Finding *RB/Rpi-blb1/Rpi-sto1*-like sequences in conventionally bred potato varieties

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The main objectives in potato breeding are increasing yield abilities and improving resistance to numerous pathogens and pests. Among them, the late blight caused by the *Phytophthora infestans* oomycete is one of the most destructive potato diseases both in Russia and worldwide. Wild relatives of cultivated potato are traditionally used in breeding as the source of valuable *R* genes conferring resistance to pathogens. Of particular interest are Mexican wild species because Mexico is the centre of origin and diversity of *P. infestans* and at the same time, it is the centre of potato species diversity. Mexican wild potato species *S. bulbocastanum* and *S. stoloniferum* are an important source of the *R* genes conferring broad-spectrum resistance against various isolates of *P. infestans* (*Rpi-blb1*, *Rpi-blb2*, *Rpi-sto1*). Recently these genes have been transferred into cultivated potato gene pool using the cisgene approach. At the same time there is a high probability of finding genotypes with the *Rpi-sto1* gene (functional homologues of *Rpi-blb1*) among conventionally bred varieties because for about 40 years *S. stoloniferum* has been used in breeding as a source of the Ry_{sto} and $Ry\text{-}f_{sto}$ genes of the extreme resistance to the most important viral pathogen PVY. In this study 188 potato varieties bred in Russia and in near-abroad countries were screened for the presence of six gene-specific markers of the *RB/Rpi-blb1 = Rpi-sto1* and *Rpi-blb2* genes conferring broad-spectrum resistance against *P. infestans*, and for the markers linked to the Ry_{sto} and $Ry\text{-}f_{sto}$ genes conferring extreme resistance to PVY. In addition, a marker for detecting male sterile mitochondrial DNA type gamma derived from *S. stoloniferum* was used. The genotypes selected through the molecular markers were divided into four groups: (A) 13 PVY resistant varieties carrying diagnostic markers of the Ry_{sto}, $Ry\text{-}f_{sto}$ genes and having sterile mt-type gamma; (B) four varieties possessing mt-type gamma and not having the markers of the *R* genes introgressed from *S. stoloniferum*; (C) eight genotypes carrying five gene-specific markers for the *RB/Rpi-blb1/= Rpi-sto1*; (D) the rest 166 (86.9 %) varieties not possessing any of the diagnostic markers associated with the *S. stoloniferum* genetic material. The sequences of the *Rpi*-sto1- and BLB1 F/R-amplicons were identical in all the genotypes of group 'C' and showed respective 99 % and 100 % similarity to the corresponding fragments of the *Rpi-sto1* and *Rpi-blb1* genes from the GenBank database. Among the genotypes of group 'C' various mt-types were detected, and some of them were male fertile.

Key words: potato; *Solanum stoloniferum*; marker assisted selection; *R* genes; *RB/Rpi-blb1/Rpi-sto1*; male sterility.

Последовательности, гомологичные участкам гена *RB/Rpi-blb1/Rpi-sto1*, у сортов картофеля, созданных методами традиционной селекции

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Традиционные задачи селекции картофеля – повышение урожайности и устойчивости к многочисленным патогенам и вредителям. Из них наибольший ущерб картофелеводству как в России, так и мире наносит фитофтороз, вызываемый оомицетом *Phytophthora infestans*. Дикие виды картофеля используются в селекции в качестве источников *R* генов устойчивости к патогенам. Особый интерес представляют мексиканские виды, поскольку Мексика – центр происхождения и разнообразия *P. infestans* и центр разнообразия видов картофеля. Дикие мексиканские виды картофеля *S. bulbocastanum* и *S. stoloniferum* являются источниками *R* генов устойчивости к широкому спектру рас *P. infestans* (*Rpi-blb1*, *Rpi-blb2*, *Rpi-sto1*). В последние годы эти гены были интрогрессированы в геном культурного картофеля с использованием методов цис-генетики. В то же время высока вероятность выявления генотипов с геном *Rpi-sto1* (функциональный гомолог *Rpi-blb1*) у сортов, созданных методами традиционной селекции, поскольку уже около 40 лет селекционеры используют *S. stoloniferum* в качестве источника устойчивости к наиболее вредоносному вирусу картофеля – PVY. В настоящей работе проведен молекулярный скрининг 188 сортов картофеля российской селекции и стран ближнего зарубежья с ген-специфичными маркерами *RB/Rpi-blb1*, *Rpi-sto1* и *Rpi-blb2*; маркерами, сцепленными с генами Ry_{sto}, $Ry\text{-}f_{sto}$, детерминирующими устойчивость к PVY, и маркером митотипа gamma, ассоциированного с мужской стерильностью *S. stoloniferum* гибридов. Отобранные в молекулярном скрининге генотипы могут быть разделены на четыре группы: (A) 13 устойчивых к PVY сортов с диагностическими маркерами генов Ry_{sto}, $Ry\text{-}f_{sto}$ и со стерильным мт-типом gamma; (B) четыре сорта с мт-типом gamma, не обладающие маркерами *R* генов устойчивости, интрогрессированых от *S. stoloniferum*; (C) восемь генотипов, у которых были детектированы все пять ген-специфичных маркеров гена *RB/Rpi-blb1/Rpi-sto1*; (D) оставшиеся 166 (86.9 %) сортов выборки, у которых не были выявлены маркеры *R* генов устойчивости *S. stoloniferum* и митотип

