. W wyniku sekwencjonowania uzyskano 9 specyficznych odczytów na matrycach DNA odmian ‘Ceres’, ‘Ojebyn’, ‘Vir’, ‘Ores’, ‘Foxendown’, ‘Ben Finlay’ i ‘Bona’.

Słowa kluczowe: CAPS, *Cecidophyopsis ribis*, marker molekularny, porzeczka czarna,

Blackcurrant gall mite (*Cecidophyopsis ribis*) is one of the most dangerous pests of blackcurrant. It causes deformation of blackcurrant buds and reduces fruit yield. The selection of the most favorable molecular markers coupled to resistance to *C.ribis*, can accelerate the breeding process and improve efficacy when choosing genotypes that are resistant to this disease. The aim of this study was to analyze genome regions conjugated with resistance of blackcurrant (*Ribes nigrum*) to blackcurrant gall mite by assessing the degree of polymorphism in coupling groups, including the Ce and P regions, and by searching for new genome fragments regulating *C. ribis* resistance. As a result of the analysis of electrophoregrams with CAPS reaction products, 119 polymorphic DNA fragments were identified, out of which 100 were selected for sequence analysis. As the end product of sequencing, 9 specific readings were obtained on DNA matrices of the varieties ‘Ceres’, ‘Ojebyn’, ‘Vir’, ‘Ores’, ‘Foxendown’, ‘Ben Finlay’ and ‘Bona’.

Key words: CAPS, *Cecidophyopsis ribis*, molecular marker, blackcurrant

Introduction

Blackcurrant gall mite (*Cecidophyopsis ribis*) is the most dangerous pests of blackcurrant. It is a vector for blackcurrant reversion virus (BRV), which reduces fruit yield and consequently leads to plantation removal (Pluta and Żurawicz, 2002; Łabanowska et al., 2015). Limiting the population of blackcurrant gall mite and production of blackcurrant varieties with increased resistance to this pest and BRV is very important for horticultural practice. Many research institutions in Europe conduct studies to identify molecular markers linked to two separate types of resistance, determined by Ce and P genes. The Ce resistance gene in these studies was sourced from gooseberry (*R. grossularia*) (Knight et al., 1974; Brennan et al., 1993), and the P gene from *R. nigrum* ssp. *Sibiricum* (Anderson, 1971). The Research Institute of Horticulture in Skierniewice works on the identification of molecular markers linked to resistance and the position of fragments that determine this resistance in the genome of blackcurrant. The aim of the presented research conducted in 2019 was to assess the polymorphism of linked groups, including regions associated with the resistance of blackcurrant to *C. ribis* by means of sequence analysis.

Material and Methods

Research material

The research was carried out on plants of 12 genotypes representing the genus *Ribes*

Managing editor: Magdalena Szechyńska-Hebda.

(6 resistant and 6 susceptible to *C. ribis*), selected based on literature data and analyses carried out as part of the project in 2015-2019 (Tab. 1).

Methods

DNA was isolated from young leaves of plants representing selected genotypes using the procedure proposed by Doyle and Doyle (1990). The quality and concentration of DNA in the obtained samples was determined based on electrophoregrams in a 0.8% agarose gel and the measurement of extinction coefficients at the wavelengths 230, 260, 280 and 320 nm (Gene Quant Pro Amersham Pharmacia Biotech).

Templates of DNA isolated from the analysed genotypes of the genus *Ribes* (Tab. 1) were used for amplification with combinations of SSR primers (Tab. 2) selected for the study from databases (following information on the location of regions closely linked to QTL determining resistance to blackcurrant gall mite: Lg2[*]; LG4[*] and LG6[**], Brennan et al., 2008[*] and Mazeikiene et al., 2012[**]) and findings from the project executed in 2015-2019.

