Segregation of DNA markers of potato (*Solanum tuberosum* ssp. *tuberosum* L.) resistance against Ro1 pathotype *Globodera rostochiensis* in selected F_1 progeny

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ABSTRACT

Marker assisted selection is the fast and objective method for detection of resistance major genes. This method is practical for identification of some candidate genes of quantitative resistance. Genetic markers based on Polymerase Chain Reaction (PCR) were used for evaluation of F_1 progenies Ornella × Mira and Tábor × Mira. Cultivar Mira has resistance against Ro1 pathotype *G. rostochiensis*. Cultivars Ornela and Tábor are susceptible to Ro1. Seedlings of F_1 generations were used for analyses. Plants were cultivated in greenhouse. DNA was isolated from tissue discs by GenElute Plant Genomic DNA Kit (Sigma, SRN). PCR marker of major gene *H1* was used for bulked analyses, according to (Niewöhner et al. 1995). Size of this marker was 760 bp. Standard infection tests with Ro1 pathotype *G. rostochiensis* according to Potoček (1987) in all of the analysed genotypes were made. Segregation ratios of F_1 progenies were determined. These ratios have described segregation of resistance markers and segregation of traits in the biological test. The both methods of evaluation of potato's resistance were compared by correlation analyse. High correlations were found between occurrence of PCR marker for *H1* and resistance to Ro1 in biological test. Coefficient of correlation $r = 0.962$ in F_1 progeny Ornella × Mira and $r = 0.964$ in hybrids Tábor × Mira. Statistical evaluation of real ratios of segregation by infection tests and DNA markers with theoretical ratios of segregation in simplex and duplex *H1* gen qualitative determined resistance was made as well. Resistant cultivar Mira as donor of simplex determined resistance was confirmed.

Keywords: potato; *Solanum tuberosum* ssp. *tuberosum* L.; DNA markers; PCR; Ro1 pathotype *Globodera rostochiensis*; resistance; major gene *H1*; bulked segregation analysis

Nematodes of *Globodera* genus can be considered as a significant quarantine pest in all of the Temperate Zone. The highest incidence of *G. rostochiensis* has been during the last 40 years because the sale of potatoes has increased rapidly. In the affected areas *G. rostochiensis* causes big loss in quantity and quality of potato tubers, which can be more than 300 millions EUR per year (Bendezu et al. 1998). The possibility of complete elimination of this pathogen is very problematic. Both of *Globodera* species are included on the list of quarantine pathogens worldwide. Both species are on list A2 (EPPO – Europe Plant Protection Organization) (Smith et al. 1992). Plant breeding for resistance is considered as very important and effective strategy of plant protection at present (Bartoš 1991, Smith et al. 1992, Bendezu et al. 1998).

Genetics determination of resistance *Solanum* genus against *G. rostochiensis*

The resistance against this parasitical nematode is monogenic respectively oligogenic or polygenic. No genes are localised in the basic genome of *S. tuberosum* ssp. *tuberosum* L. The origin of resistant genotype is based on interspecies hybridisation; somatic hybridisation or it can be some result of genetics manipulations.

From the historic view, the first was discovered dominant monogenic determined resistance against Ro1 pathotype of *G. rostochiensis* (Huijsman 1955). The gene, which controls this resistance, was called *H1*. The gene source of this resistance was *S. tuberosum* ssp. *andigena* Hawkes CPCI1673. The gene *H1* is inherited tetrasomicaly and the dominant control resistance is reflected already manifested in simplex assemblage (*H1h1h1h1*). This situation was discussed among others by Huijsman (1955), Bartoš (1991), Pineda et al. (1993). By crossing, this gene was incorporated in many cultivars of *S. tuberosum* ssp. *tuberosum* L. This gene is localised on chromosome V (Gebhardt 1994). The mechanism of resistance, which is controlled by gene *H1*, is based on nutrition deficiency and female *G. rostochiensis* ontogenesis (Piegat and Wilski 1965, Hoopes et al. 1978). The result of female larvae nutrition deficiency is their smaller size and the limited ovum production (Ross 1986). Mullin and Brodie (1988) stated that the degree of second stage juvenile's infection is markedly lower in plants with this type of resistance. Forasmuch as Ro1 pathotype *G. rostochiensis* is considered as the most expanded, this resistance can be regarded as the great part of integrated potato protection. This resistance can be found in large number of potato cultivars. *S. tuberosum* ssp. *andigena* Hawkes was found later as a donor of resistance against

