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Role of Ry_{chc} gene diversity in the resistance of wild potato relative *Solanum chacoense* to potato virus Y

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Potato virus Y (PVY) is one of the most significant pathogens in the production of cultivated potato *Solanum tuberosum* worldwide. The most efficient way to combat viral diseases is the use of resistant varieties. The Ry_{chc} gene was discovered in the tuber-bearing wild potato relative *Solanum chacoense*. This gene is able to confer extreme resistance to PVY by rapidly halting virus multiplication in plants without the development of visible symptoms. Two molecular markers were developed to predict the presence of the Ry_{chc} gene and PVY resistance with 100% accuracy. In this study, the diversity of the Ry_{chc} gene and its relationship with resistance to PVY were investigated in genotypes from the Russian potato genbank. A collection of 60 genotypes originating from different populations was examined for PVY resistance and for the presence of Ry_{chc} using molecular markers. The observed phenotypes ranged from susceptibility to hypersensitive reaction and extreme resistance to PVY. Irrespective of the type of resistance, all the PVY-resistant plants carried the Ry_{chc} gene, indicating the prevalence of this resistance mechanism in *S. chacoense*. Unexpectedly, two *S. chacoense* individuals with the Ry_{chc} gene were found to be completely susceptible to PVY. Ry_{chc} sequences were evaluated via long-read targeted sequencing in all Ry_{chc}-positive genotypes. Despite the low overall sequence variation observed in Ry_{chc} gene, identical polymorphisms were found in Ry_{chc} alleles from susceptible *S. chachoense* genotypes, demonstrating the existence of nonfunctional Ry_{chc} allele unable to confer PVY resistance.

Keywords Virus resistance, Extreme resistance, Hypersensitive response, Allelic diversity, PVY, Nanopore sequencing

Potato is the most important non-grain crop worldwide. The use of vegetative reproduction by tubers makes potato especially vulnerable to various viral diseases, since many plant viruses are unable to enter true seeds but readily accumulate in tubers[1]. The viral load can multiply in successive vegetative generations, leading to severe yield losses[2,3]. Chemical control agents commonly used to combat various plant diseases are inefficient against viruses because of their intracellular lifestyle. As a result, modern potato cultivation largely depends on the use of virus-free tubers obtained from in vitro cultivated plants[4]. This practice is cumbersome and costly, and healthy plants can be readily infected again under field conditions[2]. Among potato viruses, arguably the most important is potato virus Y (PVY). PVY is distributed worldwide and causes yield losses of up to 80% in potato[3,5]. In addition to yield reduction, tuber quality is also compromised[3]. Under field conditions, PVY is primarily distributed by aphids in a non-persistent manner, which makes control of viral spread via insecticides challenging[3,6], although some cultural practices, such as straw mulching or spraying with mineral oil, could have positive effects by impeding aphid infestation on potato plants[4,7].

The most potent approach to combat viral diseases is the use of genetically resistant varieties[8]. In different potato species, two main types of resistance genes that act either through the hypersensitive response (HR) or confer extreme resistance (ER) have been identified[9]. In contrast to the hypersensitive response, which causes the development of necrotic lesions that restrict virus spread in plant tissues, extreme resistance is associated mainly with the formation of no or only minor necrotic lesions and presumably acts through halting of viral multiplication inside plant cells[9]. Multiple genes conferring either HR or ER resistance were discovered in

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different potato cultivars and wild relatives, named with "N" or "R" first letters according to the resistance type – *Ny-1*, *Ny-2*, *Ny-Smira*, Ny_{chc}, Ny_{dms}, Ny_{tbr}, Ny_{spl}, $Ny_{(o, n)sto}$, Nc_{spl}, Nc_{tbr}, Ny_{adg} for HR and Ry_{adg}, Ry_{sto}(XI) and Ry_{sto}(XII), *Ry-f*$_{sto}$, Ry_{hou}, Ry_{chc} genes for ER[5,8,10–14]. Some novel sources of extreme resistance to PVY have been proposed recently[15,16]. While HR genes often confer strain-specific resistance, ER genes are generally effective against a broad spectrum of PVY strains. For example, the Ry_{sto} gene from *S. stoloniferum* Schlechtd. et Bché. is effective against all known PVY strains and even against another potyvirus, potato virus A[8,17,18]. Ry_{sto} protein is an immune receptor that recognizes the conserved region in potyviral coat proteins[19]. The Ry_{adg} gene from *S. tuberosum* subsp. *andigena* Hawkes confers ER to all strains of PVY[13]. The third most studied gene for extreme PVY resistance, Ry_{chc} from *Solanum chacoense* Bitter, also provides multistrain resistance to PVY[20–22]. Ry_{chc} was widely used in the selection of PVY-resistant potato[8,23]. Upon PVY infection, the phenotypic effect of the resistance conferred by Ry_{chc} gene in these cultivars varies from no symptoms to more pronounced necrotic areas developing around the site of inoculation[20,21,24]. After continuous efforts to create DNA markers and map Ry_{chc}, this gene was finally cloned from the 40–3 *S. chacoense* accession via map-based cloning and 40–3 BAC library analysis[22]. The transgenic expression of Ry_{chc} in two potato cultivars makes it resistant to PVY. Later, using hybrid genome assembly data, different Ry_{chc} allele variant was identified in the 184202-2 dihaploid potato line obtained after crossing of several Ry_{chc}-bearing potato cultivars[25]. Both identified Ry_{chc} alleles were highly similar, with few point mutations or short deletions/insertions between them, and were able to confer extreme resistance to PVY. While in the original study, the Ry_{chc} gene was suggested to have 5 exons and 4 introns[22], further analysis of RNAseq data demonstrated that Ry_{chc} mRNA comprises only 4 exons[25]. Ry_{chc} encodes a Toll/interleukin-1 receptor (TIR)-nucleotide binding site (NBS)-leucine rich repeat (LRR)-type protein that is 911 aa in length. Curiously, the Ry_{chc} gene was shown to have two splicing variants, one for the 911 aa protein and another for the severely truncated 554 aa protein[25]. Using a known Ry_{chc} sequence, two PCR markers located directly within the gene sequence were developed. These two markers demonstrated complete linkage with both the presence of Ry_{chc} in the genome and PVY resistance[22,25].

The investigation of plant *R* genes is hindered by their large number, cluster arrangement and repetitive nature of LRR domains[26]. Long-read sequencing technologies could be a valuable tool for studying *R* genes both separately and in their native genome arrangement. Recently, PacBio sequencing was used to evaluate the diversity of the Ry_{sto} gene in several *Solanaceae* species via targeted sequencing of Ry_{sto} amplicons[27]. In this study, a similar approach was used to discover the allelic diversity of the Ry_{chc} gene in a large collection of plants from the Russian potato genbank that had been previously classified as *S. chacoense*. These plants were characterized for PVY resistance, and the relationship between the presence of different Ry_{chc} allelic variants and resistance to PVY was investigated.

