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Research Paper

Studies of potato resistance to *Globodera rostochiensis* revealed novel alleles for 57R marker

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Globodera rostochiensis resistance has been an important trait in potato (*Solanum tuberosum*) breeding for decades. Our aim was to complement phenotypic testing with genetic marker analysis. We analysed the results of *G. rostochiensis* resistance greenhouse testing in 4601 tubers of 2918 breeding clones from 11 years. Applicability of *H1* gene markers TG689 and 57R was compared. We implemented the latter with the positive predictive value of 99.1% and negative predictive value of 60.0% into the breeding scheme. The 57R marker alleles of 22 Estonian cultivars and 470 breeding clones were determined. Two unique 57R alleles, 57R-887 and 57R-1155, were found in Estonian cultivar 'Anti'. The 887 bp allele has two deletions (14 bp and 490 bp) accompanied by several other indels and SNPs within the 57R marker region. The 1155 bp allele has three deletions (7 bp, 20 bp and 210 bp) accompanied by several other indels and SNPs within the same region. Partial resistance to *G. rostochiensis* in 'Anti' suggests that the newly described alleles could affect the *H1*-mediated resistance directly or indirectly.

Key Words: *Globodera rostochiensis*, *H1* locus, *Solanum tuberosum*, 57R marker, TG689 marker, marker-assisted selection.

Introduction

The potato cyst nematodes (PCN) *Globodera rostochiensis* and *Globodera pallida* are economically very important potato crop pests worldwide. PCNs infest roots of the plant resulting in significant crop yield reduction. The two species of PCNs are divided into eight pathotypes (Ro1 to Ro5 of *G. rostochiensis* and Pa1 to Pa3 of *G. pallida*). Main damage occurs due to infestation with *G. rostochiensis*, the golden cyst nematode, with pathotype 1 (Ro1) being the most widely spread in temperate regions (Schultz *et al.* 2010).

Crop rotation is not an effective way to avoid infestation, because dormant cysts of nematodes can survive for many years in the soil and hatch in the presence of the suitable host. It is possible to use pesticides to protect crops from nematode infestation, however, this method poses danger to the environment and use of many of these compounds is forbidden in the European Union (The Commission of the European Communities 2007, The Council of the European Union 2003). Farm to Fork strategy of EU's Green Deal aims to reduce the use of plant pesticides by 50% by 2030 (European Comission 2020). Brodie has reported a reduction in the number of *G. rostochiensis* populations in soil after two successive years of cultivating a resistant potato cultivar (Brodie 1996). Therefore, growing resistant potato cultivars is the most effective, promising and environmentally friendly method for nematode control. Great emphasis is currently placed on breeding cultivars resistant to a broad spectrum of PCN populations.

Approximately 17 genes and loci have been linked to PCN resistance in potato (Ramakrishnan *et al.* 2015). Three genes, *H1*, *GROVI*, *GRO1-4*, provide near-absolute resistance against *G. rostochiensis* (Finkers-Tomczak *et al.* 2011, Milczarek *et al.* 2021, Paal *et al.* 2004). The *H1* gene was discovered in 1952 and has since then been actively introgressed into commercial potato cultivars (Toxopeus and Huijsman 1953). This gene provides near-absolute resistance to pathotypes Ro1 and Ro4 (Bakker *et al.* 2004). PCR-based markers that have been developed for *H1* detection include TG689, TG689_1P, 57R, N146, N195 and CP113 (Meiyalaghan *et al.* 2018, Ramakrishnan *et al.*

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2015, Schultz *et al.* 2012).

Marker TG689 has been widely used for *H1* detection. A number of studies have demonstrated high congruence between the presence of TG689 resistance-associated allele and the phenotypic nematode resistance (Biryukova *et al.* 2008, Milczarek *et al.* 2011, Schultz *et al.* 2010). Another *H1*-associated SCAR marker 57R is closely linked to the resistance gene and has demonstrated near complete concordance between phenotype and genotype (Schultz *et al.* 2012). Both of the aforementioned markers have been previously compared in different sets of breeding clones and cultivars (Park *et al.* 2018, Schultz *et al.* 2012). The ability of either marker to predict resistance was shown to be high —99.7% and 98.3% for 57R and TG689, respectively—but the ability to predict susceptibility was much lower, 47% and 41%, respectively (Park *et al.* 2018).

G. rostochiensis was detected in Estonia for the first time in 1953 (Sarv and Riispere 1980). Data on the population structure of this nematode in Estonia is scarce with last report dating back to 1979, when it was demonstrated that the most prevalent pathotype was Ro1 (Riispere and Ehamaa 1979). It is estimated that approximately 75% of Estonian potato fields are infested with PCN leading up to 30% potato yield loss (Krall and Luik 2000). PCN resistance breeding in Estonia was initiated already in 1960's and resulted in the first nematode resistant cultivars 'Ants' and 'Piret' released in 1992 and 2001, respectively (Krall and Luik 2000, Tähtjärv 2016).

G. rostochiensis resistance continues to be a desired trait also in modern breeding programmes. Phenotypic testing of breeding material is time-consuming, sensitive to environmental factors and requires prior vernalization of tubers. Genetic marker analysis has been shown to cost over 10 times less compared to artificial inoculation with PCN (Ortega and Lopez-Vizcon 2012). Implementing genetic testing for nematode resistance in Estonian potato breeding could reduce required time, optimise resource costs and improve selection accuracy. The aim of the current study was to select a genetic marker for *G. rostochiensis* resistance breeding and verify it in Estonian breeding clones and cultivars. Additionally, we analyse the results from 11 years of phenotypic PCN testing, compare applicability of the breeding strategies with and without the use of genetic markers, and describe new alleles of 57R marker.

Materials and Methods

Plant material

The first set of samples used in the analysis included 212 Estonian potato breeding clones. The clones had been preselected by the breeder based on morphological traits and greenhouse nematode resistance testing. They were tested for the presence of 57R and TG689 markers, and compared to phenotypic *G. rostochiensis* resistance data.

The second set of samples included 258 potato breeding clones, which were not pre-selected based nematode resistance in greenhouse. These clones were analysed with 57R marker and tested for *G. rostochiensis* resistance in the greenhouse.

A selection of 22 Estonian potato cultivars from the years 1934–2017 were tested with the markers TG689 and 57R. A selection of these cultivars (including 'Anti' and 'Juku') was also tested for *G. rostochiensis* resistance in the greenhouse. Additionally, we included 14 potato cultivars of interest to compare with 'Anti' from the data set published by Ivanova-Pozdejeva *et al.* (2021) and a breeding clone from the year 2019. The plant material for 22 Estonian potato cultivars as well as for 14 others was obtained from the *in vitro* potato collection maintained by the Department of Plant Biotechnology in METK.

The nematode resistance testing was conducted altogether on 2918 Estonian potato breeding clones, from 2010–2020. The 212 breeding clones from the first sample set and 258 breeding clones from the second sample set were also part of this total number of samples analysed for nematode resistance.