Ro4 pathotype *G. rostochiensis* (Ross 1986). Among other dominant major genes controlled resistance against pathotypes *G. rostochiensis* belong gene *H2* (Dunnet 1962), genes *A* and *B* (Huijsman 1960) and genes *Fa* and *Fb* (Ross 1986). Dominant major gene *Fa* brings resistance against Ro1, Ro2, and gene *Fb* against Ro2 and Ro5. Ross (1962) described other donor of genes resistance against Ro1 pathotype *G. rostochiensis*. The source of resistance was *S. spegazzinii* Bitt. EBS510. Barone et al. (1990) supposed that it is only one major gene. This major gene was called *Gro1* and in his experiments, he localised it to the chromosome VII. Monogenic dominant controlled resistance corresponded to the segregation of backcross (1:1). This finding was repeatedly confirmed in his experiments. In the next experiments based on different DNA markers application was discovered that the resistance obtained by alloploidie with genom *S. spegazzinii* is probably determined as polygenic. Currently, this type of resistance is considered as a quantitative trait and for mapping of single loci is used strategy QTL mapping (Kreike et al. 1993, 1994, Leister et al. 1997). The next possibility of polygene determined potato resistance against Ro1 pathotype *G. rostochiensis* source is botanic species *S. vernei* Bitt. et Wittm. The resistance *S. tuberosum* ssp. *tuberosum* L. obtained from this genetic source is markered as first described quantitative – polygene determined resistance against Ro1 pathotype *G. rostochiensis* (Pleisted et al. 1962).

Detection of the gene of resistance against *G. rostochiensis* in *Solanum* genus using DNA markers

Currently, the problem of detection genes of resistance is studied by using all types of genetics markers, which are based on polymorphism of nucleic acids. Pineda et al. (1993) used the RFLP (Restriction Fragment Length Polymorphism) method for studying and localisation of gene *H1* – gene of resistance against Ro1 pathotype *G. rostochiensis* in *Solanum* genus. As primary material, he has used diploid and tetraploide genotypes. By means of genomic probe, he also has studied different combination of alleles *H1* and *h1* in the tetraploide genotypes. He has statistically summarized his results to the construed chromosome V map, which he has saturated by 21 RFLP markers. Using RFLP, the gene *H1* was also mapped method by Gebhard (1994). She has used several RFLP cDNA type probes. Niewöhner et al. (1995) have made converse of RFLP marker to specific PCR (Polymerase Chain Reaction) marker. Kreike et al. (1993) was one of the firsts, who have used the RFLP method for gene *Gro1* markering and mapping. In his analysis, he has used *S. spegazzinii* Bitt as a diploid botany donor of resistance and their hybrids with diploid *S. tuberosum* L. By means of molecular genetics markers mapping systems, he has proved that this type of resistance is not determined by one locus, which is localised in chromosome VII (Barone et al. 1990), but it is quantitative polygenic controlled type of resistant. In his experiments, he has obtained two RFLP markers, which statistic cogently proved localisation of two locus of *Gro* gene – locus *Gro* 1.2 in chromosome X and locus *Gro* 1.3 in chromosome XI. Kreike et al. (1996) present in RFLP map locus *Gro* 1.4 localised in chromosome III. Problems of polygenic determined resistance, which was brought from *S. spegazzinii* Bitt., Gebhardt (1994) studied by using RFLP markers. By means of cDNA probes, she has localised in chromosome VII the same locus as Barone et al. (1990).

Leister et al. (1997) have studied problems of locus *Gro* gene. Object of his experiments was cloning of single loci of this polygen by using YAC (Yeast Artificial Chromosome) libraries. In his work, he has made a brief of individual alleles inserted to gene group *Gro1*. In his experiments, he has evaluated 384 YAC colons. With using methods RFLP and PCR-SPLAT he has detected individual alleles. He also presents some combination of primers, which are necessary for amplification of these allele's markers. Results of crossing with *S. spegazzinii* have introduced in *S. tuberosum* some genes with resistance against: Ro1 pathotype G. *rostochiensis*, *G. pallida*, lite blight and some viruses. Leister et al. (1997) in there results found two DNA markers covered chromosome II, one marker in chromosome III, five markers in chromosome V, four markers in chromosome VII, two markers in chromosome XI and one marker in chromosome XII.