Materials and methods
Plant material
The study involved *S. chacoense* plants representing 11 accessions from the collection of the N.I. Vavilov Institute of Plant Genetic Resources (VIR). These accessions include *S. chacoense* material with a different origin and a different source of acquisition (Table 1). Expeditions throughout South America were performed by P. Zhukovskij and by L. Gorbatenko, who collected the accessions k-2732 and k-19769, respectively. Professor Jack Hawkes kindly provided accessions k-2861, k-7394 and k-22638 during his visit in VIR in 1990. Accession k-22638 was delivered as tubers and attributed by J. Hawkes as *Solanum commersonii* Dun., but later was recognized by a curator of Russian genbank as *S. chacoense*. Currently, its systematic position has not been completely resolved. The accessions k-21848, k-21849 and k-21854 were obtained from potato collection maintained in Ecuador (National Agricultural Research Institute, INIAP), and the accession k-22687 was obtained from the US Potato Genebank (USPG). Accessions of *S. chacoense* were obtained either as populations or as tubers and preserved as orthodox seeds in the VIR genebank. In 2017, the botanical seeds of 11 accessions *S. chacoense* from seed reproduction 1991–1992 were involved in a study of PVY resistance. Resistance to PVY was assessed among *S. chacoense* seedlings in the first year and their clonal plants in the second year by artificial inoculation in 2018–2019. All *S. chacoense* genotypes were subsequently maintained by the propagation of tubers annually in the VIR greenhouse. The second round of studies on PVY resistance was performed in 2021–2023. Owing to the unequal ability of the *S. chacoense* accession for clonal propagation, the number of plants that were saved for testing for molecular genetic analysis differed (Table 1). In total, 60 genotypes of *S. chacoense* were evaluated for resistance to PVY and the presence of DNA markers.

PVY infection
S. chacoense plants were tested in greenhouse by artificial inoculation. *Nicotiana tabacum* L. plants ('Samsun' variety) were used for PVY inoculum preparation. Three leaflets per one seedling of each *S. chacoense* accession were inoculated with sap from a mixture of tobacco plants displaying visible symptoms of ordinary and necrotic PVY strain infection. Ordinary strain infection displayed itself as mosaic and vein clearing, while necrotic strain infection – as vein necrosis on tobacco plants. The plant leaves identified for inoculation were powdered with carborundum powder, and the virus inoculum was then rubbed across the leaves. Symptoms of virus infection were evaluated after 10–14 days, and ELISA was carried out by taking leaf samples after 30 days. An ELISA double sandwich method[28] was used to detect PVY in the leaves of all tested plants. The diagnostic kits used were produced in the A.G. Lorch Potato Research Institute (Russia) and Bioreba (Switzerland). *S. chacoense* plants showing no symptoms and no virus accumulation according to ELISA in the seedling test were retested next year as clonal plants. *S. chacoense* individuals were considered susceptible if the virus was detected by ELISA but were considered resistant if no virus was detected over two independent trials.

№	Accessions No in VIR collection	Year	Plant ID	Source of accessions	PI number (USDA genbank)	Family number in this study	Number of genotypes	Genotypes
1	k-2732	1955	Fac.de Arg.B.634.1945	VIR expedition by P. Zhukovskij	no	537	7	W49,W70,W73,W76,W78,W79,W75
2	k-2861	1957	CHC 2430/3	Provided by J. Hawkes	no	539	5	W69,W71,W74,W86,W88
3	k-3060	1958	no	Agricultural Institute in Hungary	no	538	3	W80,W83,W84
4	k-7394	1966	HHR 3700	Provided by J. Hawkes	320,292	541	9	W30,W50,W51,W52,W53,W54,W67,W81,W82
5	k-19759	1987	Fac.de Arg.B.634.1945	VIR expedition by P. Zhukovskij	no	3	2	W35,W95
6	k-19769	1987	no	VIR expedition by L. Gorbatenko	320,286	542	14	W15,W19,W23,W26,W27,W55,W56,W57,W58,W59,W60,W61,W64,W72
7	k-21848	1991	SLE s. n.	National Agricultural Research Institute (INIAP), Ecuador	230,582	544	3	W62,W65,W66
8	k-21849	1991	COR 707 A	National Agricultural Research Institute (INIAP), Ecuador	265,576	545	5	W07,W13,W14,W20,W77
9	k-21854	1991	HHR 3198	National Agricultural Research Institute (INIAP), Ecuador	320,285	546	5	W16,W17,W21,W22,W29
10	k-22638	1992	no	Provided by J. Hawkes	no	548	6	W08,W10,W12,W25,W24,W28
11	k-22687	1993	HHR 3633	USDA-ARS (USA genbank)	320,291	8	1	W96

Table 1. Accessions and sources of *S. chacoense* genotypes from this study.

Screening the collection for the presence of DNA markers of the Ry_{chc} gene

The isolation of plant genomic DNA was performed according to Doyle and Doyle[29], with the incubation period extended to 2 h. Genomic DNA was diluted 100-fold prior to use. The PCR markers MG64-17[22] and 1648F24/1648R22[25] were used (Table S1, Figure S1). The total volume of the PCR mixture was 25 µl, which included 20 µl of water, 2.5 µl of buffer, 0.5 µl of each primer (10 µM stock solution), 0.5 µl of dNTPs (10 mM stock solution), 0.5 µl of Taq polymerase and 0.5 µl of template DNA. All PCR reagents were supplied by Evrogen (Russia). PCR was performed using a 96-well MiniAmp™ Plus Thermal Cycler (Thermo Fisher Scientific, USA). The PCR conditions for MG64-17 consisted of one cycle of 3 min at 94°C; 36 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C; and a final elongation of 10 min at 72°C, according to Li et al.[22]. The PCR conditions for 1648F24/1648R22 consisted of one cycle of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, and a final elongation of 10 min at 72°C. PCR products were separated via electrophoresis on 1.5% agarose gels in 1× TAE buffer (40 mM Tirs, 20 mM acetic acid, and 1 mM EDTA) and visualized via ethidium bromide DNA gel stain (Biolabmix, Russia) and UV transillumination. All *S. chacoense* genotypes were evaluated with both markers at least twice.

Amplification of the full-length Ry_{chc} gene

The primer pairs STRG1648f-F/STRG1648f-R and RyCDS-F/Ry5-R (Table S1) were used to amplify the full-length Ry_{chc} gene from genomic DNA. The total volume in the PCR assay was 20 µl, including 12.9 µl of water, 4 µl of buffer, 1 µl of each primer (10 µM stock solution), 0.4 µl of dNTPs (10mM stock solution), 0.2 µl of Phusion High-Fidelity DNA polymerase and 0.5 µl of template DNA. All PCR reagents were supplied by NEB (USA). The PCR conditions consisted of one cycle of 40 s at 98°C, followed by 32 cycles of 20 s at 98°C, 20 s at 63°C, and 2 min at 72°C, and a final elongation of 5 min at 72°C. For some difficult-to amplify samples, LongAmp Taq DNA polymerase (NEB, USA) was used instead of Phusion. All PCR products were separated via electrophoresis on 1.0% agarose gels in 1× TAE buffer and visualized through ethidium bromide DNA gel stain and UV transillumination.

