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Usefulness of RAPD, RFLP and SCAR molecular markers and *AGPaseB* gene methylation level in the screening of resistance to the golden cyst nematode (*Globodera rostochiensis*) pathotype Ro1 in different Polish potato genotypes

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Abstract

The aim of this study was to assess the application of various DNA markers as well as the ADP-glucose pyrophosphorylase small subunit (*AGPaseB*) gene methylation for the screening of potato cultivars and breeding lines with different resistance to *Globodera rostochiensis*. Tetraploid genotypes were included. The 2 kb and 0.7 kb random amplified polymorphic (RAPD) fragments as well as a PCR-amplified 1.6 kb fragment of *AGPaseB* gene positioned close to the *Gro1* locus were tested. Two novel sequence characterised amplified region (SCAR) fragments were generated from 2 kb RAPD product of susceptible and resistant genotypes. Significant correlation (r=0.32) was detected only between the presence of SCARI and SCARII fragments markers in all the investigated genotypes. Three different allelic forms of the *AGPaseB* gene were detected in tetraploid potato genotypes. One of these allelic forms may be closely linked with *Gro1*. A strong methylation occurred at promoter region and the 5' part of *AGPaseB* gene which was generally absent at its 3' UTR region. The methylation pattern of *AGPaseB* investigated by restriction with *MspI* and *HpaII* varied between some *G. rostochiensis* resistant and sensitive genotypes. We concluded that methylation profiling of *AGPaseB* gene should be promising for the development of practical assays for resistance to nematodes in potato.

Keywords: Allelic forms; breeding; *Gro*; marker selection; pest resistance; *Solanum tuberosum.*
Abbreviations: AFLP_amplified fragment length polymorphism; *AGPaseB*_ADP-glucose pyrophosphorylase small subunit gene; BSA_bovine serum albumin; cv(s)_cultivar(s); HELP_*HpaII* tiny fragment enrichment by ligation-mediated PCR; Indels_Insertion/deletion; PVP_polivinylpyrrolidone 40; RAPD_random amplified polymorphic DNA; RFLP_restriction fragment length polymorphism; RLGS_restriction landmark genome scanning; SCAR_sequence characterised amplified region; SDS_sodium dodecyl sulphate; SSC_NaCl- sodium citrate buffer; TAE_Tris- acetic acid- ethylenediamine tetraacetic acid buffer.

Introduction

The golden cyst nematode (*Globodera rostochiensis*) and white cyst nematode (*G. pallida*) are both serious quarantine pests of cultivated potato (*Solanum tuberosum* ssp. *tuberosum*). The discovery of nematode resistance genes in several wild diploid potato species and their successful transfer to the cultivated tetraploid potato is an important achievement in modern resistance breeding. Nevertheless, the inheritance of nematode resistance is complex. According to recent data (Finkers- Tomczak et al., 2011), 14 potato cyst nematode resistance gene loci have been mapped on eight linkage groups in diploid or tetraploid potato genotypes. Four resistance loci (*H1*, *GroVI*, *Gro1* and *Gpa2*) are responsible for near-immunity to one or more nematode pathotypes, whereas some others participate in the penetration of partial resistance (Bakker et al., 2004). The inheritance of Ro1 nematode resistance is polyallelic (Jacobs et al., 1996). A major Ro1 resistance gene originating from *S. spegazzinii* is localised to the *Gro1* locus on chromosome VII (Barone et al., 1990; Gebhardt et al., 1993). *Gro1* is inherited as a single dominant allele conferring resistance to the *Globodera rostochiensis* pathotypes Ro1 (most common in Polish potato genotypes) and Ro5. However, some members of *Gro1* family may also be functional as nematode *R* genes and influence resistance to pathotypes other than Ro1 (Paal et al., 2004). Several molecular markers linked to *Gro1* have been identified mostly based on random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism

(AFLP) assays (Kreike et al., 1993; Ballvora et al., 1995; Paal et al., 2004).

The aim of the present study was to compare the association between the categorisation of Polish tetraploid potato genotypes by means of a biological test of resistance to *G. rostochiensis* and a few DNA fragments amplified with markers flanked or close to the *Gro1* locus. Most studied cultivars (cvs) as well as breeding lines were new and not investigated previously by molecular markers. In addition, we intended to estimate whether the methylation pattern of one of the analysed fragments (*AGPaseB* gene) is altered in resistant genotypes.