The reaction for PCR assays contained: 10 ng

Tabela 1
Table 1

Wykaz genotypów rodzaju Ribes, wytypowanych do analiz molekularnych

List of genotypes of the genus Ribes selected for molecular analysis

Lp. No.	Odmiana Variety	Rodowód Parentage	Wrażliwa/Odporna[1] Susceppribility/Resistant	Kraj Country
1.	Ores	(Ojebyn × S24) × Ceres	odporna/resistant	Polska/Poland
2.	Ceres	(*R. Dikuscha* x Barchatnaja) × samozapylenie	odporna/resistant	Polska/Poland
3.	Polares	S12/3/83 x EMB (1834)/113	odporna/resistant	Polska/Poland
4.	Foxendown	Ben LomondD x (BC3 z agrestu × BC2 *R. Glutinosum*	odporna/resistant	Szkocja/Scotland
5.	Vir	nieznany/unknown	odporna/resistant	Rosja/Russia
6.	Ben Finlay	[(SCRI P10/9/13 × Ben Alder) × EM B1834-67]	odporna/resistant	Szkocja/Scotland
7.	Ben Hope	Westra x (SCRI 238/36 × EM 21/15)	wrażliwa/susceppribility	Szkocja/Scotland
8.	Bona	Ojebyn × (*R.Dikuscha* × Climax)	wrażliwa/susceppribility	Polska/Poland
9.	Gofert	Gołubka × Fertodi 1	wrażliwa/susceppribility	Polska/Poland
10.	Gołubka	*R. Nigrum* × *R. Dikuscha*.	wrażliwa/susceppribility	Rosja/Russia
11.	Ojebyn	Seedling selection of *R. Nigrum* from north sweden	wrażliwa/susceppribility	Szkocja/Scotland
12.	Riasnaja	nieznany/unknown	wrażliwa/susceppribility	Rosja/Russia

[1] *dane z literatury*

Tabela 2
Table 2

Wykaz starterów SSR użytych do analiz molekularnych
List of SSR primers used for molecular analysis

Starter Primer	Sekwencja startera (5'-3') Starter sequence (5'-3')		Lokalizacjana chromosomach Location on the chromosome
g1-G06F/g2-J08R	AAAACACACATCTCTCACCCC	ATAGCCCATGCCCATATTCA	LG 2[*]
g2-J08F/g1-G06R	CGCCGAGCTCTAATCACTGT	TCGAATCTGAACCACGATGA	LG 2[*]
g2-J08F/g1-B02R	CGCCGAGCTCTAATCACTGT	CCATTGATTTGGTGAGGGT	LG 2[*]
g1-B02F/g2-J08R	CGCTTCATCGCTCTCCTCT	ATA GCC CAT GCC CAT ATT CA	LG 2[*]
g1-G06F/g1-B02R	AAAACACACATCTCTCACCCC	CCA TTG ATT TGG TGA GGG T	LG 2[*]
g1-B02F/g1-G06R	CGACTTCATCGCTCTCCTCT	TCG AAT CTG AAC CAC GAT GA	LG 2[*]
e1-O21F/g2-L17R	TCTCTCCAACTGAGAAGGAAA	GAG CTG TTG CTG TTG CCA TA	LG 4[*]
g2-L17F/e1-O21R	TTTGGA AAA CCT CCC CTT TT	GAT TTG TTC TTG TGC AGC GA	LG 4[*]
g1-I02F/g1-D11R	TGA ATA TCA GAC CGC CAT CA	AAT CGA ATG GAA TCG TCC	LG6[**]
g1-D11F/g1-I02R	GAA GAC GAC AAA GCC TCC	TCC AAG ATG AAG CTT CTC AAA TC	LG6[**]

[*]- *Brennan i in. 2008;* [**]- *Mazeikiene i in. 2012*

DNA, 0.5U Taq Platinum® polymerase (Invitrogen), 10 × PCR-buffer, 1.5 mM $MgCl_2$, 1.25 mM dNTP (Applied Biosystems) and 0.35 µM SSR primer. The thermal profile of the reaction was as follows: initial denaturation -94°C/2 min, 4 cycles (denaturation 94°C/50 s, primer annealing 55–50°C/50 s (in each subsequent cycle the annealing temperature was reduced by 1°C), DNA strand elongation 72°C/60 s), 30 cycles (denaturation 94°C/50 s, primer annealing 55–50°C/90 s, DNA strand elongation 72°C/60 s), final elongation 72°C/10 min. The reaction was performed in a Biometra Basic thermocycler.

The SSR-PCR reaction products were digested with 4 restriction enzymes (Tab. 3), which were separated on a 2% agarose gel stained with ethidium bromide, then DNA fragments characteristic for the analysed genotypes were isolated from agarose gels using a commercial FastGene Gel/PCR Extraction Kit (Nippon Genetics)..