Sequencing of the full-length Ry_{chc} gene sequence on the MinION platform

The full-length Ry_{chc} gene sequences were isolated from the PCR mixture via the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, USA). The quality of the isolated DNA was assessed by electrophoresis and spectrophotometry. The nanopore sequencing library was prepared using SQK-NBD114.96 (Oxford Nanopore Technology, Oxford, UK) and sequenced on FLO-MIN114 flow cell on a MinION sequencer. FAST5 data were base called and demultiplexing was performed via Guppy ver. 6.5.7 in high-accuracy mode with an NVIDIA GPU (RTX3060 12 GB).

Sequence analysis of the full-length Ry_{chc} gene

The adapter sequence was trimmed by guppy_barcoder. Quality control of the sequencing was performed by pycoQC (ver. 2.5.2). To obtain the complete sequence of the Ry_{chc} gene, the following programs were used:

minimap2 (ver. 2.24-r1122) for raw data mapping, samtools (ver. 1.21) for data filtering and sorting, CLAIR3 (ver. v1.0.7) for SNP calling, WhatsHAP (ver. 2.2) for identifying the allelic state of the Ry_{chc} gene in samples, and amplicon_sorter.py to obtain the Ry_{chc} gene consensus sequence in its allelic state[30]. The protein sequences were obtained by annotating the gene exons according to data provided by Akai et al.[25] (GenBank: LC726345.1) and then translating them according to the standard genetic code via the Geneious ver. 10.0.5 program. Domain annotation was performed by InterPro online software[31]. To make dendrograms, Ry_{chc} gene sequences or protein sequences were aligned using MUSCLE with default settings. Phylogenetic trees were generated using IQ-TREE 3.0.1[32], taking into account the optimal model selected by the program. All trees were visualized in iTOL[33].

Results

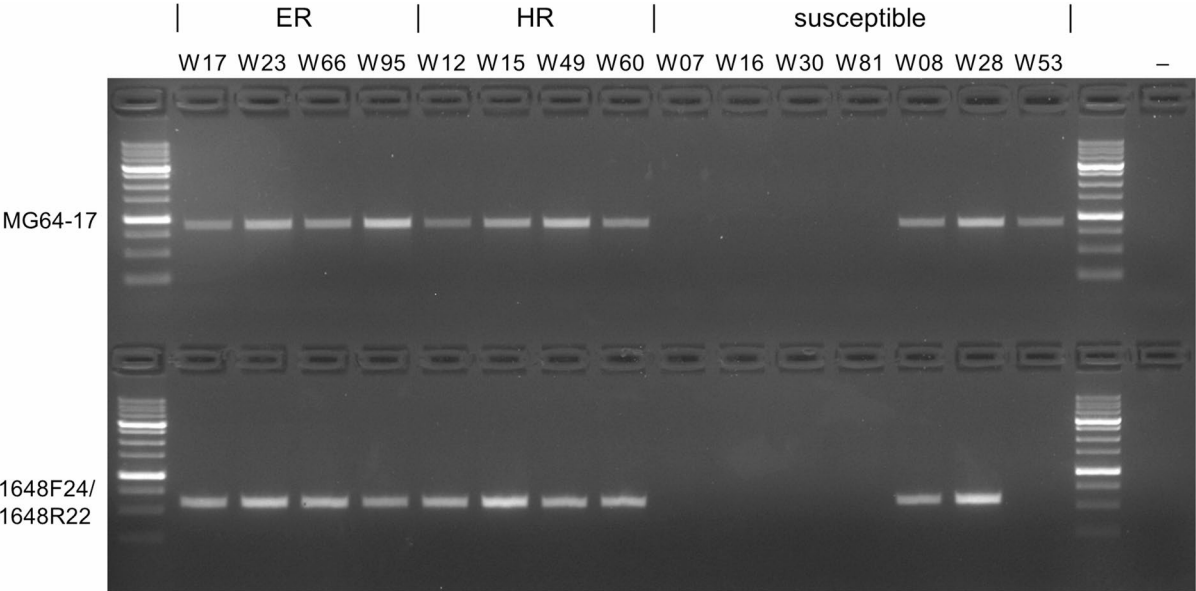### Collection of *S. chacoense* genotypes evaluated for PVY resistance

Sixty *S. chacoense* individuals belonging to 11 accessions from the VIR (Table 1) were grown in the greenhouse to evaluate PVY resistance under artificial inoculation. Infection results from inoculation with the mixture of ordinary and necrotic PVY strains were symptoms in the aerial parts of the plants and data of ELISA double-antibody sandwich (DAS) test. A variety of phenotypic responses were observed in *S. chacoense* plants, ranging from no visible symptoms or small point (tiny) necroses to necrotic rings or spots on the upper side of the leaflets, necrosis along the veins of the underside of leaflets, mild mottle or rugosity, leaf distortion or collapse and dropping of intermediate leaves, which remained clinging to the stem. All *S. chacoense* individuals were divided into three groups according to phenotype evaluation and ELISA assessment. Twenty-nine plants presented no visible symptoms or tiny necrotic lesions (Fig. 1) and no PVY accumulation according to ELISA analysis were considered to be extremely resistant to PVY (Table 2). Twenty-two PVY-susceptible plants demonstrated diverse
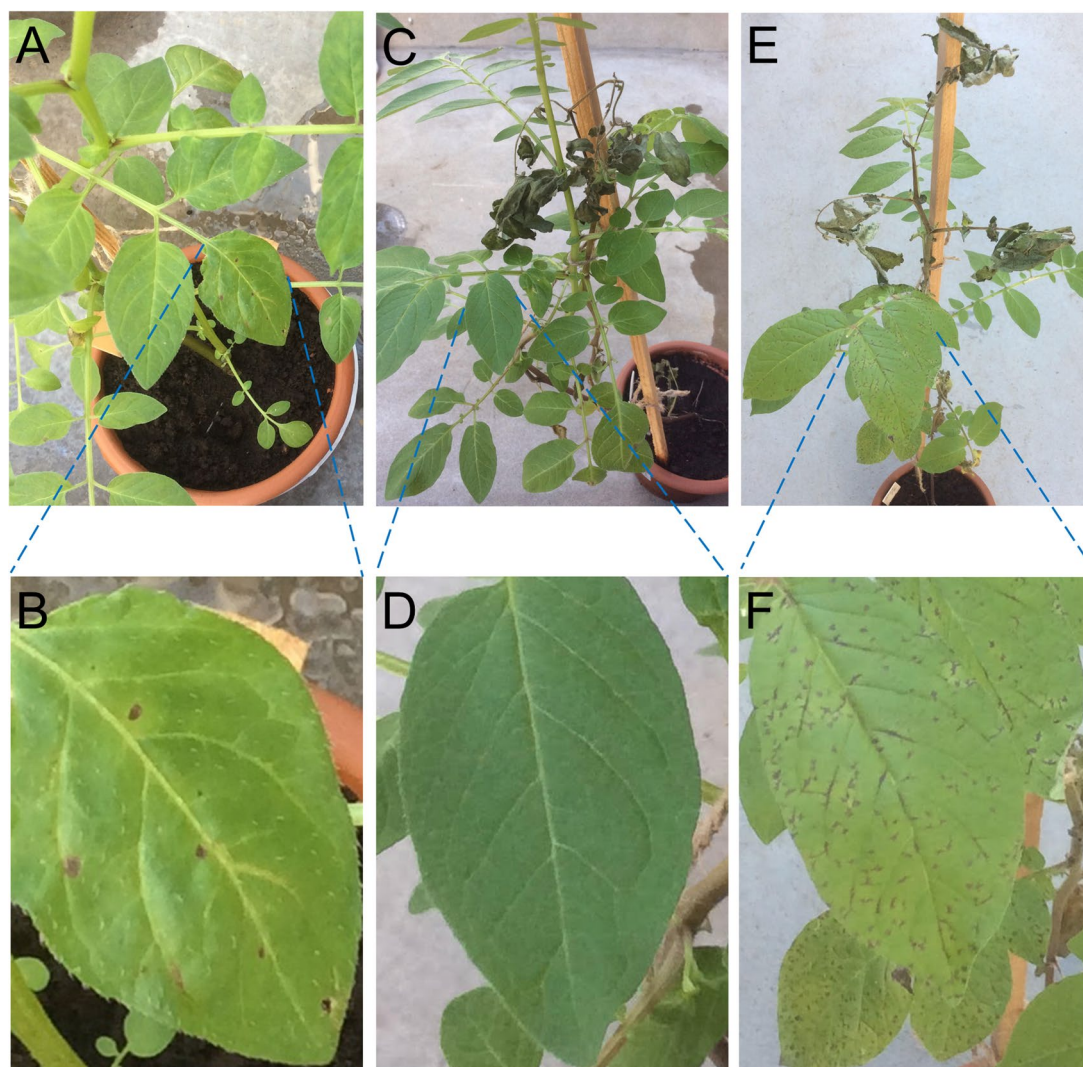
Fig. 1. Reaction of *S.chacoense* plants on PVY infection following mechanical inoculation. Extreme resistance – no systemic infection (**A**) with no lesions (not shown) or tiny necrosis on the inoculated leaf (**B**). Hypersensitive reaction – stem necrosis (**C**) with no systemic infection (**D**). Susceptibility – excessive stem necrosis (**E**) and systemic infection on leaves (**F**).