Phenotypic testing for G. rostochiensis resistance

The nematode resistance testing of 2918 breeding clones was conducted during a period of 11 years, from 2010–2020. In total, 4601 tubers were tested. The method of choice was adapted from Koppel *et al.* (1998) for its affordability, robustness and applicability in breeding selection. A single tuber was included for each genotype in the first testing year, and in the retesting phases two tubers were included. Retesting was performed for those genotypes that had no cysts or up to three cysts in the first testing year.

Soil infested with *G. rostochiensis* Ro1 pathotype was originally collected from an infested site in Jõgeva county, Estonia in the 1990s and subsequently propagated with susceptible genotypes in the soil and maintained at METK. Tubers were grown in the substrate containing one-third of infested soil as the bottom layer, covered with two-thirds of fresh soil as the upper layer. A single tuber was planted in a non-transparent 700 ml plastic pot in mid-May and grown in the greenhouse environment. Varieties of local relevance 'Juku', 'Anti', 'Vigri' or 'Sulev' were used as susceptible controls in every test. At least 10 replicates for the susceptible control variety were included. After 8–10 weeks, the plants were removed from the pots and the cysts were visually assessed and counted. For large numbers of cysts, the count was occasionally rounded to 100, 200, or 300. Clones with three or fewer cysts were considered 'resistant' and those with four cysts or more were considered 'susceptible'.

DNA extraction

Genomic DNA was extracted from fresh material of *in vitro* plantlets, tubers or leaves from the field using an adapted method described by Weigel and Glazebrook (2002) or by modified CTAB method (Doyle 1990). For the Weigel and Glazebrook method, plant material was crushed

by micropestle in a 1.5-ml tube filled with 600 µl DNA-extraction buffer (200 mM Tris pH 8.5, 250 mM NaCl). The tubes were centrifuged for 5 minutes at maximum speed. The supernatant was transferred into a clean 1.5-ml tube, followed by isopropanol precipitation of equal volume. For the modified CTAB method, plant samples were crushed by two 3 mm metal beads with Qiagen Tissuelyser II (30 Hz for 1 minute) in 2 ml tubes. CTAB buffer (2% CTAB (w/v), 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% PVP-40) was added, followed by incubation at 60°C for 30 minutes and then centrifuged for 5 minutes at maximum speed. The supernatant was transferred into a clean 1.5-ml tube and precipitated with an equal volume of isopropanol. For both methods DNA precipitation was done by centrifuging isopropanol-containing solution for 10 minutes at maximum speed, followed by washing with 70% ethanol and centrifuging for 5 minutes at maximum speed. The supernatant was discarded and dry DNA was resuspended in 50 µl of water.

PCR analysis

Two separate PCR reactions were run for TG689 and 57R markers with primers listed in **Table 1**. The reaction for TG689 also included primers for β-carotene hydroxylase (*BCH*) gene as the internal control (**Table 1**) (Brown *et al.* 2006, Milczarek *et al.* 2011, 2014). The amplification was performed in the final volume of 20 µl of reaction mix containing: 0.4 µM for 57R or 0.4 µM for TG689 primers and 0.2 µM for BCH, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 U FirePol Taq Polymerase (Solis Biodyne, Estonia) or 0.5–1 U DreamTaq Polymerase (Thermo Fisher Scientific, USA) and 1 µl of DNA (30–150 ng). Respective PCR annealing temperatures are also shown in **Table 1**. For the marker TG689 resistance is indicated by the presence of 141 bp product, while for the marker 57R by the presence of 450 bp fragment.

Additional analysis of breeding clones negative for both 57R and TG689 was made with *GRO1-4* gene-linked marker Gro1-4-1 (Asano *et al.* 2012). The primer sequences, fragment size and annealing temperature are indicated in **Table 1**. The marker was amplified in the final volume of 15 µl as a part of multiplex-PCR according to the programme described by Rogozina *et al.* (2019). The reaction contained 1X DreamTaq Green Buffer and 0.75 U of DreamTaq DNA Polymerase (Thermo Fisher Scientific, USA), 0.2 µM dNTPs, 0.4 µM of each forward and reverse primer and 1 µl of DNA (30–150 ng).

Sequencing and alignment of new 57R alleles from cv 'Anti'

The PCR fragments from cv 'Anti' were sequenced with 57R forward and reverse primers (**Table 1**) by Sanger sequencing at the core laboratory of Estonian Biocentre (Tartu, Estonia). Additional primers were designed to obtain better quality of 5′ and 3′ ends of the fragment (57R_seq_5prime: AGG TGT CCA ATT TCG TGT GC, 57R_seq_3prime: TAA TTT CCA CCC GCT GCA TG). The sequencing samples were either purified directly from agarose gel with FavorPrep GEL/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan) or cloned beforehand into *Escherichia coli* with CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA).

The newly sequenced 57R alleles were aligned with the 57R fragment from the susceptible haplotype RH89-039-16 retrieved from NCBI (AC239963) and 57R from the resistant haplotype SH83-92-488 retrieved from NCBI (HQ223091) (Finkers-Tomczak *et al.* 2011). Alignment was created using BioEdit (Hall 1999) and the MAFFT algorithm in Benchling ("Benchling [Biology Software]" 2021). The sequence of the susceptible haplotype was

Table 1. Primer names, nucleotide sequences, annealing temperatures, product sizes and references for the markers used in this study

Marker	Primer name	Primer sequence 5′-3′	Annealing T (°C)	Product size (bp)
57R[a]	57R_F	TGCCTGCCTCTC CGATTTCT	63	450 (R), 887 (57R-887), 1155 (57R-1155), 1393 (S)
	57R_R	GGTTCAGCAAAAGCAAGGACGTG		
57R-887[b]	57R_F_Anti	GGCCTGTGTACTATCGATAACCAAG	57	414 (57R-887)
	57R_R	GGTTCAGCAAAAGCAAGGACGTG		
TG689[c], internal control BCH[d]	TG689 forward	TAAAACTCTTGGTTATAGCCTAT	55	141 (R), 290 (internal control)
	TG689 reverse	CAATAGAATGTGTTGTTTCACCAA		
	BCH forward	CATGACATAGTTTGAATTTTGAGTC		
	BCH reverse	CGTTTGGCGCTGCCGTAAGTT		
Gro1-4-1[e]	Gro1-4-1 F	AAGCCACAACTCTACTGGAG	68 (5 cyc) + 58 (35 cyc)[f]	602 (R)
	Gro1-4-1 R	GATATAGTACGTAATCATGCC		

[a] Finkers-Tomczak *et al.* 2011.
[b] This study.
[c] Milczarek *et al.* 2011, 2014.
[d] Brown *et al.* 2006.
[e] Asano *et al.* 2012.
[f] Rogozina *et al.* 2019.
R - resistant genotype, S - susceptible.

handled as the reference. The sequence data of the two newly described alleles 57R-887 and 57R-1155 was deposited in the NCBI database (OP740234 and OP740235, respectively).