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gamma. Последовательности ПЦР продуктов, полученные при амплификации с ген-специфичными праймерами Rpi-sto1 и BLBF/R, у всех генотипов группы С были идентичны и имели 99 и 100 % сходства с соответствующими фрагментами референсных последовательностей генов *Rpi-sto1* и *Rpi-blb1* из GenBank. В группе С выявлены генотипы с различными мт-типами, среди них – образцы с мужской фертильностью.

Ключевые слова: картофель; *Solanum stoloniferum*; маркер-вспомогательный отбор; *R* гены; *RB/Rpi-blb1/Rpi-sto1*; мужская стерильность.

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The common potato (*Solanum tuberosum* L.) is the first non-grain food crop in the world and in Russia. Potato varieties are susceptible to many diseases and pests so the main objectives in potato breeding are increasing yield abilities and resistance to numerous fungal, viral, bacterial pathogens and pests. Among them, late blight caused by the *Phytophthora infestans* oomycete remains the most destructive disease that decrease potato production both worldwide (Haverkort et al., 2008, 2009, 2016) and in Russia (Elanskij, 2015). To control late blight and prevent yield losses, potato cultivation requires frequent fungicide treatments, e. g. in Northern Europe such treatments are performed from 10 to 15 times a year and up to 25 times – in humid summers (Fact Series..., 2014). Growing of potato varieties with durable resistance against late blight is one of the main strategies of reducing the use of chemical fungicides.

Breeding potatoes resistant to *P. infestans* has been continuing for about 100 years. Various wild potato species have been found to be resistant to late blight and some of them have been actively used in breeding (Hanneman, Bamberg, 1986; Zoteyeva et al., 2012). Among them Mexican potatoes are of particular interest because the central regions of Mexico are considered to be a center of genetic diversity both for *P. infestans* and for wild potato species (Hawkes, 1990; Fry et al., 1993; Goss et al., 2014). *Solanum demissum*, indigenous to Mexico, served as an initial source of late blight resistance genes (named *R1* to *R11*) in potato breeding. These *R* genes conferring race-specific resistance against *P. infestans* were introgressed from *S. demissum* into the gene pool of *S. tuberosum* through interspecific crosses and conventional breeding in the first part of the last century (Malcolmson, Black, 1966). However, this race-specific resistance was quickly overcome by specific *P. infestans* strains (Wastie, 1991; Fry, Goodwin, 1997; Fry, 2008). In the subsequent decades, the efforts of breeders were aimed at a search for new sources of race-non-specific durable resistance against *P. infestans* that were found in many wild potato species (Ross, 1986). However, breeding of new varieties with race-non-specific resistance is time-consuming and laborious because of polygenic inheritance, as QTLs for this type of resistance were mapped on all the twelve potato chromosomes (Simko, 2002).

The *R* genes conferring broad-spectrum resistance against various *P. infestans* isolates have been found in several Mexican species that are highly resistant to late blight but less easy crossable with cultivated potato in comparison with *S. demissum*, e. g. broad spectrum resistance genes were identified in Mexican diploid species *S. bulbocastanum* – the *RB* gene also known as *Rpi-blb1* (Naess et al., 2000; Song et al., 2003; van der Vossen et al., 2003) and the *Rpi-blb2* (van der Vossen et al., 2005), both encode CC-NB-LRR proteins (Vleeshouwers et al., 2011). The interspecific incompatibility between *S. bulbocastanum* and *S. tuberosum* was overcome using ploidy manipulations, interspecific bridge crosses with the other wild species and subsequent backcrossing of the complex hybrids (Hermsen, Ramanna, 1973). As a result of long-term conventional breeding process that lasted for 46 years, the *Rpi-blb2* gene from *S. bulbocastanum* has been introgressed into a common potato gene pool and two late blight resistant varieties carrying this gene – Bionica and Toluca – have been developed (Haverkort et al., 2009). The *RB/Rpi-blb1* gene was introgressed into the *S. tuberosum* genome through somatic hybridization (Helgeson et al., 1998).