MATERIAL AND METHODS

Plant material

Methods of detection of *H1* gene based on PCR were used on the collection of potato cultivars with declared resistance against the Ro1 pathotype of *G. rostochiensis* (Accent, Arnika, Berber, Bettina, Darwina, Granola, Gusta, Hilta, Ilse, Indra, Lyra, Mira, Olga, Ponto, Tanja, Ute) and declared susceptibility to Ro1 (Ornella, Tábor). Those genotypes were cultivated *in vitro*, on the modified medium according to Murashige and Skoog (1962). The segregation of the specific PCR marker was studied on the F_1 progeny of crosses Ornella × Mira and Tábor × Mira. The seedlings of hybrid progenies were cultivated in the greenhouse.

Detection of seedling resistance against the Ro1 pathotype of *G. rostochiensis* by infection test

For the detection of resistance (susceptibility respectively) of seedlings against the Ro1 the method according to Potoček (1987) was used. The test is based on the artificial infection of plants by the standard pathogen (Ro1 pathotype) and by quantification of abundance of nematodes after the cultivation.

DNA extraction

DNA was obtained from stems *in vitro* plants and from foliar pieces of greenhouse cultivated seedlings. For ex-

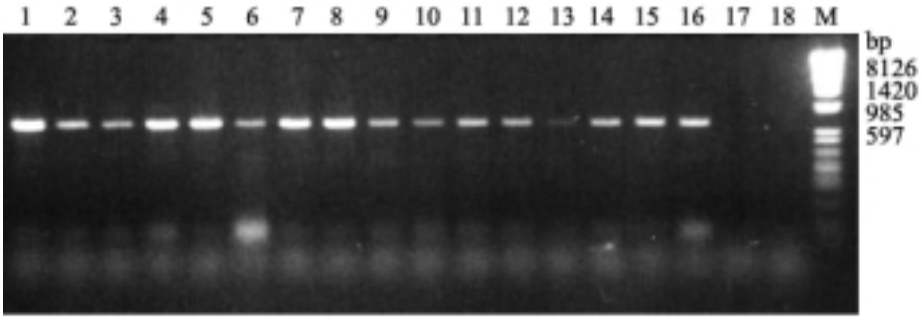

Figure 1. 760 bp PCR marker in collection of potato cultivars with declared resistance (1–16) and susceptibility (17–18) against the Ro1 pathotype of *G. rostochiensis*

Resistant cultivars: 1 – Accent, 2 – Arnika, 3 – Berber, 4 – Bettina, 5 – Darwina, 6 – Granola, 7 – Gusta, 8 – Hilta, 9 – Ilse, 10 – Indra, 11 – Lyra, 12 – Mira, 13 – Olga, 14 – Ponto, 15 – Tanja, 16 – Ute
Susceptible cultivars: 17 – Ornela, 18 – Tábor, M – the leader λDNA/*Eco*47I/*Ava*II

traction of DNA the GenElute Plant Genomic DNA Kit (Sigma, Germany) was used.

PCR detection of dominant *H1* allele

The pair of primers according to Niewöhner et al. (1995) for amplification of dominant *H1* allele marker was used. The sequences of the primers were: F 5'GCG TTA CAG TCG CCG TAT 3' and R 5'GTT GAA GAA ATA TGG AAT CAA A 3'. The melting temperature (T_m) was fixed 56°C for both of primers according to Sambrook et al. (1998). The thermocycler T-Gradient (Biometra, Germany) was used for amplification of DNA. Composition of the 25 µl reaction was: 100 ng of genomic DNA, 2.5mM $MgCl_2$, 0.3mM of both primers, 0.2mM dNTP and 0.7 U of the Red *Taq* polymerase (Sigma, Germany). Conditions of amplification were: 1 × (93°C, 180 s), 35 × (93°C 30 s, 51°C 45 s, 72°C 90 s) and 1 × (72°C 90 s). Amplified markers were analysed in 1.5% agarose electrophoretic gel in TBE buffer. Electrophoretic diagrams were visualized by ethidium bromide.