Predictive values

Predictive values were determined for both 57R and TG689 marker as described by Trevethan (2017). Positive predictive values describe the ability to correctly predict resistance to *G. rostochiensis* using genetic marker screening test. It was calculated as the percentage of potato breeding clones with the resistance allele out of all clones resistant to PCN. Negative predictive values describe the ability to correctly predict susceptibility to *G. rostochiensis* using genetic marker screening test. It was calculated as the percentage of potato breeding clones without the resistance allele out of all clones susceptible to PCN.

Statistical analysis for concordance

To evaluate the significance of the relationship between the presence/absence of both markers (TG689, 57R) and resistant/susceptible bioassay results, the two-tailed Fisher's exact test was used.

Plotting of the results

Scatter plots were generated using R packages ggplot2 (Wickham 2016) and gghighlight (Yutani 2017). Results were plotted in R-studio (RStudio Team 2020).

Results

Interpreting nematode infestation test results

Firstly, we aimed to assess the variability of the nematode resistance testing results obtained with our custom protocol. We analysed the results from 11 years of phenotypic PCN resistance testing. On average, 418 tubers (SD ±25.9) were tested each year, altogether 4601 tubers. In total, 2918 breeding clones were grown in nematode-infested soil and developed cysts were counted. Out of all tested tubers, majority (88.8%) developed no cysts during infestation period (**Fig. 1A**). The number of cysts on infested tubers varied from 1 to over 300 (**Fig. 1A**, **Supplemental Fig. 1A**). Most of the breeding clones (82.7%) were tested once and culled thereafter. Percent of tubers with cysts was calculated for all tested breeding clones, including those tested only once (**Fig. 1B**). There were two big groups—resistant breeding clones (n = 2522), where only 0%–10% of tubers developed cysts, and susceptible breeding clones (n = 320), where 91%–100% of tubers developed cysts (**Fig. 1B**). Phenotype of 76 breeding clones (2.6%) remained unclear even after several tests, since 11–90% of their tested tubers had one cyst or more (**Fig. 1B**, **Supplemental Fig. 1B**).

To determine whether tubers with 1–5 cysts belong to resistant, partially resistant or susceptible breeding clones, we analysed the distribution of the number of cysts per tuber

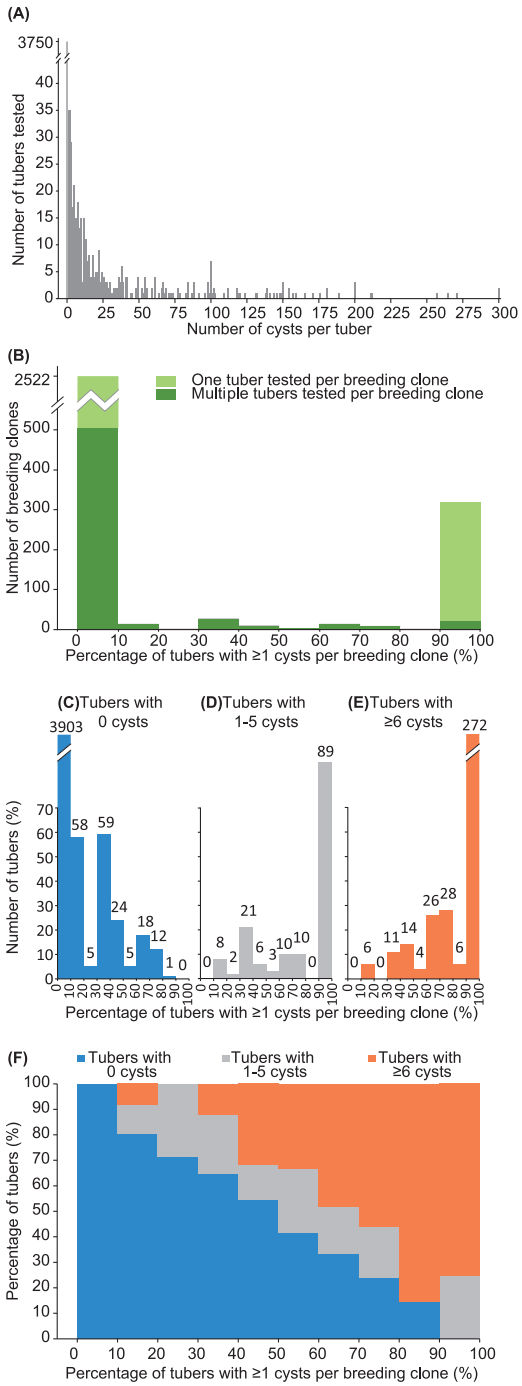

Fig. 1. Analysis of the phenotypic test results of resistance to *G. rostochiensis* based on the data obtained between the years 2010–2020. (A) Distribution of counted cysts per tuber in the data set (n = 4601 tubers). No cysts were counted for the majority of tubers, but the highest number of cysts per tuber was 300. (B) Histogram of breeding clones (n = 2918) according to the percentage of tubers with one or more cysts. Dark green bars indicate breeding clones with at least two tubers tested, light green bars indicate breeding clones with a single tuber tested. (C–E) Number of tubers with no cysts (blue), with 1–5 cysts (grey) and with six or more cysts (orange) grouped according to **Fig. 1B** (n = 2918). (F) Percentage of tubers with no cysts (blue), with 1–5 cysts (grey) and with six or more cysts (orange) grouped according to **Fig. 1B** (n = 2918).

in more detail (**Fig. 1C–1F**). We divided the tubers into three groups according to the number of cysts counted (0 cysts, 1–5 cysts, ≥6 cysts) and found that the tubers with 1–5 cysts predominantly belong to susceptible breeding clones that always have cysts (89 out of 149 tubers, 59.7%) (**Fig. 1D**). However, 60 out of 149 tubers (40.3%) with 1–5 cysts presumably belonged to partially resistant breeding clones (**Fig. 1D**). In order to improve the efficiency of PCN resistance breeding, we aimed to implement genetic marker analysis and marker-assisted selection.

Comparing markers 57R and TG689 with phenotypic tests

Two different *G. rostochiensis H1* resistance gene markers—57R and TG689—were evaluated in order to select and verify a suitable genetic marker for *G. rostochiensis* resistance breeding. In total 234 samples, including 22 cultivars and 212 breeding clones from years 2011–2015 and 2017, were analysed with 57R and TG689 markers (**Table 2**, **Supplemental Table 1**). Additionally, 258 breeding clones from the year 2018 were tested with the 57R marker only (**Supplemental Table 2**). All breeding clones were phenotypically tested for PCN resistance.