Phenotype	Number of plants	Genotypes	MG64-17	1648F24/ 1648R22
extreme resistance (ER)	29	W17, W19, W21, W23, W24, W26, W35, W50, W55, W56, W57, W59, W66, W67, W69, W70, W71, W72, W73, W74, W76, W78, W79, W80, W83, W84, W86, W88, W95	+[a]	+
hypersensitive reaction (HR)	9	W12, W15, W22, W25, W49, W51, W60, W64, W75	+	+
susceptibility	22	W07, W10, W13, W14, W16, W20, W27, W29, W30, W52, W54, W58, W61, W62, W65, W77, W81, W82, W96	-	-
		W08, W28	+	+
		W53	+	-

Table 2. Reaction of *S. chacoense* plants on PVY infection, and detection of Ry_{chc} using molecular markers. [a]«+» means the amplification of the marker, whereas «-» means its absence.

Fig. 2. Evaluation of some *S. chacoense* genotypes using molecular markers MG64-17 and 1648F24/1648R22. 897 bp band is expected for MG64-17 and 594 bp for 1648F24/1648R22. Marker - DNA Ladder 1 kb (Evrogen, Russia).

symptoms of viral infection and PVY accumulation in plant tissues. The remaining nine plants that developed stem necrosis without PVY accumulation were suggested to be resistant through a hypersensitive response mechanism (HR). Therefore, almost two-thirds of the investigated *S. chacoense* individuals were resistant to PVY. The ratio of resistant and susceptible individuals varied across the different *S. chacoense* accessions; 11 of the 14 plants in the 542 family (accession k-19769) were resistant, whereas none of 6 plants of 545 family (accession k-21849) and only 2 of the 9 plants in the 541 family (accession k-7394) were resistant, in accordance with previous study[34].

Evaluation of *S. chacoense* genotypes using molecular markers to Ry_{chc} gene

According to the phenotypical evaluation, the *S. chacoense* genotypes were relatively clearly divided into PVY-susceptible and PVY-resistant ones, whereas the latter presented no PVY accumulation and either a lack of symptoms or a hypersensitive response to infection. Since most resistant plants do not show any symptoms of infection after artificial inoculation and Ry_{chc}-mediated extreme resistance is the only known mechanism of PVY resistance in *S. chacoense* characterized molecularly, *S. chacoense* collection was screened for the presence of the Ry_{chc} gene using two PCR markers, MG64-17[22] and 1648F24/1648R22[25]. Both markers are located in the Ry_{chc} sequence, and a perfect correlation between the presence of the marker and PVY resistance was obtained in previous studies. Regardless of the type of resistance – hypersensitive response or extreme resistance – all PVY-resistant *S. chacoense* plants were uniformly positive for both MG64-17 and 1648F24/1648R22 (Table 2), which suggests that Ry_{chc} is present in these plants. The majority of the PVY-susceptible genotypes didn't demonstrate the amplification of neither MG64-17 nor 1648F24/1648R22, indicating the absence of Ry_{chc}. Unexpectedly, 2 susceptible genotypes, W08 and W28 (both originate from accession k-22638), were positive for both markers, whereas in another susceptible genotype, W53 (accession k-7394), only MG64-17 but not 1648F24/1648R22 was amplified (Table 2; Fig. 2). To check the specificity of the PCR, the marker amplicons were purified and Sanger sequenced. In W53, the MG64-17 sequence was similar but not identical to the previously published Ry_{chc}

sequence from 40−3 *S. chacoense*[22] and was clearly the result of misamplification of distinct *R* gene. In contrast, for both the MG64-17 and 1648F24/1648R22 primer pairs, the amplicon sequences from W08 and W28 plants were identical to the sequence of *Ry*$_{chc}$ from the 40−3 *S. chacoense* genotype (Figure S2). Therefore, *Ry*$_{chc}$ or at least its fragment is present in two PVY-susceptible genotypes from the investigated collection, W08 and W28. These genotypes belong to the k-22,638 accession with a contradictional taxonomy (see Materials and methods).