RESULTS

The optimisation of chemical composition and conditions of amplification to the successful obtaining of specific PCR markers was necessary for completion. Selection of the thermal gradient during amplification, that is one of the functions of the thermocycler, was used. The cultivars with declared resistance and susceptibility against the Ro1 pathotype of the *G. rostochiensis* to optimisation of the PCR detection of the dominant *H1* allele were used. The optimised method has enabled amplification of the 760 bp fragment in the case of the resistant genotypes. No product of amplification of the susceptible cultivars was detected. This fact manifests the applicability of the described method of detection of dominant allele of *H1* gene. This is demonstrated in Figure 1.

F_1 progeny genotypes from described assortment of cultivars were selected. Crossings of cultivars: Ornella × Mira, Tábor × Mira were made. Ornella and Tabor are susceptible and Mira has declared resistance against Ro1. In Ornella × Mira hybridisation 365 seedlings and in Tábor × Mira hybridisation 223 seedlings were acquired.

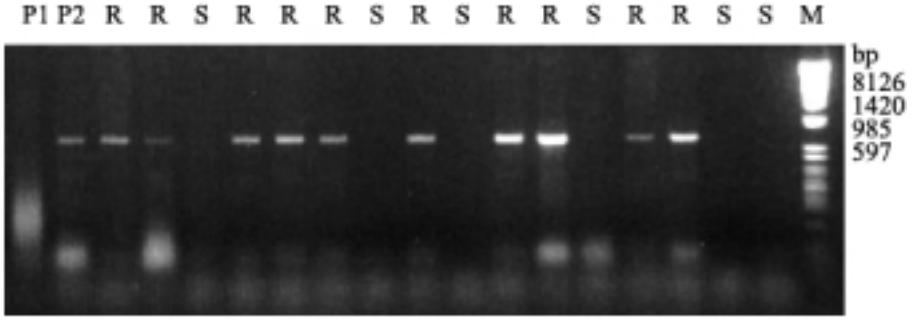

Figure 2. Segregation of 760 bp PCR marker in F_1 progeny of Ornella × Mira

P1 – parent – susceptible cultivar Ornella, P2 – parent – resistant cultivar Mira, R – resistant genotypes of F_1 progeny, S – susceptible genotypes of F_1 progeny, M – the leader λDNA/*Eco*47I/*Ava*II

Table 1. Segregation of infection tests and PCR markers in evaluated F_1 progenies and their statistical evaluation

Crossing	Real ratio of segregation		Theoretical ratio of segregation		Critical value of *t*-test	Value of *t*-test	*P*
	infection test		infection test				
	resistant plants	susceptible plants	resistant plants	susceptible plants	$\alpha = 0.05$		
Ornella × Mira	198	167	1	1	1.96	1.11	0.95
Tábor × Mira	110	113	1	1	1.96	0.47	0.95
	PCR marker		PCR marker				
	presence 760 bp band	absence 760 bp band	presence 760 bp band	absence 760 bp band	$\alpha = 0.05$		
Ornella × Mira	205	160	1	1	1.96	1.63	0.95
Tábor × Mira	112	111	1	1	1.96	0.28	0.95

Presumptive genotypes: Ornella: *h1h1h1h1*, Tábor: *h1h1h1h1*, Mira: *H1h1h1h1*

All seedlings that were in the model hybridisation were used in infection test. Ratios between resistant and susceptible genotypes are described in Table 1.

Linear regression and correlation analysis have characterised the closeness of dependence between presence of 760 bp PCR marker and resistance of seedlings against the Ro1 detected by infection test. In both cases, there was very high correlation between presence of marker of dominant allele of major gene *H1* and resistance detected by infection test. The index of correlation in F_1 progeny of Ornella × Mira was $r = 0.962$ and in Tábor × Mira was $r = 0.964$.