From 22 Estonian cultivars, released between 1934 and 2017, six showed the presence of *H1* resistance allele, 16 showed the absence of *H1* resistance allele and cv 'Anti' yielded PCR products of unexpected size (**Fig. 2**, **Table 2**). The results of the two markers TG689 and 57R were in complete agreement with each other for all tested cultivars as well as with the resistance phenotype, except for cv 'Anti' (**Table 2**). Cultivar 'Anti' showed partial resistance to *G. rostochiensis* with 66% (42/64) of the tubers being resistant (0–3 cysts) and 34% (22/64) being susceptible. The oldest cultivar with the *H1* resistance gene appeared to be 'Ants', which was released in 1992 (**Table 2**). All cultivars released later than the year 2000 showed presence of *H1* resistance gene (**Table 2**). Thus, it can be concluded that *H1* gene is common in recent Estonian potato cultivars and has successfully been bred into new cultivars with the use of phenotypic testing for nematode resistance.

We analysed 212 pre-selected breeding clones to test if there is any difference in the predictability of TG689 and 57R to detect the *H1* resistance gene (**Supplemental Table 1**). These breeding clones had been culled prior to genotyping analysis with only 13 susceptible clones remaining for further breeding. All of those 13 breeding clones were negative for both TG689 and 57R markers (negative predictive values 100%). Out of the 199 nematode resistant breeding clones, 195 (positive predictive value 98.0%) were positive for 57R, and 188 (positive predictive value 94.5%) were positive for TG689 (**Fig. 3**). The difference between the number of mismatches between phenotype and genotype (4 for 57R and 11 for TG689) was not statistically significant (two-tailed Fisher's exact test $p = 0.1115$). We continued our work with the marker 57R for its more stable performance in our conditions and for its high concordance between phenotype and genotype in previous studies (Park *et al.* 2018, Schultz *et al.* 2012). In our study, 57R marker also gave less discrepancies with the phenotype.

Table 2. Presence of *G. rostochiensis* resistance markers TG689 and 57R in Estonian potato cultivars. The cultivars are presented according to the order of the release year. Empirically measured resistance to *G. rostochiensis* is also shown

Cultivar	Year of release	TG689	57R	Resistance to *G. rostochiensis*
Kalev	1934	–	–	Susceptible
Virulane	1941	–	–	Susceptible
Jõgeva kollane	1942	–	–	Susceptible
Olev	1953	–	–	Susceptible
Talvik	1953	–	–	Susceptible
Sulev	1960	–	–	Susceptible
Viru	1961	–	–	Susceptible
Varmas	1965	–	–	Susceptible
Ando	1973	–	–	Susceptible
Ere	1973	–	–	Susceptible
Ane	1979	–	–	Susceptible
Sarme	1984	–	–	Susceptible
Vigri	1984	–	–	Susceptible
Mats	1987	–	–	Susceptible
Ants	1992	+	+	Resistant
Anti	1995	–	a	Partial resistance
Juku	1997	–	–	Susceptible
Piret	2001	+	+	Resistant
Maret	2003	+	+	Resistant
Reet	2007	+	+	Resistant
Teele	2013	+	+	Resistant
Tiina	2017	+	+	Resistant

'+' resistant allele, '–' susceptible allele, 'a' corresponds to partially resistant allele.

Concordance of the 57R marker with resistance to PCN

To assess how 57R results match the phenotype, another population of breeding clones with no previous preselection was used. 258 breeding clones from 50 different crosses were analysed for 57R marker and phenotypic *G. rostochiensis* resistance. Resistant 57R allele was detected in 228 samples, of which 226 were also phenotypically resistant (positive predictive value 99.1%), whereas two (0.9%) were susceptible (**Table 3**). There were 30 breeding clones, which were homozygous for the susceptible allele of 57R (**Table 3**). Subsequent biological tests showed that 18 of them acquired cysts (negative predictive value 60.0%), while 12 (40.0%) had no cysts (**Table 3**). PCR analysis revealed that three out of these 12 samples without 57R resistance allele that had no cysts have the *GRO1* resistance gene, possibly explaining their phenotype.

In conclusion, we confirmed that the marker 57R correlates very strongly with PCN resistance (99.1%), but it is not able to predict all resistant clones in the breeding population. Hence, utilising multiple PCN genetic markers for *H1* and other resistance genes is encouraged in order to be

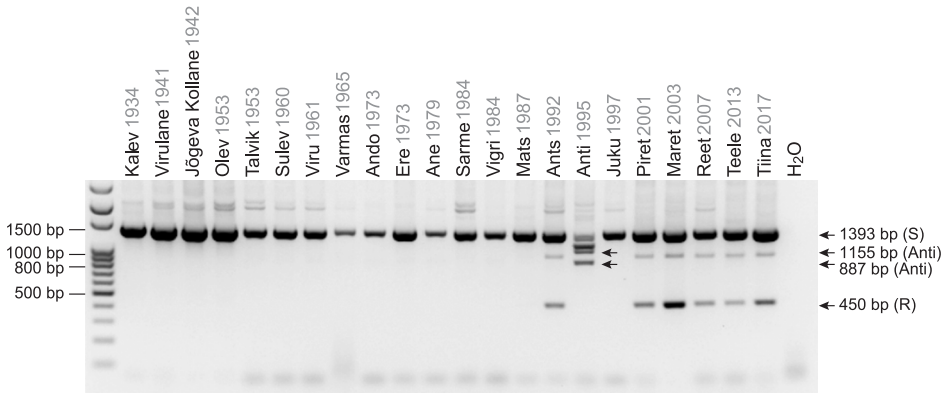

Fig. 2. The presence of *G. rostochiensis* resistance marker 57R in Estonian cultivars released since 1934. The cultivar names are followed by the year of release (grey text). The fragments were separated on a 1.5% agarose gel. Size of the fragments was compared to 100 bp DNA ladder. The 1393 bp fragment corresponding to the susceptible allele, 450 bp to the resistant allele, the 887 bp and 1155 bp fragments (novel alleles characterised in cultivar 'Anti') are depicted with arrows.

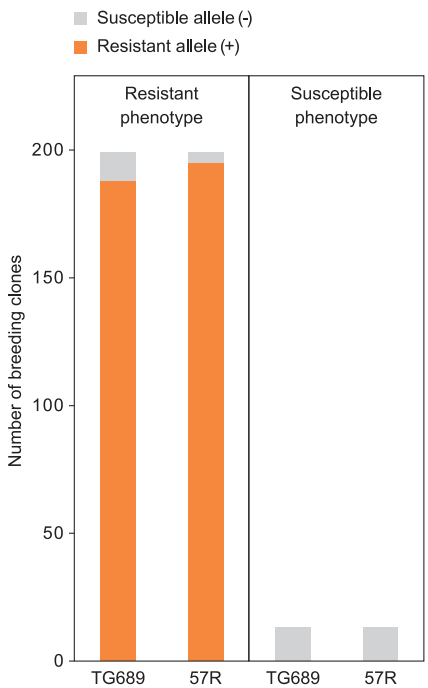

Fig. 3. The presence of *G. rostochiensis* resistance markers 57R and TG689 is shown in 212 breeding clones, grouped according to the phenotypic resistance to *G. rostochiensis*.