Results

RAPD and SCAR analyses

Our previous results showed that the application of *H1*-linked markers with the resistance to Ro1 pathotype of *G. rostochiensis* appeared promising for the fast screening of potato cvs and breeding lines (Galek et al., 2011). In the present approach, fifty-eight potato cvs. and breeding lines-resistant and susceptible to *G. rostochiensis* (Supplementary Table 1.) were used for the screening of two DNA fragments amplified with markers linked with *Gro1* allele, 2 kb RAPD and 0.7 kb (Ballvora et al., 1995) fragments. It appeared that 2 kb RAPD fragment was presented in 60% of resistant cvs. and in 52% of resistant breeding lines, and also in 31% of susceptible genotypes (Table 1; Fig 1a). Partial sequences of 2kb RAPD product of cvs. 'Desirée' and 'Bóbr' were registered at GenBank under accession numbers KC818429 and KC818430. The 5' terminal sequences obtained from 2kb RAPD product from cvs. 'Desirée' and 'Bóbr' were 269 and 453 bps in length; and in 3' terminal sequences, 458 and 498 bp, respectively. They contained *OPI19* primer at both ends. The sequence of the 2kb RAPD product from the two potato cvs. showed two nucleotide substitutions and two insertion/deletion (indels) between aligned 5' regions and two other substitutions, accompanied by a single indel between aligned 3' regions. A BLAST search revealed that they contain regions homologous to *S. lycopersicum* N-acetyl-D-glucosamine kinase-like (LOC101249937) mRNA (accession no. XM004243575) at positions 545 to 739 of KC818429 and 215 to 430 and 769 to 963 of KC818430, respectively. The gene encoding for this protein is located on chromosome VII, which confirms the localisation of RAPD locus (Ballvora et al., 1995).

In attempt to detect correlation between the resistance and marker penetration, the SCAR markers were developed from 2kb RAPD product by taking into account the differences in the nucleotide sequence of 2kb RAPD product between cvs. 'Desirée' and 'Bóbr'. As 2kb RAPD appeared both in resistant and susceptible cultivars, we developed respective SCAR markers such as SCARI from the susceptible cv. 'Desirée' and SCARII from the resistant cv. 'Bóbr'. SCAR I was present in 73 and 96% of resistant cvs. and breeding lines (i.e. 88% for all resistant genotypes), respectively, whereas SCARII was present in 80% of resistant cvs. and 81% of breeding lines (81% for all resistant genotypes). Both SCAR I and SCAR II were also distinguished in 75% of susceptible cvs. and 83% of breeding lines (i.e. 81% for all susceptible genotypes) and 75% of all the analysed genotypes contained both markers (Table 1.).

In the present study the next RAPD amplified fragment (0.7 kb) is linked with a part of chromosome VII; therefore, it was also included in the analysis. This product appeared in potato resistant cvs. and breeding lines with a relatively high frequency (87 and 52%, respectively). However, its presence among susceptible genotypes was even higher in breeding lines (Table 1.).

The statistical analysis did not reveal any significant association between the resistance of genotypes to *G. rostochiensis*, assayed by biological tests, and the presence of all the investigated RAPD fragments, because the correlation coefficient values were very low. However, a small, but significant association (0.32) was detected between the presence of SCARI and SCARII fragments in all the genotypes (Supplementary Table 2.).

PCR analysis of RFLP locus of AGPaseB (a)

Using a specific primer pair, we were able to amplify *AGPaseB (a)* locus (named after the protocol used in Ballvora et al. 1995) in various potato genotypes. The presence of a 1.6 kb fragment of *AGPaseB* gene was assayed due to the fact that this fragment, along with 2kb RAPD product, was linked with resistance in diploid potato (Ballvora et al., 1995). The 1.6 kb fragment of *AGPaseB* gene was present in 55% of tested resistant (and in 25% of susceptible) cvs and in 50% of breeding lines (Table 1; Fig 1.b). Due to the relatively low number of tested genotypes, no significant correlation between the resistance to *G. rostochiensis* and the presence of 1.6 kb fragment was detected (Supplementary Table 2.).