Described hybrid progenies were used for PCR detection of dominant allele of *H1* as well. Segregation of optimised PCR marker is described in Figure 2.The ratio between presence and absence of PCR marker in studied F_1 progenies is shown in Table 1.

The dominant *H1* allele controlled resistance indicates in simplex configuration in the tetraploid potato genome. The chosen PCR marker is not able to concretise a number of dominant alleles in studied locus. For this reason the statistical analyse of segregation ratios by *t*-test according to Myslivec (1957) was completed. Obtained segregation ratios that describe segregation of resistance in infection test and segregation of PCR markers were compared with theoretical ratios of segregation of F_1 progeny, when the resistant parent could prove the resistance determined in simplex (*H1h1h1h1*), duplex (*H1h1h1h1*), triplex (*H1h1h1h1*) and quadruplex (*H1h1h1h1*) composition. Only for the simplex determined resistance of Mira was detected significance of *t*-test on the value $\alpha = 0.05$. This test has confirmed that there it is possible to characterise the resistance of Mira as simplex determined in probability $P = 0.95$. Detailed statistical evaluation is described in Table 1.

DISCUSSION

The possibility of using PCR markers for detection of dominant allele *H1*, which controls the resistance against Ro1 pathotype *G. rostochiensi* was unequivocally confirmed by this experiment. Breeding of nematode resistant potatoes can be considered as effective strategy of protection. This fact is supported by the reality that there was 75% cultivars with declared resistant against Ro1 pathotype *G. rostochiensis* in the List of cultivars registered in the State Book of Cultivars in 2001.

This work presents results of experiments leading to application of DNA markers, which make the production of new resistant cultivars of potatoes more effective. The kit GenElute Plant Genomic DNA Kit (Sigma, SRN) was used for isolation of DNA, which allows performance of DNA isolating from different parts of plants.

Conditions of amplification according to Niewöhner et al. (1995) were not proved as suitable for conditions in laboratory of The Czech University of Agriculture in Prague. Parameters of PCR, which the author used, did not allow amplifications of any fragments. By means of back-cross optimisation tests concentration of some components of reaction mixture was adapted. Concentration of genomic DNA was increased for 20 ng in volume 25 µl, concentration of dNTP was increased six times, and concentration of $MgCl_2$ was increased 1.7 times. Concentration of *Taq* polymerase was conversely decreased for 0.3 U in 25 µl reaction. The reason of these changes was probably other type of used *Taq* polymerase and other strategy of DNA isolation.

By using couple of specific primers value T_m 56°C was determined. For calculation of T_m method according to Sambrook et al. (1989) was used. Annealing temperature (60.6°C), which Niewöhner et al. (1995) recommended did not allow amplification of PCR fragments with size 760 bp. This fact was obviously induced by disharmony between values of T_m and used annealing temperature. Decrease of annealing temperature to 51.0°C allowed specifics amplification of desiderative PCR marker. In set cultivars with declared resistance or sensibility against Ro1 pathotype *G. rostochiensis* was confirmed that the decrease of annealing temperature did not influence the specificity of used PCR marker. For example De Jong et al. (1997) in

studying of potato genome has used lower annealing temperature to value T_m of specifics PCR primers. Necessary changes of amplification profile were induced by modified composition of reaction and by technical parameters of used thermocycler.

In set of model genotypes, it was confirmed that elected optimisation has led to highly sensitive and reliable detection of dominant allele of *H1* gene in tetraploid potato genome. This reality was confirmed by analyses of regression and correlation, which was undertaken F_1 generations genotypes. For optimisation of experiments an analogous assortment of world potato cultivars was purposely chosen, the same as used by Niewöhner et al. (1995). It was proved, that amplification of 760 bp PCR fragment markering the resistance of potato against Ro1 pathotype *G. rostochiensis* has run in the same genotypes.