Table 3. Number and percentage of breeding clones with and without 57R genetic marker among the data set of 258 breeding clones. Breeding clones were grouped according to the phenotypic resistance to *G. rostochiensis*

Phenotype	57R +	57R –
Resistant	226 (99.1%)	12 (40.0%)
Susceptible	2 (0.9%)	18 (60.0%)
Total	228 (88.4%)	30 (11.6%)

'NA' - not assessed.

able to genetically explain a wider range of nematode resistance and to overcome the effect of linkage disequilibrium.

Comparison of two breeding scenarios

We aimed to compare two different resistance breeding scenarios based on the practices applied at METK: 1) only greenhouse testing for the resistance; 2) genetic marker analysis combined with greenhouse testing for the resistance. In the first scenario, on average 258 (SD = ±9.1) breeding clones (one tuber per clone) are selected for the first greenhouse testing each year (**Fig. 4A**). This is repeated for up to five years with a decreasing number of clones, resulting in a total of 418 (SD = ±26) tubers to be tested yearly (**Fig. 4A**).

In the second scenario, where MAS is combined with phenotypic testing, PCR analysis of 258 (SD = ±9) breeding clones with 57R marker precedes the greenhouse nematode cyst counting (**Fig. 4B**). In the first year, only breeding clones negative for 57R (~30 tubers) and in the third year, all the promising breeding clones are tested phenotypically (**Fig. 4B**). This results in a total of 84 phenotypic tests per year (**Fig. 4B**). Breeding clones tested negative for 57R had only 60.0% correlation with the phenotype. All these samples will be included in the biological testing in the first year, except for when they were culled due to other traits. Thus, the second scenario leads to five times fewer greenhouse tests compared to the first scenario and requires less time for initial results (**Fig. 4**).

'Anti' and 15 other potato genotypes possess two novel 57R alleles

As mentioned earlier, cultivar 'Anti' stood out with its two unexpected PCR products when we analysed it with the marker 57R (**Fig. 2**). What is more, we observed another interesting phenomenon when analysing the phenotypic PCN resistance of cv 'Anti'. Out of 64 tubers of the cv 'Anti' tested between years 2010–2020, we observed no cysts in 35.9% of tubers, 1–5 cysts in 32.8% of tubers, and

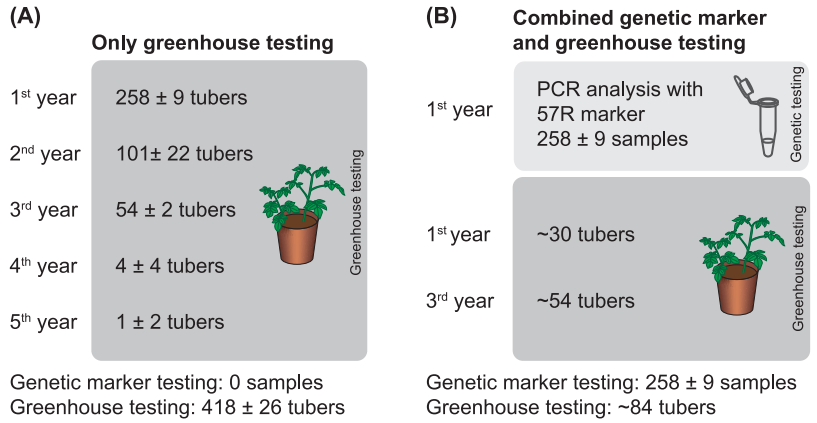

Fig. 4. Schematic comparison of two strategies for the nematode resistance potato breeding showing the number of greenhouse tests for *G. rostochiensis* resistance without (A) and with (B) genetic marker analysis. (A) The right panel involves only greenhouse testing (altogether 418 ± 26 tubers tested and no genetic samples tested). (B) The left panel depicts a breeding strategy, where greenhouse testing is combined with genetic marker testing (altogether approximately 84 tubers tested and 258 ± 9 genetic samples tested). The analysis was based on data collected between 2010–2020 in Estonia, with 258 ± 9 3rd year breeding clones as the starting material.

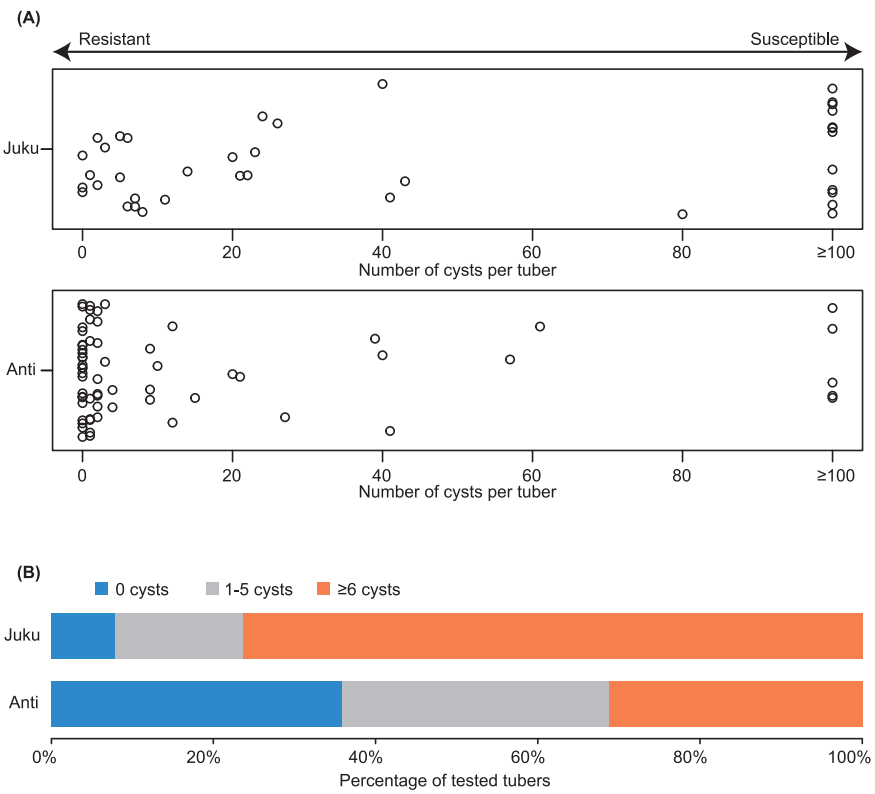

Fig. 5. Cultivar 'Anti' shows partial resistance to *G. rostochiensis* compared to susceptible cultivar 'Juku'. (A) Distribution of nematode test results for cultivars 'Juku' (susceptible phenotype; n = 38) and 'Anti' (partial resistance phenotype; n = 64). (B) Percentage of tubers observed with no cysts (blue), with 1–5 cysts (grey) and with six or more cysts (orange) in cultivar 'Anti' and susceptible cultivar 'Juku' (n = 38–64).

over five cysts in 31.3% of tubers (**Fig. 5**). For comparison, susceptible cultivar 'Juku' had zero cysts in 7.9% of the cases and all the other tubers (92.1%) tested showed one or more cysts (**Fig. 5**). As the PCN resistance test results showed partial resistance for 'Anti', we further analysed the sequences of its new 57R alleles.