AGPaseB methylation

For the estimation of the methylation pattern of *AGPaseB* gene we applied a further restriction fragment length polymorphism (RFLP) analysis using genomic DNA from 7 resistant and 2 susceptible genotypes restricted with *MspI* and *HpaII* enzymes with differential sensitivity to methylated DNA regions. Unlike *HpaII*, *MspI* cuts methylated 5'Cm^5CGG3'restriction sites. A longer *HpaII* restriction fragments could be expected, where the *AGPaseB* methylated. Results of Southern hybridisation are shown in Fig 2. We noticed three main hybridisation signals (0.3, 0.7 and 0.9 kb), when the 1.6 kb probe (exon 6- exon 9) for *AGPaseB* gene fragment of resistant breeding line no. PS-646 was used. This indicates the presence of at least three different allelic forms of ADP- glucose pyrophosphorylase in all the analysed genotypes of tetraploid potato. However, the same hybridisation pattern observed for *HpaII* and *MspI* restriction fragments, suggests a general lack of methylation of the 3' part of *AGPaseB* gene. Nonetheless, some additional polymorphic bands of higher molecular mass and often weaker intensity (denoted by arrows in Fig 2.a) than the adjacent hybridisation ones resulted from the altered methylation profile of *AGPaseB* gene only in three potato genotypes (S-39403, Ceza and K.95-3227), in which two of them (Ceza and K.95-3227) were susceptible. The second genotype was obtained from the crossing of PS-646 and 'Ceza'. This indicates that the inheritance of *AGPaseB* methylation profile depends on the origin of progeny in some crosses (Supplementary Table 1). The application of another 0.7 kb hybridisation probe generated from line no. PS-646 (promoter region, exon 1 and intron 1), revealed that, in general, strong methylation occurs at the promoter region

Table 1. The percentage of potato genotypes with the presence of the analysed DNA fragment.

Genotypes	R/S	Genotype no.	2 kb RAPD	SCARI	SCARII	1.6 kb *AGPase B*	0.7 kb RAPD
Breeding lines	R	27	52	96	81	50	52
	S	12	17	83	83	100	67
Cvs	R	15	60	73	80	55	87
	S	4	75	75	75	25	100
All genotypes	*R*	*42*	*55*	*88*	*81*	*53*	*64*
	S	*16*	*31*	*81*	*81*	*60*	*75*

All the genotypes were categorised either as resistant (R) or susceptible (S). The presence of PCR-based markers was indicated in percentage (%). The number of analysed genotypes within each category is also given. The low number of genotypes analysed for the presence of the *AGPaseB* gene fragment resulted in the appearance of that fragment in all the analysed susceptible cvs. and breeding lines.

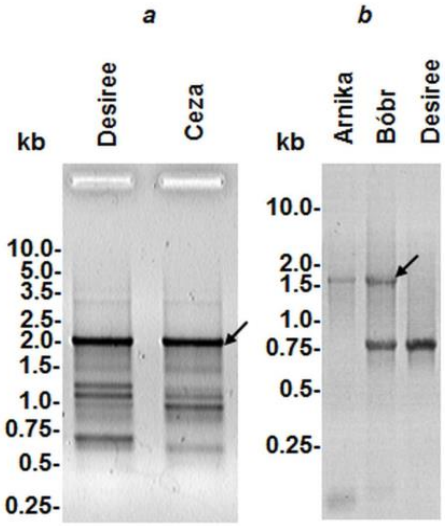

Fig 1. Electrophoretical pattern (on 1% agarose gel) of chosen analysed markers among different potato genotypes. PCR amplification of (a) 2 kb RAPD marker and (b) 1.6 kb *AGPaseB* fragment. As the calibrator of DNA fragments size, Gene Ruler 1 kb DNA Ladder (Fermentas) was used. The size of DNA fragments was indicated in kb. The arrows indicate the DNA fragments used as the investigated markers.

and the 5' part of this gene, and that some polymorphic *HpaII* fragments indeed varied among the tested genotypes (Fig 2.b). For instance, some susceptible genotypes ('Ceza', K.95-3227) showed doubled *HpaII* hybridisation signals, whereas some resistant ones (S-39392 and S-39399) revealed single signals.