Amplification and SMRT sequencing of the full-length *Ry*$_{chc}$ genes

Despite the presence of the *Ry*$_{chc}$ gene, W08 and W28 plants were clearly susceptible to PVY, demonstrating virus accumulation in plant tissues, systemic necrosis at the whole-plant level and growth inhibition. Apparent non-functionality of *Ry*$_{chc}$ in some plants could be due to modifications of gene sequences, such as point mutations, deletions or recombinations. It is known that *R* genes are especially prone to diverse modifications and rearrangements, which make important contributions to the rapid evolution of these genes[35−37]. Targeted SMRT sequencing could be used to study full-length gene sequences from multiple plants using different barcodes. To amplify the full-length *Ry*$_{chc}$ sequences from *Ry*$_{chc}$-bearing *S. chacoense* plants, the previously developed primer pair STRG1648f-F/STRG1648f-R was used[25]. These primers flanked the whole *Ry*$_{chc}$ ORF, approximately 90 bp before the start codon and 900 bp after the stop codon. After optimization of the PCR conditions, the PCR fragment of the expected size was amplified from the most marker-positive *S. chacoense* genotypes (Table S2). However, for few marker-positive plants, no band or only a very weak band was obtained, possibly due to the difficulty of long fragment amplification from plant DNA samples contaminated with different PCR-inhibiting components. More surprisingly, in some *S. chacoense* genotypes that were negative for *Ry*$_{chc}$ markers, PCR products with sizes similar to the *Ry*$_{chc}$ amplicon length were amplified (Table S2). To improve the robustness and specificity of PCR, alternative primers were developed to amplify *Ry*$_{chc}$, with the forward primer at the beginning of the first exon and the reverse primer in the 3'-UTR (Figure S3). Amplification with this primer pair, named RyCDS-F/Ry5-R, was also suboptimal, with no bands in some *Ry*$_{chc}$-bearing *S. chacoense* plants, and amplification of the product of the correct size in some marker-negative PVY-susceptible plants (Table S2). However, using both the STRG1648f-F/STRG1648f-R and RyCDSF/Ry5-R primer pairs, for every marker-positive *S. chacoense* plant including two PVY-susceptible genotypes W08 and W28, amplicon of the correct size was obtained with at least one primer pair. We also tried some more primer pairs for full-length *Ry*$_{chc}$ amplification, but with poor efficiency and specificity (data not shown).
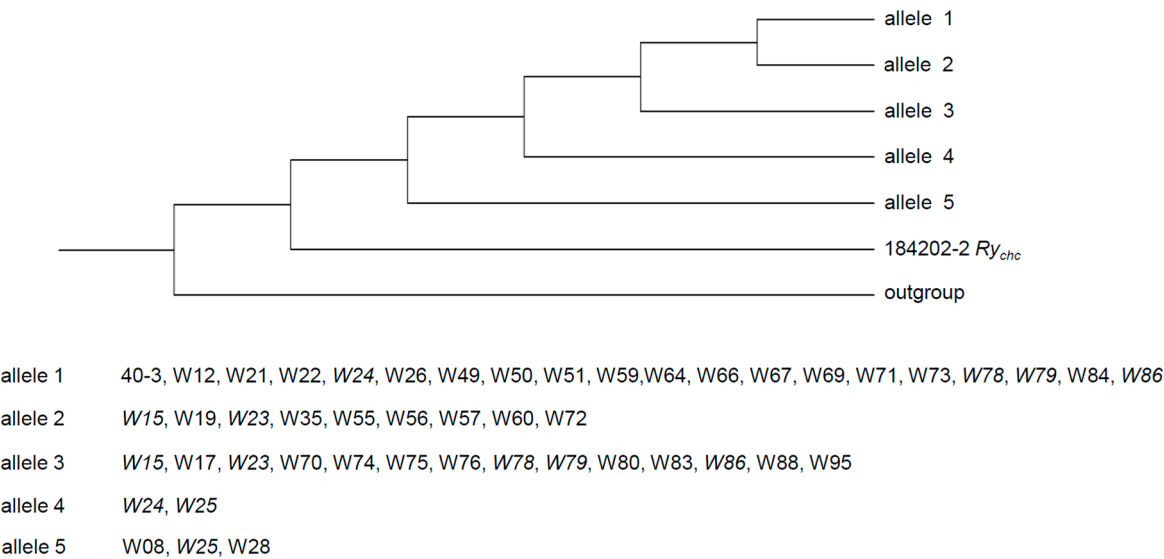
All PCR products of the correct size from either marker-positive or marker-negative plants were purified, individually barcoded and sequenced in parallel via MinION. In total, 88 PCR products from either one or two primer pairs were sequenced for 50 plants — all 38 PVY-resistant and 12 PVY-susceptible plants — including W08, W28 and ten marker-negative *S. chacoense* genotypes. Sequencing data were sorted to individual *S. chacoense* genotypes using the barcodes and analysed. Sequences very similar to those of previously published *Ry*$_{chc}$ genes were obtained from all marker-positive *S. chacoense* plants, either extremely resistant or hypersusceptible to the PVY, and also from PVY-susceptible genotypes W08 and W28. In contrast, no *Ry*$_{chc}$-like sequences were found in any of the 10 marker-negative *S. chacoense* plants. All sequences obtained from these PVY-susceptible genotypes were somewhat similar to *Ry*$_{chc}$ and represented another *R* genes (data not shown), which were apparently misamplified due to the sequence similarity with *Ry*$_{chc}$. Such superfluous *R* genes were also identified in sequenced amplicons from some *Ry*$_{chc}$-bearing plants, suggesting co-amplification of the *Ry*$_{chc}$ gene and some homologous *R* genes from these plants. However, the implemented approach was suitable to obtain *Ry*$_{chc}$ gene sequences from all marker-positive *S. chacoense* plants and to clearly distinguish them from any co-amplified genes at the stage of data analysis.

Diversity of *Ry*$_{chc}$ sequences in *S. chacoense* genotypes

Five alleles of *Ry*$_{chc}$ were found in this study (Fig. 3). Most genotypes from different *S. chacoense* accessions carry allele 1, which is equal to *Ry*$_{chc}$ from 40−3 *S. chacoense*[22]. Allele 2 has the single SNP relative to allele 1 (Supplementary File 1). It was discovered in fewer genotypes limited to accessions k-19759 and k-19769 (Table 1; Fig. 3). Allele 3 which was abundant and distributed between several accessions has several SNPs relative to allele 1 and small polymorphic region in the first intron with two adjacent deletions of 4 bp and 7 bp and three nucleotide substitutions (Supplementary File 1, Figure S4). *Ry*$_{chc}$ alleles 4 and 5 were detected in a small number of plants from the single accession k-22638. *Ry*$_{chc}$ alleles from all *S. chacoense* genotypes showed marked similarity. Besides the region in the first intron, no deletions or insertions were identified in the whole amplified *Ry*$_{chc}$ sequences, and only 19 SNPs were discovered between all identified genes in the whole *Ry*$_{chc}$ ORF (Supplementary File 1). Curiously, exons were enriched in SNPs relative to introns. Apart from the aforementioned polymorphic region in the first intron, as few as three SNPs were found in all three *Ry*$_{chc}$ introns with a total length of 701 bp, with two SNPs in the first intron and one in the third intron. In contrast, in four *Ry*$_{chc}$ exons with an overall length of 2730 bp, 16 SNPs were found. Among these 16 SNPs in exones, 9 were nonsynonymous, suggesting mild positive selection operating on the *Ry*$_{chc}$ sequence (dN/dS = 1,286).