DNA fragments isolated from the gel and purified were cloned in bacterial plasmids (TOPO® TA Cloning® Kit for Sequencing (Invitrogen). Next, the plasmids were inserted into One Shot TOP10 chemically competent *E. coli* (Invitrogen), following the protocol specified by the manufacturer. Selected bacterial colonies containing the plasmid with the inserted DNA fragment of the analysed *Ribes* genotypes were transferred to LB liquid culture medium. The bacterial suspension was incubated (37°C/16 h) and bacterial plasmids were isolated using a commercially available FastGene Plasmid Mini Kit (Nippon Genetics).

Bacterial plasmids containing characteristic genomic fragments were PCR-labelled with the M13 universal primer using a DTCS Quick Start Kit (Beckman Coulter), and then sequenced using a CEQ (8000) Genome Analyzer (Beckman Coulter).

Sequences were analysed with CEQ (8000) software v.9.0. Contigs from reads were assembled in SeqMan software (Lasergen v.7 package). Nucleotide sequence diversity was analysed using MegAlign software (Lasergen v.7 package). Obtained DNA sequences were compared with NCBI data using Blast-nt bioinformatics software.

Specific oligonucleotides were designed for the obtained DNA sequences (PrimerSelect software, Lasergen v.7 package).

Results

After identifying polymorphisms between genotypes susceptible/resistant to *C. ribis*, the obtained sequences were analysed with MegAlign software (Lasergen v.7 package) for the potential transformation of polymorphism into the CAPS primer. The analysis revealed four restriction enzymes: PstI, Tru1I, AseI, SmaI, which met the following criteria: the length of the products obtained after the cleavage clearly distinguishing alleles, the analysed sequence has no more than three cleavage sites (Tab. 3).

A total of 1,440 PCR amplification reactions with 10 pairs of primers flanking Ce and P regions in various combinations were carried out (Tab. 2), 2). Primers were digested with selected restriction enzymes, electrophoregrams were analysed, 119 polymorphic DNA fragments identified, and 100 sequences were selected for further analysis (Tab. 4). Sequencing produced 9 specific reads with a band size of 351 to 490 bp (Tab. 5).

The comparative analysis of the obtained sequences showed a diverse sequence arrangement obtained for the investigated resistant and susceptible genotypes, most likely being single-nucleotide polymorphisms (SNP). One pair of specific oligonucleotides was designed for each obtained sequence (Tab. 6)

Discussion

Due to the nutritional value of blackcurrant, research is carried out worldwide to find varieties suitable for cultivation in a specific climate and with increased tolerance to low temperature and diseases (mainly blackcurrant reversion virus) and pests (blackcurrant gall mite). Limiting the population

Tabela 3
Table 3

Wykaz enzymów restrykcyjnych użytych do analizy CAPS
List of restriction enzymes used for CAPS analysis

Enzym Enzyme	Pochodzenie Origin	Rozpoznawana sekwencja Recognized sequence	Warunki reakcji Reaction conditions	Inaktywacja Inactivation
PstI	*Providencia stuarti*	5'…CTGCA G…3'	37°C/ 16 h	80°C/ 20min
Tru1I	*Thermus ruber*	5'…T TAA…3'	65°C/ 16 h	80°C/ 20min
AseI	*Vibrio species*	5'…AT TAA…3'	37°C/ 16 h	65°C/ 20min
SmaI	*Serratia marcescens*	5'…CCC GGG…3'	30°C/ 16 h	65°C/ 20min

Tabela 4
Table 4

Wykaz fragmentów DNA wytypowanych do analizy sekwencji.
List of DNA fragments selected for sequence analysis.