Discussion

Study design

So far, mainly 3-year biological tests have been used for the assessment of resistance of potato genotypes to *G. rostochiensis*. Currently, application of alternative methods such as molecular markers for assessment of reaction of potato cultivars to nematode invasion in early stages of the breeding process is one of the most important challenges for breeders. The use of molecular markers for genotype selection could significantly accelerate the development of new varieties. The screening of genotypes resistant to the Ro1 pathotype of *G. rostochiensis* may be based either on single or multiplexed PCR markers (Galek et al., 2011; Milczarek et al., 2011; Milczarek, 2012). Experiments performed with molecular markers by Kreike et al. (1993) proved that resistance to the pathotype Ro1 from diploid *S. spegazzinii* is quantitatively polygenic. We made an attempt to verify the applicability of chosen genomic DNA fragments amplified with molecular markers for the quick screening of *G. restochiensis* resistance and susceptibility in novel plant material. The tests involved Polish tetraploid potato genotypes which had not been investigated previously for the presence of DNA fragments amplified by all RAPD and SCAR markers of our interest (Galek et al., 2011; Milczarek, 2012). We based our study on the results obtained from a diploid mapping population. The presence of 2 kb and 0.7 kb RAPD markers was previously evaluated by Barone et al. (1990) and Ballvora et al. (1995).

Factors influencing the performance of PCR-based markers

The broad spectrum of *G. rostochiensis*-resistance genes introgressed into the cultivated potato often brings false positive results for newly investigated genotypes with unknown genetic background (Chełkowski and Stępień, 2001). In searching for successful markers, every study on the identification of proper markers is important. Markers which were identified on the basis of mapping populations different from the tested gene pool of our plant material may have presented a limited quality for nematode resistance genotyping, especially when they were transferred from diploid potato to tetraploid cvs. and breeding lines. Not all the markers developed by Barone et al. (1990) in diploid material were successful in the study provided by Ballvora et al. (1995), even among diploid lines. Indeed, this also happened in our study. For instance, 2kb RAPD fragment seemed to be promising in the resistant genotype selection. However, it was present also in some susceptible genotypes. Ballvora et al. (1995) noticed that the presence of RAPD molecular markers is connected with the repulsion of the *Gro1* resistance allele, which contains hot-spots for mutations. All these factors make the discussion of our results challenging. Moreover, the differences in the nucleotide sequence of 2 kb RAPD product from the analysed resistant and susceptible genotypes may be associated with putative high recombination events of the whole genomic region. Together with 2kb fragment amplified with the RAPD marker, SCAR products appeared to be of very low quality for the genotyping

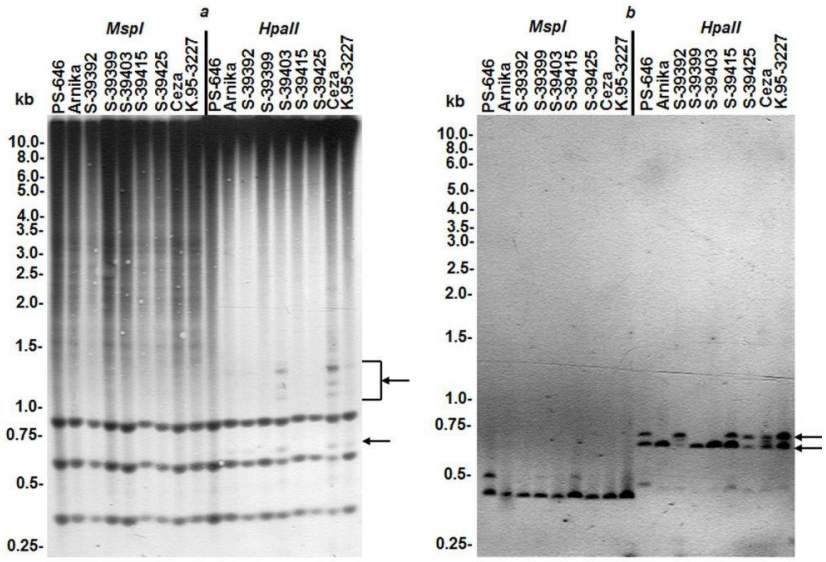

Fig 2. Southern blot hybridisation of total DNA from different potato genotypes with (a) 1.6 kb *AGPaseB* fragment or (b) 0.7 kb *AGPaseB* fragment used as hybridisation probes. Restriction enzymes used for DNA digestion are indicated (*MspI, HpaII*). As the calibrator of DNA fragments size, Gene Ruler 1 kb DNA Ladder (Fermentas) was used. The size of DNA fragments was indicated in kb. Polymorphic fragments are indicated by arrows.

of potato materials, because they were present in resistance and susceptibility genotypes. This might have been due to putative recombination events, in which regions amplified by SCAR primers are engaged; and/or PCR amplification of heterogeneous loci in tetraploid potato genotypes. The presence of a positive correlation between two SCAR fragments may reflect the fact that they descend from very similar 2kb RAPD products (despite the differences discussed above) from two potato genotypes which displayed contrasting sensitivity to *G. rostochiensis*.