Five *Ry*$_{chc}$ alleles encoded three protein variants, with three most widespread alleles 1, 2 and 3 encoded for the same protein. The predicted protein sequences of the different Ry$_{chc}$ variants were compared with each other and with previously published ones (Table 3, Supplementary File 2). There are 14 polymorphic amino acid residues between alleles from 40−3 *S. chacoense* accession[22] and 184202-2 dihaploid potato line[25]. Only four new polymorphic positions were identified in Ry$_{chc}$ protein variants from this study. 18 polymorphic positions were discovered in all currently investigated full-length Ry$_{chc}$ protein variants (Table 3). All protein sequences from this study were more similar to 40−3 Ry$_{chc}$ than to 184202-2 Ry$_{chc}$ (Table 3). Among the 3 identified protein variants, one was prevalent, whereas other two were minor. The most common variant was found in 37 out of 40 studied *S. chacoense* plants and in 37 of 38 PVY-resistant plants, both extremely resistant and hypersusceptible to PVY. It was equal to Ry$_{chc}$ previously identified in 40−3 *S. chacoense* accession (Table 3)[22]. The remaining 2

allele 1   40-3, W12, W21, W22, *W24*, W26, W49, W50, W51, W59,W64, W66, W67, W69, W71, W73, *W78*, *W79*, W84, *W86*

allele 2   *W15*, W19, *W23*, W35, W55, W56, W57, W60, W72

allele 3   *W15*, W17, *W23*, W70, W74, W75, W76, *W78*, *W79*, W80, W83, *W86*, W88, W95

allele 4   *W24*, *W25*

allele 5   W08, *W25*, W28

Fig. 3. Phylogenetic tree of all currently discovered Ry_{chc} alleles. Genotypes bearing each Ry_{chc} allelic variant are listed below the tree. Heterozygotes are highlighted in italics. Solyc09g092410.3 (Ensembl Plants database) was used as outgroup.

Genotypes	Position of amino acid substitutions																	
	124	128	131	135	149	159	161	168	173	210	217	233	376	502	518	587	624	719
40-3[a] W12, W15, W17, W19, W21, W22, W23, W24 (first allele), W25, W26, W35, W49, W51, W50, W55, W56, W57, W59, W66, W67, W69, W70, W71, W72, W73, W74, W76, W78, W79, W80, W83, W84, W86, W60, W64, W75, W88, W95	G	S	K	V	E	R	I	K	V	Y	I	A	K	R	Q	R	S	S
W8, W25 (first allele), W28	–	–	–	–	–	–	–	–	I	C	V	–	E	L	L	G	–	–
W24 (second allele), W25 (second allele)	–	–	–	–	–	K	–	–	I	C	V	–	E	–	–	–	R	–
184202-2[b]	D	E	Q	I	K	–	V	R	I	C	V	E	E	–	–	G	–	C

Table 3. Polymorphic amino acid positions in the predicted Ry_{chc} proteins identified in this and previous studies. [a]from Li et al.[22]. [b]from Akai et al.[25].

protein variants were identified for the first time. The next most frequent Ry_{chc} variant was found only in three *S. chacoense* plants, two of which, W08 and W28, were susceptible to PVY, and one, W25, mounted hypersensitive response on PVY infection. This variant has seven SAAPs (single amino acid polymorphisms) relative to the common Ry_{chc}, five of which are shared with 184202-2 Ry_{chc}, while two, R502L and Q518L, are unique (Table 3). Both W08 and W28 plants were homozygous for this allele, whereas W25 was heterozygous. Since both *S. chacoense* plants with this Ry_{chc} allele present in the homozygous state were susceptible to PVY, whereas all the other Ry_{chc}-bearing genotypes, and they only, were resistant, we concluded that this Ry_{chc} protein variant was nonfunctional against PVY infection. The remaining Ry_{chc} protein variant was found in two heterozygous W24 and W25 plants (Table 3), both of which were resistant to PVY. It differed from the most common Ry_{chc} variant with 6 SAAPs, of which 4 were shared with 184202-2 Ry_{chc}, while R159K and S624R polymorphisms were found only in this allele. While in W24 genotype this allele was in combination with common Ry_{chc} variant typical for resistant plants, in W25 it coexisted with apparently non-functional Ry_{chc} allele. W25 plant was resistant to PVY through HR, similarly to several plants homozygous for common Ry_{chc} allele (Table 1), which suggests that Ry_{chc} variant identified in genotypes W24 and W25 was functional against PVY infection despite of several polymorphisms relative to common Ry_{chc}. Collectively these results indicate that among two new Ry_{chc} protein variants identified in this study, one was functional against PVY infection while the other was nonfunctional. While multiple SAAPs were observed in 184202-2 Ry_{chc} protein and in several alleles in this study, only two polymorphisms R502L and Q518L found in the single Ry_{chc} allele apparently have drastic negative influence on Ry_{chc} functionality. According to the annotation of the domain organization of Ry_{chc}, the R502 and Q518 residues were located between the NB-ARC domain and the LRR domains of this protein (Figure S5). Since PCR markers MG64-17 and 1648F24/1648R22 locate in the end of the last exon and in 5'-UTR far outside the gene region bearing polymorphisms (Figures S1,S5), both markers cannot provide any discrimination between functional and nonfunctional Ry_{chc} alleles.
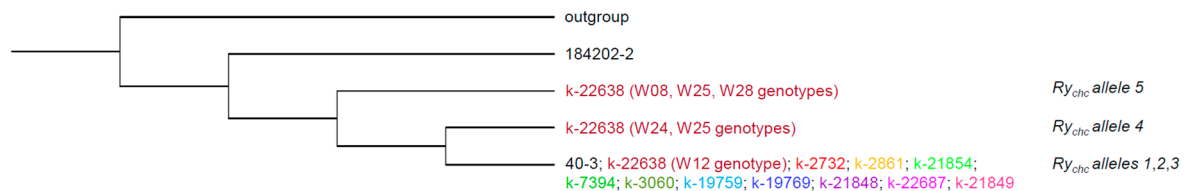
Fig. 4. Phylogenetic tree showing the distribution of different Ry_{chc} protein variants between various families of *S. chacoense* plants (different colors for different accessions). Proteins from the 40−3 *S. chacoense* and 184202-2 dihaploid potato line are also included. All families, except for the k-22638 family which comprise several protein variants, have a single Ry_{chc} variant. A0A3Q7I9U8 (UniProt database) was used as outgroup.