Starter/enzym Primer/enzyme	Długość [pz] Fragmentów DNA Band size of DNA fragments [bp]	Analizowane genotypy Analyzed genotypes											
		Ores	Ceres	Polares	Foxendown	Vir	Ben Finlay	Ben Hope	Bona	Gofert	Gołubka	Ojebyn	Riasnaja
g1-G06F/ g2-J08R/ *PstI*	360	+[1]	+	+	+	+	+	+	+	+	+	+	+
	390	+	+		+		+	+			+	+	+
	430	+	+	+	+	+	+	+	+	+	+	+	+
	490	+	+			+			+	+	+		
g1-B02F/ g1-G06R/ *AseI*	300	+		+	+	+	+					+	+
	340	+	+	+	+	+	+	+	+	+	+	+	+
g1-G06F/ g2-J08R/ *AseI*	350		+			+	+	+	+	+			
	460	+	+	+	+	+	+	+	+	+	+	+	+
	510	+	+	+	+	+	+	+	+	+	+	+	+
g1-B02F/ g1-G06R/ *SmaI*	430		+			+	+	+	+				
	580	+	+	+	+			+	+	+			+

[1] – „ + ” – *fragment wytypowany do sekwencjonowania*

Tabela 5
Table 5

Wykaz specyficznych sekwencji uzyskanych w wyniku sekwencjonowania
List of specific sequences obtained as a result of sequencing

Lp. No.	Nazwa sekwencji Sequence name	Pochodzenie/genotyp Origin/ Genotype	Kombinacje starterów Flankujących regiony ce i p/enzym Primers flanking regions ce and p/enzyme	Wielkość zsekwencjonowanego fragmentu dna [pz] DNA band size [bp]
1	Seq.73	‘Ceres’	g1-G06F/g2-J08R/ *PstI*	363
2.	Seq.74	‘Ojebyn’	g1-G06F/g2-J08R/ *PstI*	390
3.	Seq.75	‘Vir’	g1-G06F/g2-J08R/ *PstI*	490
4.	Seq.76	‘Ceres’	g1-G06F/ g2-J08R/ *AseI*	351
5.	Seq.77	‘Ores’	g1-G06F/g2-J08R/ *AseI*	460
6.	Seq.78	‘Foxendown’	g1-G06F/g2-J08R/ *AseI*	462
7.	Seq.79	‘Vir’	g1-G06Fg2-J08R/ *AseI*	461
8.	Seq.80	‘Ben Finlay’	g1-B02F/ g1-G06R/ *SmaI*	435
9.	Seq.81	‘Bona’	g1-B02F/ g1-G06R/ *SmaI*	436

Tabela 6
Table 6

Wykaz sekwencji oligonukleotydów specyficznych
List of specific oligonucleotide sequences

Lp. No.	Oligonukleotyd Oligonucleotide	Reverse	Forvard
1.	2.73	CTCGCTGAGCGGATTTGGAT	ACGGCGGTTAGGAAGGTGTGGGT
2.	2.74	CGATAGCGGGACTGTAAGAG	TTATGAGCCCCAAAGCCCTAAG
3.	2.75	CGTGGTATGACATCAGACTACAC	ACGGACTGGACCGGGAC
4.	2.76	T AGTGAAGAACGGAGCTCAGGAG	TTTATTGCGGAGGGAGAGGCGCT
5.	2.77	CCGACCATACTTGAGTCGTAATAG	ACTGATTTCCCCCGAGCTCTTG
6.	2.78	GAGGCTAAGAAGGACGTGTATGGG	TAGGATGCTTCCCGTCAATACGA
7.	2.79	GCCGAGTTTCTGTATCGTGCA	CGAATAAGTCAGGGCGCATAGGC
8.	2.80	TCTTGTCAACTCGGCTCCCAGACT	CAGGGCAGAAAGCTGTGGAGGGAA
9.	2.81	TGAGTTCATCGCCATCAGACATG	GAAGGGCGAATGGAGGGCTGATAT

of blackcurrant gall mite and the creation of blackcurrant varieties with increased resistance to this pest and BRV is very important for horticultural practice. So far, genetic and breeding studies have led to the identification of two distinct types of resistance to blackcurrant gall mite (Brennan et al., 2008; Mazeikiene et al., 2012), the source of which are genes from gooseberry (*R. grossularia*) (Knight et al., 1974) and *R. nigrum* ssp. *Sibiricum* (Anderson 1971).

The use of molecular markers in horticultural studies improves the efficiency of conventional breeding by selecting plants with markers linked to economically important performance traits (Gupta et al. 1999). The search and identification of such markers is aimed at shortening the breeding cycle, because the plant material can be selected as early as at the seedling stage, regardless of the stage of the plant's development at which a given trait is revealed. Breeding for resistance is oriented at finding markers linked to resistance traits, and a single marker or a set of markers linked to this trait may be sufficient for marker-assisted selection (MAS).