Based on the BAC clones used by Finkers-Tomczak *et al.* (2011), the size of resistant 57R allele is 450 bp. The 57R susceptible allele has previously been shown to be approximately 1500 bp in size (Milczarek *et al.* 2011, Park *et al.* 2018), however, in this study we specified the size of susceptible 57R allele to be 1393 bp according to the sequence of BAC RH009O14 (Finkers-Tomczak *et al.* 2011). Two new alleles of approximately 900 and 1200 bp in size

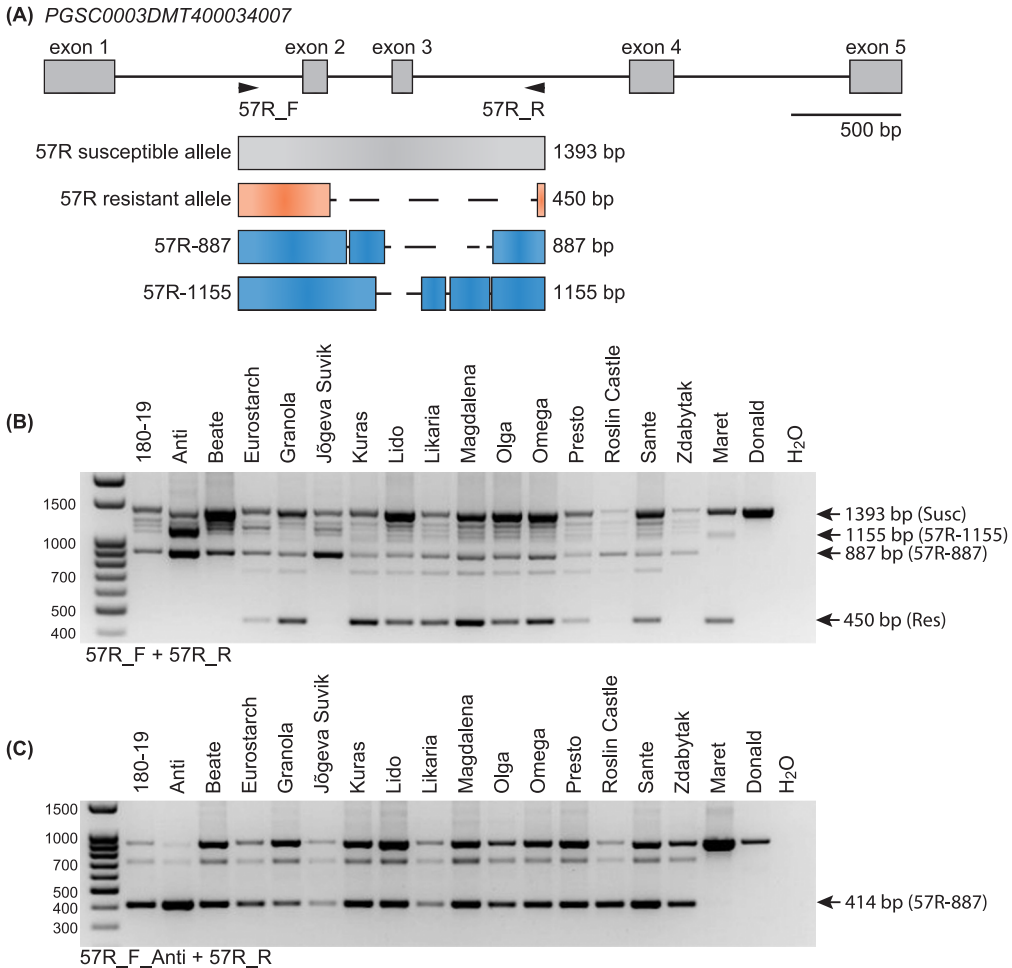

Fig. 6. Cultivar 'Anti' and 15 other potato genotypes possess the novel 57R allele of the size 887 bp. (A) Schematic representation of the alignment of 57R alleles and the gene *PGSC0003DMG401013075* (transcript *PGSC0003DMT400034007*) as annotated in Ensembl Plants. The susceptible allele was based on the sequence of haplotype RH89-039-16 and the resistant allele was based on the sequence of haplotype SH83-92-488 as published by Finkers-Tomczak *et al.* (2011). The novel alleles 57R-887 and 57R-1155 from cultivar 'Anti' were of the size 887 bp and 1155 bp, respectively. (B) Agarose gel electrophoresis image of 57R marker PCR products. The size of resistant 57R allele (R) is 450 bp, susceptible 57R allele (S) 1393 bp and two novel alleles 887 bp and 1155 bp. Genotypes with the novel allele 57R-887 and without the resistant 57R allele are 'Anti', 'Beate', 'Jõgeva Suvik', 'Roslin Castle', 'Zdabytak' and '180-19'. Cultivars with the novel allele 57R-887 and with the resistant 57R allele are 'Eurostarch', 'Granola', 'Kuras', 'Lido', 'Likaria', 'Magdalena', 'Olga', 'Omega', 'Presto', and 'Sante'. (C) Agarose gel electrophoresis image of PCR products (414 bp) amplified with the forward primer specific to the novel allele 57R-887 (57R_F_Anti) and marker 57R reverse primer (57R_R) confirms the presence of this allele in the selected 16 genotypes.

from cultivar 'Anti' were further analysed by sequencing. DNA sequences of the novel 57R alleles from cv 'Anti' revealed fragments of 887 bp and 1155 bp in length, which correspond neither to resistant nor to susceptible allele (**Fig. 6A**, **Supplemental Fig. 2**).

By aligning the novel allele 57R-887 from cv 'Anti' against the susceptible haplotype RH89-039-16, we found 19 SNPs, 1 single nucleotide insertion, 3 single nucleotide deletions, as well as a 14 bp and a 490 bp deletion (**Fig. 6A**). These two deletions are located within the same region as the 942 bp deletion site of the known resistant allele. The alignment of the 57R-1155 allele from cv 'Anti' with the abovementioned reference sequence revealed 42 SNPs, two single nucleotide insertions and one 3-nucleotide insertion, two single nucleotide and two 2 bp deletions, as well as a 7, 20 and 210 bp deletion (**Fig. 6B**).