All *S. chacoense* genotypes bearing Ry_{chc} protein variants other than the most common, W08, W24, W25, and W28, belong to the same *S. chacoense* family 548, which was raised from the seeds of accession k-22638 (Fig. 4; Table 1). This family also comprises the W12 genotype, which has a "common" Ry_{chc} allele, and the W10 genotype, which lacks Ry_{chc}. Hence, all the biodiversity of the Ry_{chc} protein sequences identified in this study was limited to individuals of a single *S. chacoense* accession. K-22638 accession originated from tubers that were kindly provided to VIR collection by Prof. J. Hawkes, and this accession was attributed by him as *S. commersonii* Dun. This accession was subsequently reclassified as *S. chacoense* on the basis of its distinctive features, such as the petiolulate acute to acuminate leaflets, the terminal leaflet hardly larger than the laterals, and the uniformly white corolla. Certain forms of *S. chacoense* were erroneously identified as *S. commersonii* and have been reported in previous studies[38]. Variation of *S. chacoense* and *S. commersonii* complicates their taxonomy and several features common for both species have been reported in natural habitat. Both *S. chacoense* and *S. commersonii* are spread through several provinces of Argentina and Uruguay, and their representatives were included within diploid microspecies described by Bukasov and Hawkes[39,40]. The rare events of hybridization between *S. chacoense* and *S. commersonii* observed under natural and experimental conditions[39,40] indicate possible gene flow between these wild potato relatives. Taking this into account, the k-22638 accession seems to be an outlying *S. chacoense* variant genetically distinct from more typical plants of this species and close to *S. commersonii*, which could be a reason for the presence of nontypical Ry_{chc} alleles in this accession. To examine the possible presence of Ry_{chc} in *S. commersonii*, published genomes of this species (GenBank numbers GCA_001239805.1; GCA_018258275.1; GCA_029007595.1; GCA_029582365.1; GCA_029582665.1) were searched for the presence of Ry_{chc}-like genes. Gene very similar to Ry_{chc} was found in none of them. In contrast, in the published *S. chacoense* genome M6_v5.0 (SpudDB) the Ry_{chc} gene was found (Table S3). It is also cannot be excluded that the k-22638 accession is related to another wild potato species *S. malmeanum* Bitter. A recent report by Nicolao et al.[41] brings new light to this wild relative. Former taxonomic classification of *S. malmeanum* was rather complicated, due to geographical distribution patterns that partially overlap with *S. commersonii* and *S. chacoense* and several shared morphological traits[41]. Morphological variability of *S. malmeanum*, *S. commersonii* and *S chacoense* and non-consistent criteria used by different botanists could have leaded a mistake in classifying accessions of those sympatric species. Currently, there are no high-quality genome assemblies available for *S. malmeanum* to check for the presence of Ry_{chc} gene.

Discussion

In this study, phenotypic and genotypic evaluation of *S. chacoense* plants from multiple accessions demonstrated that the PVY resistance trait is widespread in *S. chacoense* and that this resistance is due to the presence of the Ry_{chc} gene. The abundance of PVY-resistant plants in this species is in accordance with previously published data ([42] and references therein). In addition to Ry_{chc}, the existence of some genes conferring HR to PVY was previously proposed in *S. chacoense*[43]. In this study, the presence of Ry_{chc} in all the PVY-resistant plants and the susceptibility of all the Ry_{chc}-negative plants strongly suggest that Ry_{chc} is the only factor associated with PVY resistance in the investigated *S. chacoense* collection. Since this gene was identified in 40 out of 60 *S. chacoense* plants of diverse origins, Ry_{chc}-mediated resistance could be the widespread mechanism of PVY resistance in this potato species. It was shown earlier that the most common mode of Ry_{chc} action is extreme resistance with a lack of visible necrosis, although in some cases, a hypersensitive response also occurs[20,22,25]. Both types of defense responses to PVY infection in Ry_{chc}-bearing *S. chacoense* plants were observed in this study. Extreme resistance was the most common, since less than one-fourth of the plants with resistance to PVY (9 of 38) developed significant necrotic lesions on leaves or the stem necrosis, indicating the hypersensitive response. A comparison of Ry_{chc} sequences from plants displaying either ER- or HR-type resistance to PVY revealed that Ry_{chc} genes were identical between them. Differences in the observed phenotype could therefore be related to the local variabilities in plant growing conditions, unequal infection or some inter-genotype variations influencing the mounting of defence responses. The dependence of *R* gene-based resistance on environmental conditions is a well-studied phenomenon[44,45]. Since the genes associated with extreme resistance belong to the same NB-LRR gene family as classical *R* genes conferring HR and seem to operate through at least partially similar mechanism[5,45–47], unfavourable conditions could also hinder the mounting of the ER, leading to delayed action, which cannot rapidly block virus spread. This results in the generation of visible necrosis instead of mounting resistance at the single-cell level[48].

In contrast to individuals with a hypersensitive response to PVY that demonstrated large necrosis but no PVY accumulation in plant tissues, two Ry_{chc}-bearing genotypes, W08 and W28, displayed systemic PVY

infection. We believe that this is the first time that plants with the Ry_{chc} gene were found to be susceptible to PVY. An earlier study indicated that some PVY multiplication could be observed in Ry_{chc}-bearing potato plants; however, this multiplication was detected only under especially unfavourable conditions, such as permanent high temperature, and systemic infection was rarely detected by ELISA[49]. Both the PVY-susceptible W08 and W28 genotypes had the same Ry_{chc} variant in the homozygous state. Apparently, two adjacent non-conserved amino acid changes, R502L and Q518L, which are unique to this allele, perturbed the functionality of Ry_{chc} protein to a sufficient extent to make it ineffective against PVY. It was previously shown that even a single amino acid change could have a drastic influence on the activity or specificity of NB-LRR proteins[44,50,51]. The reason for the existence of the apparently nonfunctional Ry_{chc} allelic variant is elusive. Among hundreds of NB-LRR genes present in the genomes of higher plants, many nonfunctional variants exist, which accumulate mutations that disturb their functionality[52]. This process of R gene attenuation is at least partially promoted by the pleiotropic effects inherent to some R genes, which makes the selection of such non-functional variants favourable in pathogen-free conditions[53–55]. Alternatively, the identified apparently inactive Ry_{chc} variant could have altered specificity and play a role in resistance to viruses other than PVY or to some specific PVY strains. The specificity of pathogen recognition by NB-LRR proteins is mainly defined by the LRR domain, which is the least conserved part of this class of proteins[56]. According to domain prediction, the R502L and Q518L polymorphisms from the susceptible Ry_{chc} allele are located adjacent to the LRR domain but outside of it. Ry_{chc} sequences were especially conserved in the second part of this gene (Supplementary file 1), and C-terminal LRR domain was almost identical between different Ry_{chc} variants (Table 3, Supplementary File 2). It is uncertain could the R502L and Q518L polymorphisms have any effect on pathogen recognition specificity, or they rather modify Ry_{chc} functionality in a different way. Curiously, the single amino acid change in the region between NB-ARC and LRR was sufficient to make the Arabidopsis R gene $SNC1$ constitutively active without interaction with pathogens[57].