In 2019 research was continued to assess the degree of polymorphism in linked groups of genes, including regions associated with resistance of blackcurrant to *C. ribis* by means of sequence analysis. The operation of most popular sequencers used in research and diagnostics relies on a modified method developed by Sanger and Coulson in 1975. In our study the *Ribes* genomes were sequenced with a CEQ 8000 system (Beckman Coulter). Sequencing is often used for comparative analysis, and identification of single nucleotide polymorphisms within the same fragment (SNP) (Bang et al., 2007; Thiel et al., 2004). Such comparisons provide detailed information on the genetic similarity/polymorphism, origin and evolution of the tested organisms.

Preliminary comparative analysis of DNA sequences specific for *Ribes* genotypes with a different degree of resistance to blackcurrant gall mite carried out in our study extended the database of blackcurrant polymorphisms for susceptible and resistant cultivars necessary to investigate the regulation of resistance to *C. ribis*.

Comparative analysis of the sequenced DNA fragments identified in these genotypes revealed a strong homology (88-90%) with a nucleotide sequence for the Ce marker presented by Brennan et al. (2008). Currently, studies are focused on the identification of inheritance of resistance genes (*Ce* and *P*) in genotypes obtained by hybridization of species in which they were found.

In our work, we are also searching for markers linked to *Ce* and *P* resistance genes, which would enable the characterization of *Ribes* genotypes different in terms of resistance. At the present stage, our preliminary studies verifying the selected marker sequences have not confirmed their suitability for the selection of genotypes resistant and susceptible to blackcurrant gall mite.

Conclusions

Results of the research analysing the polymorphism of fragments differentiating the genotypes of *Ribes* (resistant/susceptible) obtained for DNA templates from plants selected from the collection are used to establish a database of sequences linked to resistance against *C. ribis*.

References

Anderson, M. M. (1971). Resistance to gall mite (Phytoptus ribis Nal.) in the Eucoreosma section of Ribes. Euphytica (20), 422–426.

Bang, H., Kim, S., Leskovar, D., King, S. (2007). Development of a codominant CAPS marker for allelic selection between canary yellow and red watermelon based on SNP in lycopene β-cyclase (LCYB) gene. Molecular Breedin (20), 63–72

Brennan, R., Jorgensen, L., Gordon, S., Loades, K., Hackett, C., Russell, J. (2008). The development of a PCR-based marker linked to resistance to the blackcurrant gall mite (Cecidophyopsis ribis Acari: Eriophyidae). Theor. Appl. Genet. (118), 205–211.

Brennan, R. M., Lanham, P. G., McNicol, R. J. (1993). Ribes reeding and research in the UK. Acta Horticulturae (352), 267–275. https://doi.org/10.17660/ActaHortic.1993.352.38.

Doyle, J. J., Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus (12),13–15.

Gupta, P. K., Varshney, R. K., Sharma, P. C., Ramesh, B. (1999). Molecular markers and their applications in wheat breeding. Plant Breed (118),369–390.

Knight, R. L., Keep, E., Briggs, J. B., Parker, J. (1974). Transference of resistance to black currant gall mite Cecidophyopsis ribis, from gooseberry to black currant. Ann. Appl. Biol. (76),123–130.

Łabanowska, B. H., Piotrowski, W., Gruchała, M. (2015). Blackcurrant gall mite – Cecidophyopsis ribis (Westw.), harmfulness and possibility to control with fenpyroximate (Ortus 05 SC). Prog. Plant Prot. 55 (3), 305–311.

Mazeikiene, I., Bendokas, V., Stanys, V., Siksnianas, T. (2012): Molecular markers linked to resistance to the gall mite in blackcurrant. Plant Breeding. (131),762–766.

Pluta, S., Żurawicz, E. (2002). Effect of reversion virus

on the yield and fruit size in blackcurrant Ribes nigrum L. Proceedings 8th International Symposium Rubus and Ribes. Scotland, Dundee. July 2001. Acta Horticulturae 585 (1): 393–398.

Sanger, F., Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol. 94(3): 441–448.

Thiel, T, Kota, R, Grosse, I, Stein, N, Graner, A. (2004). SNP2CAPS: a SNP and INDEL analysis tool for CAPS marker development. Nucleic Acids Res; (32),e5.