Using Ensembl Plants database, we searched for a gene annotation in the location of 57R marker and found that it corresponds to a gene coding for a protein of unknown function (PGSC0003DMT400034007). The fragment of the 57R susceptible allele is identical to the sequence of *PGSC0003DMT400034007* and encloses the second and the third exons (**Fig. 6A**). The 57R resistant allele and the two novel alleles all carry a deletion of the third exon among other allele-specific mutations (**Fig. 6A**). Interestingly, a single nucleotide substitution from T to A in the allele 57R-887 from cv 'Anti' was detected, which results in a premature stop codon in the second exon of *PGSC0003DMT400034007* (**Supplemental Fig. 2**). The 14 bp deletion detected in allele 57R-887 is located in the

second intron and the 490 bp deletion includes the whole third exon and parts of the surrounding introns. The allele 57R-1155 also has a larger deletion of 210 bp that includes the full third exon, and two smaller deletions in the intron region.

Next, we included 15 additional genotypes that showed the presence of the novel 57R alleles. However, amplification of the allele 57R-1155 was very weak in those cultivars (**Fig. 6B**). We found five additional genotypes with 887 bp and 1155 bp alleles, but without the 450 bp resistant allele, showing similar profile to cultivar 'Anti' (**Fig. 6B**). These genotypes were cultivars 'Beate', 'Roslin Castle' and 'Zdabytak', local accession 'Jõgeva Suvik' and the breeding clone 180-19. Moreover, we found ten genotypes, which in addition to the novel alleles possess the 450 bp resistant allele (**Fig. 6B**). The breeding clone 180-19 is the progeny of cultivar 'Magdalena' as the female parent and 'Impala' as the male parent. We found that the novel alleles of 180-19 were inherited from cv 'Magdalena'.

Since the allele 57R-887 clearly amplified more efficiently, we designed a new forward primer specific to this allele in order to distinguish it in particular (**Table 1**). The primer 57R_F_Anti spans over the 14 bp deletion and when used together with 57R reverse primer amplifies a PCR product of 414 bp from the novel allele. We verified that all the 15 genotypes together with cv 'Anti' amplify the 414 bp fragment with the above-mentioned primers (**Fig. 6C**). In conclusion, here we show for the first time the existence of two novel 57R alleles, 57R-887 and 57R-1155, in 16 potato genotypes.

Discussion

Phenotypic testing of G. rostochiensis resistance

Resistance to *G. rostochiensis* is an important and highly desired trait in potato breeding, hence, phenotypic characterization of nematode resistance is a valuable tool in potato breeding. There is a well-established and internationally agreed nematode resistance testing protocol by OEPP/EPPO (2006). However, it has its limitations such as being time-consuming and cost-intensive. Our protocol was chosen based on its robustness and affordability, again, with its own limitations. Over 4500 phenotypic tests for *G. rostochiensis* resistance were conducted at METK since the year 2010. Testing has been initiated each year at the end of May and results are obtained usually at the beginning of August, the duration of the test being approximately 2.5 months. Throughout the testing period, weekly observations were needed and a suitable environment was to be maintained.

Unclear phenotypes and varying infestation level for susceptible control cultivars have made the nematode testing challenging to interpret with our protocol. **Fig. 1C–1F** shows that when only 1–5 cysts appear on tubers, it can refer to either susceptible or partially resistant clones. Misleading results can also be a consequence of the phenotypic testing methodology. False negative results can occur as a consequence of inoculation inefficiency, hatching differences of the nematode and difficulties in visually detecting cysts in the soil. Few cysts may sometimes still appear on the tubers of resistant genotypes and debris such as small stones may be mistaken for cysts, resulting in false positive counts. Thus, we conclude that phenotypic nematode resistance testing with our method does not always give clear definite answers and hence additional data is required in order to assess nematode resistance.

Suitability of genetic markers for G. rostochiensis resistance screening

The initial aim of our research was to decrease the amount of nematode resistance phenotypic tests, to reduce the time needed to get the results and to enable all-the-year-round testing. Genetic analysis has been reported to be an efficient tool for screening for PCN resistance in a number of studies (Asano *et al.* 2021, Biryukova *et al.* 2008, Milczarek *et al.* 2011, 2014, Schultz *et al.* 2012, Zoteyeva *et al.* 2020). PCR-based methods are less time-consuming than phenotypic tests, require less plant material, are easily repeatable and also more informative with allelic dosage (Meiyalaghan *et al.* 2018). We chose to analyse the markers for gene *H1*, which provides near-absolute resistance against *G. rostochiensis* and is widely implemented in commercial potato cultivars (Finkers-Tomczak *et al.* 2011, Toxopeus and Huijsman 1953). There are two SCAR markers often used for *H1* detection, TG689 and 57R that are located 700 kb away from each other (Biryukova *et al.* 2008, Finkers-Tomczak *et al.* 2011, Milczarek *et al.* 2014, Park *et al.* 2018, Schultz *et al.* 2010, 2012).

The two aforementioned *H1*-linked PCR markers were applied on 212 potato breeding clones and 22 Estonian cultivars. PCR analysis of phenotypically resistant breeding clones revealed no statistically significant differences between 57R and TG689 (**Fig. 3**). These results indicate that both markers are useful for resistance detection in the given set of samples. A strong allelic association between 57R and *H1* gene compared to other markers for this gene was previously demonstrated by several studies (Khiutti *et al.* 2020, Milczarek *et al.* 2014, Park *et al.* 2018, Schultz *et al.* 2012, Zoteyeva *et al.* 2020). In addition, the reliability of marker TG689 was reported to be pedigree-specific (Schultz *et al.* 2012). This in turn poses a risk that for certain breeding clones or parents this marker could result in contradictions between biological tests and molecular analysis. The results of the given study demonstrated 57R as an effective marker for detection of resistant potato breeding clones. Thus, we chose to further analyse the possibilities of utilising 57R in marker-assisted selection.

Genetic factors affecting resistance to G. rostochiensis

Among the 258 unculled breeding clones, there were 12 (4.7%) phenotypically resistant clones negative for 57R marker. It cannot be excluded that a crossing-over event

caused the resistance gene *H1* to be inherited without the 57R marker. When the marker is present, plants can sometimes still develop cysts. One of the reasons could possibly be that the expression of *H1* can be activated or deactivated depending on environmental conditions (Lavrova *et al.* 2015). Hence, on some occasions resistant genotypes still acquire 1–5 cysts in biological testing.

In addition, resistance to *G. rostochiensis* not only depends on the *H1* gene, but can also be conferred by other genes, such as *GROVI* and *GRO1-4* that originate from *Solanum vernei* and *Solanum spegazzini*, respectively (Barone *et al.* 1990, Jacobs *et al.* 1996). *GRO1-4* provides resistance against all pathotypes of *G. rostochiensis* (Paal *et al.* 2004). However, the introgression of those genes is expected to be rare (Schultz *et al.* 2012). Additional analysis of the 12 breeding clones that did not have the resistant allele of 57R, but were phenotypically resistant to *G. rostochiensis*, revealed the presence of *GRO1-4* gene in three of them (**Table 3**).