Applicability of methylation profiling of AGPaseB gene

In addition, we estimated whether the methylation pattern of one of the analysed markers (*AGPaseB* gene fragment) is altered in resistant genotypes. The importance of genomic methylome for potato breeding programs was recently highlighted by the discovery that the level of methylation heterozygosity may be connected with heterosis (Nakamura and Hosaka, 2010). The methylation pattern of 5' part of the *AGPaseB* sequence varied at least between some *G. rostochiensis* resistant and sensitive genotypes. Hence, it appeared more promising for the genotyping of the plant material used in our study than PCR-based assays. Further attempts should be undertaken in order to use hybridisation probes specific to various allelic forms of the analysed gene (Fig 2a). Interestingly, one of these allelic forms may be expected to be more closely linked with *Gro1* than the currently investigated probes. It is also known that methylation of promoter regions may affect gene transcriptional activity and that epialleles may be inherited, leading to plant environmental adaptations (Zhang et al., 2010). In the future, it would be worth checking whether the level of *AGPaseB* transcript accumulation between potato genotypes correlates with their resistance to *G. rostochiensis.* It is important to evaluate the methylation extent in the genome of the investigated material more accurately by using alternative methods, including detection of methylation-sensitive RAPD products (Nakamura and Hosaka, 2010), restriction landmark genome scanning (RLGS) or *HpaII* tiny fragment enrichment by ligation-mediated PCR (HELP).

Materials and Methods

Plant material and genomic DNA isolation

The whole material obtained was initially screened by breeders and the number of resistant as well as susceptible genotypes was scored. The biological tests in the pots were conducted in 3 consecutive years according to the procedure for screening all breeding lines on the early potato breeding stage, fulfilled at the Laboratory of the Plant Breeding and Acclimatisation Institute in Bydgoszcz (Stefan and Malinowska, 2000). For the presence of mature cysts, roots of plants were visually scored and the number of cysts was calculated. Plants with 3 or less cysts were considered resistant. Plants showing more than 3 cysts were considered susceptible. The number of plants used for the analysis ranged from 5 to 40 per given genotype, depend on the number of available tubers for performing biological tests (Stefan and Malinowska, 2000). Nineteen potato cultivars and 39 breeding lines originating from Strzekęcin, Krokowa and Dybowo Polish Plant Breeding Stations were evaluated using PCR-based markers.

The plant material for our study was propagated at the Experimental Station of Wroclaw University of Environmental and Life Sciences (Swojec, Poland) under field conditions. Genomic DNA was extracted from flash-frozen young leaves collected from 3-month-old plants growing in the field, using DNA Plant MiniKit (Qiagen) or Junghans and Metzlaff (1990) protocols.

PCR- based assays

The analyses were performed at two independent labs on Hybaid and Biometra thermocyclers, at least three times, using 20 ng total DNA, 0.25 mM dNTPs, 0.45 μM each primer, and 1 U *Taq* DyNAzyme polymerase in the respective reaction buffer (Thermo Fisher Scientific)