Based on these informations the model crossings of resistant cultivar Mira with susceptible cultivars Ornella and Tábor were carried out. The objective of this hybridisation was evaluation of the allele composition of *H1* of resistant strain Mira. Parental components Ornella and Tábor declared susceptibility to Ro1 pathotype of *G. rostochiensis* and for this reason the allelic composition in *H1* locus is (*h1h1h1h1*). The PCR marker described according to Niewöhner et al. (1995) is dominant and is able to detect only dominant *H1* allele in tetraploid potato genome. The marker does not allow distinguishing the simplex, duplex, triplex and quadruplex configuration of dominant *H1* allele. Forasmuch as that is very advantageous for breeders to know compositions of allele *H1* gene, two bulked analyses were made. Standard bulked tests and their combination with infection tests were described by several authors (Huijsman 1955, Pineda et al. 1993). Parallel evaluation of F_1 progeny segregation of described crossings by using of infection tests and using of modified PCR marker according to Niewöhner et al. (1995) were made in this study. The statistical analyses confirmed at the level $\alpha = 0.05$ that the segregation of infection tests and PCR marker correspond with model *H1h1h1h1* × *h1h1h1h1*. The donor of the dominant *H1* allele was predominantly cultivar Mira. Small differences between ratios of segregation of infection tests and PCR marker that are shown in Table 1 can be explained by interaction of genotype of resistant plant and external conditions. These conditions could affect accuracy of appraisal of resistance (susceptibility respectively) of evaluated plant. The possible mistake of evaluation refers for sample Bartoš (1991), Becker (1993) and Odenbach and Sacristan (1997).

The molecular-genetic part of this work is the model of the Marker Assisted Selection's (MAS) application in potato breeding to qualitative resistance against Ro1. The method allows view resistance only on the base of evaluation of genetic information, which is not encumbered by influences of external environs.

This study was sponsored by projects NAZV – MZe ČR – EP9107 Analysis of donor of resistance against *Globodera* sp. in potato's genome by means of DNA markers, FRVŠ G4 – 1347/2001 Detection of genes of potato's resistance against genus *Globodera* nematodes and MSM 412100002 Stabilizing and limiting factors of yield formation and quality of crop production.

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Received on May 14, 2002

ABSTRAKT

Segregace DNA markerů rezistence bramboru (*Solanum tuberosum* ssp. *tuberosum* L.) vůči Ro1 patotypu *Globodera rostochiensis* ve vybraných F_1 generacích

Selekce s využitím DNA markerů je rychlou a objektivní metodou detekce konkrétních majorgenů rezistence. Tuto metodu lze použít rovněž pro identifikaci některých kandidátních genů polygenně determinované rezistence. Genetické markery založené na polymerázové řetězové reakci byly použity pro hodnocení vybraných F_1 generací křížení Ornella × Mira a Tábor × Mira, kde odrůdy Ornella a Tábor mají deklarovanou senzitivitu a odrůda Mira rezistenci vůči Ro1 patotypu *G. rostochiensis*. Izolace DNA byla provedena z listových terčíků semenáčků kultivovaných ve skleníku s využitím GenElute Plant Genomic DNA Kit (Sigma, SRN). Pro segregační analýzu byl použit PCR marker majorgenu *H1* (Niewöhner et al. 1995) o velikosti 760 bp. U všech analyzovaných genotypů byly provedeny standardní biologické infekční testy s Ro1 patotypem *G. rostochiensis* dle Potočka (1987). U genotypů F_1 generace byly stanoveny štěpné poměry charakterizující segregaci DNA markerů i segregaci v rámci biologických infekčních testů. Obě metody stanovení rezistence bramboru byly statisticky porovnány korelační analýzou. U obou křížení byly nalezeny těsné korelace mezi výskytem PCR markeru dominantního majorgenu *H1* a rezistencí detekovanou infekčním testem. U F_1 generace kříženců Ornella × Mira byl zjištěn korelační koeficient $r = 0{,}962$ a u kříženců Tábor × Mira $r = 0{,}964$. Rovněž bylo provedeno statistické porovnání skutečných štěpných poměrů infekčních testů a DNA markerů s předpokládaným štěpným poměrem simplexně a duplexně determinované rezistence řízené dominantním majorgenem *H1*. Statistickou analýzou bylo potvrzeno, že použitý rezistentní genotyp (odrůda Mira) je donorem simplexně založené rezistence.

Klíčová slova: brambor; *Solanum tuberosum* ssp. *tuberosum* L.; DNA markery; PCR; Ro1 patotyp *Globodera rostochiensis*; rezistence; majorgen *H1*; segregační analýza

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