The interaction between a nematode and its host plant is complex and regulated on different levels. In *G. pallida* it was reported that plant's immune response triggered by co-expression of genes *GPA2* and *RBP-1* can be suppressed by nematode's *Gp*SPRY-414-2 (Mei *et al.* 2018). Similar plant defence mechanisms could also apply to interactions between *G. rostochiensis* and its host.

Marker-assisted selection (MAS) as a tool to reduce greenhouse testing

Our study showed that 99.1% of 57R positive samples also had a nematode resistant phenotype. This in turn means that almost all clones that are detected by 57R as resistant will develop no cysts, and the pot test can be omitted for those samples in the first year of testing. Postponing phenotypic analysis for all 57R positive samples results in reduction of pot tests from approximately 418 to 84, which saves resources and time needed for phenotypic cyst counting. What is more, in the second scenario greenhouse tests are planned only in the first and third year instead of testing in five consecutive years in the first scenario (**Fig. 4B**). This significantly accelerates the breeding process as well as reduces workload and costs required for greenhouse testing. Altogether, we favour the second scenario as the breeding process is sped up and the acquired data is more informative due to the combination of genotypic and phenotypic analyses.

Presence of G. rostochiensis resistance in Estonian potato cultivars

Potato cyst nematode resistance was not targeted in Estonian potato breeding before 1960's (Krall and Luik 2000). As expected, the older cultivars are susceptible and do not possess *H1* gene (**Table 2**). The first nematode-resistant Estonian cultivar 'Ants' was released in 1992. Resistance allele of *H1* gene is present in all cultivars released since the year 2001. This demonstrates that it was successfully introgressed into all modern Estonian cultivars already before genetic testing was started. Introgression of the resistance alleles was achieved by selecting and crossing *G. rostochiensis* resistant parents, which resulted in at least 70% of resistant offspring (Koppel and Tsahkna 2003). It is clearly an advantage that modern Estonian potato cultivars possess the *H1* gene with nearly-absolute resistance to pathotypes Ro1 and Ro4, as it helps to reduce the use of pesticides and assists to implement more sustainable agricultural practices.

In our analysis, Estonian cultivar 'Anti' stood out with its partial resistance to PCN in the pot tests (**Figs. 4**, **5**), although, in the breeding report it is considered susceptible to nematode infestation (Koppel 1997). Of all pot tests with 'Anti', 35.9% showed resistant phenotype, 31.3% had more than five cysts and 32.8% had 1–5 cysts (**Fig. 5B**), indicating partial resistance to *G. rostochiensis*. Cultivar 'Anti' was initially bred for resistance to late blight, and for this purpose breeding clone from VIR (*Solanum demissum* × *Solanum infundibuliforme*) and selected elite cultivars together with resistance testing were used (Koppel 1997). The VIR breeding clone was first crossed with 'Unikat', then with 'Bellona', and left to self-pollinate (**Supplemental Fig. 3**). Last, it was crossed with 'Super' (**Supplemental Fig. 3**). As the historic data for PCN resistance of this pedigree is insufficient, the origin of partial resistance phenotype of 'Anti' cannot be concluded.

Identical profile of 57R alleles in 'Anti' and five other genotypes

In addition to partial PCN resistance, cultivar 'Anti' also exhibits a unique 57R allele profile, including two novel alleles and missing the usual 57R resistant allele. We found identical allele profile in five other potato accessions. Their PCN resistance phenotype has not been tested in our greenhouse. Nevertheless, Norwegian cultivar 'Beate' has been shown to have intermediate resistance to PCN (Holgado and Magnusson 2012). Also, Belarusian cultivar 'Zdabytak' was reported to be moderately resistant to PCN (Antonova *et al.* 2017). Our results from 'Anti' together with the data reported for 'Beate' and 'Zdabytak' suggest that the novel 57R profile might be attributed to the partial resistance to *G. rostochiensis*.

Often wild relatives are the source of disease resistance genes in potato. This is also the case for nematode resistance. The resistant allele of *H1* locus was originally derived from *S. tuberosum* ssp. *andigena* and the nematode resistance gene *GRO1-4* from *S. spegazzinii* (Barone *et al.* 1990, Gebhardt *et al.* 1993). There are two wild relatives, *S. demissum* and *S. infundibuliforme*, in the pedigree of 'Anti' (**Supplemental Fig. 3**). Estonian accessions 'Jõgeva Suvik' ('Mulk' × 'Flava') and '180-19' ('Magdalena' × 'Impala') are not direct progenies of 'Anti', hence, the source of these alleles is unclear. Based on the Potato Pedigree Database (Hutten and van Berloo 2001, van Berloo *et al.* 2007) the use of wild relatives in breeding of 'Beate',

'Roslin Castle' and 'Zdabytak' cannot be confirmed. Nevertheless, it cannot be excluded that the source of the novel 57R alleles could track back to a wild ancestor.

The marker 57R is located within the *H1* locus, interspersed with many genes relevant to disease resistances (Finkers-Tomczak *et al.* 2011). A possible explanation for the partial resistance of 'Anti' and the presence of the novel 57R alleles can be that this marker is linked to novel alleles of *H1* gene or another R-gene located nearby. However, it cannot be excluded that the novel alleles of 57R marker directly affect the phenotype. Annotation search for the genomic region of 57R demonstrated that it is located within a gene coding a protein of unknown function PGSC0003DMT400034007. Premature stop codon in exon two together with the deletion of exon three in allele 57R-887 is expected to lead to no protein synthesis. The deletion of a complete exon in the allele 57R-1155 is likely to alter the structure and function of the hypothetical and uncharacterised protein. Hence, the novel alleles of 57R influence gene expression differently and their effect on partial nematode resistance cannot be excluded.

To sum up, we analysed the results from 11 years of phenotypic *G. rostochiensis* resistance greenhouse testing (a total of 4601 tubers of 2918 breeding clones). The primary aim of this research was to reduce the number of phenotypic tests by introducing the use of genetic marker analysis. We compared predictive values of *H1* gene markers TG689 and 57R, and chose to continue with 57R. The positive predictive value of 57R was 99.1%, whereas the negative predictive value was 60%. Here we report the 57R marker alleles of 22 Estonian cultivars and 470 breeding clones. We discovered and described two novel alleles of 57R marker in 'Anti' and later found the same allele in several other potato accessions. 'Anti' and five other genotypes carry identical 57R allele profile without the dominant 57R resistance allele. Cultivar 'Anti' and reportedly also 'Beate' and 'Zdabytak' are partially resistant to *G. rostochiensis*, implying that the abovementioned 57R allele profile might contribute directly or indirectly to the phenotype.

Author Contribution Statement

Study concept and design: KL, AIP, LJ, KI. Material preparation: LKü (*in vitro* plants), TT (potato tubers). Analysis: AIP, LJ, KI, AK, JA, LKa, KL. Writing: AIP, KL, LJ, KI. Review and editing: all authors contributed to the manuscript review and editing.

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