containing 1.5 mM $MgCl_2$ in 20 µl total volume. A 2 kb RAPD product amplification with *OPI19* primer (5'AATGCGGGAG3'; Ballvora et al., 1995) was performed using the following thermal profile: 97°C, 7 min; next: 45 cycles of 95°C, 1 min, 35°C, 1.5 min and 72°C, 2.5 min. The last step included 72°C for 5 min. The 0.7 kb RAPD marker was localised with 2 kb marker on chromosome VII close to *Gro1* locus. It was amplified with *OPR10* primer (5'CCATTCCCCA3'; Ballvora et al., 1995). The PCR conditions were the same as above, except the initial denaturation step (95°C), which lasted 7 min and the last step at 72°C – 10 min. The 2 kb RAPD product from two potato cvs ('Desirée' and 'Bóbr') was eluted from the agarose gel by Qiaex II Gel Extraction Kit (Qiagen), cloned into pGEM-T Easy (Promega) vector and bi-directionally sequenced employing the Big Dye Tv 3.1 sequencing reaction kit (Applied Biosystems) with the appropriate primer, and run on an automatic capillary sequencing system (ABI Prism 31.30 XL, Applied Biosystems). The nested primers were designed for the subsequent amplification of sequence characterized amplified region (SCAR) markers from 2 kb RAPD products of cvs. 'Desirée' and 'Bóbr'. The 1.5 kb SCAR I fragment from the susceptible cv 'Desirée' was amplified with the primers: forward 5'AAATGGCCAATAACAAAGT3' (nucleotide positions: 230 to 249 at KC818429 accession) and reverse 5'GGACATGGCTGGGGAATA3' (nucleotide positions: 553 to 570 at KC818429 accession) and the 1.5 kb SCAR II fragment, from the resistant cv 'Bóbr' with another primer pair: forward 5'TTGGTTATCGAGGGTACG-AAGAGA3' (nucleotide positions: 12 to 35 at KC818430 accession) and reverse 5'AATCAAGGGAAAGAAACTTA-GCGG3' (nucleotide positions: 740 to 763 at KC818430 accession). The thermal profile was as follows: 95°C, 7 min; next: 30 cycles of 95°C, 1 min, then 55°C, 1 min for SCAR I or 51°C, 1 min for SCAR II and 72°C, 1 min. The last elongation included 72°C for 10 min. The conditions for the amplification of 1.6 kb fragment of ADP-glucose pyrophosphorylase small subunit gene (*AGPaseB*; nucleotide positions: 3732 to 5409 at L36648 accession), encoding components of enzyme converting glucose-1-phosphate into ADP-glucose during starch biogenesis were the same as described by Ballvora et al. (1995). However, for the amplification of *AGPaseB* gene 0.7 kb fragment (nucleotide positions: 601 to 1265 at L36648 accession) we used the primers: forward 5'TCCCCAAAATAGCCTCAATCA3' and reverse 5'AAACCGCCACAATCCGACAA3', and the thermal profile: 96°C, 5 min; next: 30 cycles of 95° C, 1 min, 56°C, 1 min and 72°C, 1.5 min. The last step included 72°C for 10 min. PCR products were separated on 1 to 1.5 % agarose gels in Tris-acetic acid-ethylenediamine tetraacetic acid (TAE) buffer and visualised with ethidium bromide.

Methylation analysis

15 µg of total genomic DNA from 7 resistant and 2 susceptible genotypes (denoted by asterisks in Supplementary Table 1. and listed below the table summary) restricted with *MspI* and *HpaII* was fractionated on 0.8% agarose gel and transferred onto Hybond N^+ membrane (GE Healthcare) by capillary method using 20x NaCl/ sodium citrate buffer (SSC, pH 7.0; Sambrook and Russell, 2001). After UV-crosslinking of DNA fragments to the membrane, filters were submitted for the pre-hybridisation step at 42°C for 1h. Hybridisation, in the presence of the denatured 1.6 kb or 0.7 kb *AGPaseB* amplification product used as the probe ^{32}P-labeled by random priming, lasted 16 h at 42°C. The pre-hybridisation and hybridisation mixture contained 6x SSC (pH 7.0), 0,1% Ficoll 400, 0.1% polivinylpyrrolidone 40 (PVP-40), 0.1% bovine serum albumin (BSA), 100 µg mL^{-1} salmon sperm DNA, and 0.5% sodium dodecyl sulphate (SDS). Afterwards the membranes were washed in 2x SSC (pH 7.0), 0.1% SDS for 5 and 15 min at room temperature and then in 0.1x SSC (pH 7.0), 0.5% SDS for 1h at 37 °C and for 30 min at 42°C. Finally, membranes were washed in 0.1x SSC (pH 7.0) for 10 min at room temperature and exposed to RTG film.

Statistical analysis

To show the relationship between the results of biological tests and typical fragments amplified by chosen molecular markers, correlation coefficient values were calculated. Each amplified fragment was treated as a unique character and scored in terms of binary code (1/0=+/-). Calculations were done using STATISTICA (v 10.0) software.

Conclusion

Currently developed RAPD and SCAR markers that seem to be associated with important genetic traits do not always work well on breeding material with diverse genetic background. Moreover, many alterations at the genomic level may occur during breeding programs connected with the introgression of genes. Based on the results of present study, either the development of distinct mapping populations (based on crosses including presently investigated breeding lines) or even the application of association subsequent mapping would be required for the accurate genotyping of Polish potato genotypes. From our study, methylation profiling of *AGPaseB* gene seems to be more promising for the first recognition of Polish potato genotypes resistant to nematodes. Further work is needed to find more successful markers closely related to the genes conferring resistance to Ro1 pathotype of *G. rostochiensis*.

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