S. chacoense is the most vigorous and adaptable of all the wild potato species in South America. Its natural distribution range is very extensive, from sea level to high mountains in Argentina, Paraguay, Uruguay, Brazil and Bolivia. There is morphological evidence for gene introgression from certain mountain species, such as *Solanum microdontum* Bitt., *Solanum tarijense* Hawkes, *Solanum spegazzinii* Bitt., *and Solanum kurtzianum* Bitt. et Wittm. ex Engl., that have resulted in a wider variety of *S. chacoense* habitats. Variation of *S. chacoense* and *S. commersonii* complicates their taxonomy and several features common for both species have been reported in natural habitat[39]. Despite the study of multiple *S. chacoense* plants obtained from diverse sources, which led to the identification of the unanticipated "susceptible" Ry_{chc} allele, the overall diversity of Ry_{chc} was low at both the gene and protein levels. Besides the single polymorphic region in the first intron, few SNPs were found in the whole Ry_{chc} ORF, especially in the non-coding regions. Only five Ry_{chc} alleles were found, with the most common allele was equal to the one previously discovered in 40–3 *S. chacoense*[22]. Moreover, three major Ry_{chc} alleles encoded the same protein. Two other minor Ry_{chc} alleles were discovered in only 4 out of 40 genotypes, all of which belong to the same accession k-22638. The pronounced allelic diversity of Ry_{chc} gene in plants of k-22638 accession apparently reflects its overall genetic distance from the other studied accessions, since k-22638 either represents an intermediate form between species or even belongs to species *S. commersonii* or *S. malmeanum* rather than to *S. chacoense*. Two minor Ry_{chc} protein variants had 6 or 7 polymorphisms relative to the common Ry_{chc} allele, whereas the previously identified 184202-2 Ry_{chc} had 14 polymorphisms (Table 3). In accordance with Akai et al.[25], an 184202-2 dihaploid line was obtained after crossing of several potato cultivars harboring the Ry_{chc} gene from the 'Konafubuki' cultivar which, in turn, received it from a doubled *S. chacoense* plant[24]. Among all Ry_{chc} protein variants identified to date, Ry_{chc} from 184202-2 is the most distinct from the common Ry_{chc} variant. Even in *S. chacoense* plants of the accession k-22638, which seems to be rather unique and close to *S. commersonii* or *S. malmeanum*, Ry_{chc} variants were less different from the common Ry_{chc} than 184202-2 Ry_{chc} was (Table 3). This could suggest that the ancestral doubled line used in the development of 'Konafubuki' was rather unusual among *S. chacoense* species. Alternatively, 184202-2 Ry_{chc} sequences readily accumulated multiple point mutations after transfer from ancestral doubled *S. chacoense* line to potato cultivars. Rapid accumulation of mutations could be related to any pleiotropic effects, such as autoimmunity, that are frequently observed for plant R genes and largely depend on the genetic background[55,58]. Several polymorphisms were shared between 184202-2 Ry_{chc} and alleles from the k-22638 accession (Table 3), suggesting their common origin. The low overall diversity of Ry_{chc} sequences and the prevalence of the most common Ry_{chc} allele, which is effective against multiple PVY strains in different genetic backgrounds, favour the use of *S. chacoense* in potato breeding programs as a source of PVY resistance. Additional studies are needed to find out the taxonomic determination of the k-22638 accession from Russian potato germplasm collection and to check the possible involvement of Ry_{chc} genes in resistance to PVY in species close to *S. chacoense*, namely *S. malmeanum* or *S. commersonii*.

One of the most serious drawbacks of the use of molecular markers in plant breeding is the lack of full linkage between the marker and trait of interest because the marker locus and trait locus are typically located proximally but are not the same. However, for the Ry_{chc} gene, two recently introduced PCR markers are located within its sequence[22,25], ensuring full linkage between the marker and the gene. This was confirmed in this study because all marker-positive *S. chacoense* plants were also Ry_{chc} positive, except for the single W53 genotype, in which one marker provided misleading results due to the misamplification of different R gene. However, the use of markers was insufficient to reliably predict PVY resistance in *S. chacoense* plants because of the existence of an apparently non-functional Ry_{chc} allele. To address this concern, modern sequencing technologies provide relatively simple and easy approach for studying full-length genes from multiple biological samples simultaneously. Molecular markers could be used to initially identify genes of interest in individual plants before attempting to amplify the full-length gene sequences. If closely linked markers were not developed but the expected gene sequence is available, several primer pairs could be used to amplify short gene fragments, as was recently done to identify the Ry_{sto} gene in multiple accessions of different *Solanum* species[27]. After this initial evaluation, full-length

amplification of target genes, such as Ry_{sto} or Ry_{chc} using several primer pairs is applicable to isolate the gene of interest from different plants ([27] and this study). Both primer pairs used in this study lead to some false-positive Ry_{chc} amplification from plants that do not contain this gene and to co-amplification of some additional *R* genes together with Ry_{chc}. However, such misamplified genes could be identified and omitted at the stage of data analysis. Although PacBio is frequently used for targeted long-fragment sequencing, the use of more assessable MinION allows similar results to be achieved[59]. Since both technologies allow the sequencing of full-length genes as single molecules without fragmentation on small pieces, there are no hurdles related to the sorting of short DNA pieces between highly homologous genes upon data analysis.

Conclusions

In this study, the allelic diversity of Ry_{chc} gene was examined in relation to resistance to PVY in a diverse collection of potato plants related to *S. chacoense* and, in case of k-22638 accession, possibly to other potato species. This investigation revealed that resistance to PVY is widely distributed in this species and is associated with the Ry_{chc} gene. Overall allelic variation of Ry_{chc} was low and in terms of coding sequence was restricted to the single *S. chacoense* accession, which is closely related to another wild potato species *S. commersonii* or *S. malmeanum*. In a few plants, a Ry_{chc} allele incapable to provide PVY resistance was discovered. While previously developed molecular markers cannot discriminate this allele, amplicon targeted sequencing was applied to investigate it. This approach allows for easy and robust identification of Ry_{chc} sequences in multiple plant genotypes.

Our results emphasize the importance of the relevance, accuracy and completeness of information in plant gene bank management. Challenges related to differences between potato classification systems have been noticed in the Global Strategy for the Conservation of Potato, which was developed through a collaborative effort of potato collection curators from 32 genebanks and their partners in potato research[60]. The international community has recognized the evaluation and characterization of potato accessions as a priority measure towards a harmonization of potato taxonomy. A comprehensive study of k-22638 accessions remains to be conducted in future research. Plant morphology and genetic variability of *S. chacoense* and related wild species preserved at the Russian potato genebank require a more thorough characterization. The sequence data obtained in our study linked to passport information are essential for gaining a deeper understanding of the diversity of potato genes involved in virus resistance.

Data availability

Gene and protein sequences are available under GenBank numbers PV815852, PV815853, PV815854, PV815855 and in Supplementary Files 1 and 2.

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NZ and ER conceived and designed the experiment. ER and AG participated in sample preparation. AA and NZ performed molecular studies. AA analysed the sequencing data. NZ and ER wrote the manuscript. AG and AA corrected the manuscript. All authors read the manuscript and approved it for publication.

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Declarations

Competing interests

The authors declare no competing interests.

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