The functional homologues of the *S. bulbocastanum RB/Rpi-blb1* gene were detected in Mexican allotetraploid species *S. stoloniferum* (= *S. papita*, *S. polytrichon*): *Rpi-sto1*, *Rpi-plt1*, *Rpi-pta1*, *Rpi-pta2* (Vleeshouwers et al., 2008; Wang et al., 2008; Lokossou et al., 2010). M. Wang with colleagues (2008) suggested that *S. bulbocastanum* is one of the progenitors of *S. stoloniferum*.

The *RB*/*Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* and *Rpi-sto1* from *S. stoloniferum* were mapped and cloned (Song et al., 2003; van der Vossen et al., 2003, 2005; Vleeshouwers et al., 2008). After that a transgenic approach using genes from crossable species (cisgenesis) was developed to improve late blight resistance in cultivated potato (Haverkort et al., 2009, 2016). Genetically modified (GM) cisgenic potato clones carrying a single *Rpi*-gene demonstrated only partial resistance to the aggressive isolates of *P. infestans*, whereas cisgenic GM clones containing *Rpi* gene combinations had a high level of broad-spectrum resistance (Haverkort et al., 2016). However, cultivation of such resistant genotypes (e. g. of cisgenic *Phytophthora*-resistant variety ‘Fortuna’ having the *Rpi-blb1* and *Rpi-blb2* genes) has still been a questionable issue because cisgenic plants remain under GMO regulation in the EU (Haverkort et al., 2016; van Hove, Gillund, 2017). At the same time, conventionally bred varieties Bionica and Toluca with the introgressed *Rpi-blb2* gene can be cultivated in the EU without any limitations.

It is interesting to note that *S. stoloniferum,* a wild Mexican species with functional homologues of the *RB/Rpi-blb1* gene, can be directly crossed with cultivated potato (Jackson, Hanneman, 1999), but this wild species has not been actively involved in a breeding program the directed on broad-spectrum late blight resistance. The efforts of breeders were usually focused on *S. stoloniferum* as a source of extreme resistance to potato virus Y (PVY) to be the most important viral pathogen of cultivated potato (Ross, 1986). Many West-European varieties have been developed based on interspecific hybrids *S. stoloniferum* × *S. tuberosum*. They inherit the Ry_{sto} and/or $Ry\text{-}f_{sto}$ genes from *S. stoloniferum*, both conferring extreme resistance to PVY (Flis et al., 2005; Song, Schwarzfischer, 2008). According to literature, the gene Ry_{sto} has always been associated with mitochondrial type (mt-type) gamma and with maternally inherited male sterility (Song, Schwarzfischer, 2008).

In the Russian Federation are mainly two centers bred potato varieties from interspecific hybrids with *S. stoloniferum.* These are (1) A.G. Lorkh All-Russian Potato Research Institute (VNIIKH) located in Moscow region, whose efforts are focused on developing PVY resistant material and (2) Leningrad Scientific Research Institute "Belogorka" (LenNIISKh 'Belogorka') located in north-west region of Russia whose efforts are concentrated on developing of late blight resistant material. Recently, we screened the 39 cultivars and breeding clones developed in the LenNIISKh 'Belogorka' and selected five genotypes carrying gene-specific markers for *RB/Rpi-blb1* = *Rpi-sto1*; three of these genotypes were bred up to the variety level (Gavrilenko et al., 2018).

The objectives of the present study were to screen a wider subset of 188 potato varieties with the markers of the *R* genes originating from *S. stoloniferum* and to provide evidences of the presence of *RB/Rpi-blb1* = *Rpi-sto1*-like sequences in the selected varieties and breeding clones.

Material

The one hundred eighty five varieties chosen for this study were obtained from the national potato germplasm collection maintained at VIR. Special attention was paid to the varieties having *S. stoloniferum* hybrids in their pedigrees. The pedigree records were received from different sources (Simakov et al., 2007; Yashina et al., 2010; Russian Varieties..., 2011; Kostina, et al., 2016; Potatoes…, 2016). According to the published data *S. stoloniferum* was involved in the pedigrees of 28 varieties (their names are underlined – see below); most of them show extreme or high resistance to PVY. It was also possible that *S. stoloniferum* had participated in the origin of more than these 28 varieties because many cultivars had unknown ancestors or their pedigree records indicated interspecific hybrids of unknown origin. The following cultivars were subjected to molecular screening: Aksamit, Al'pinist, Alena, Alisa, Ametist, Amur, Antoshka, Arhideja, Arlekin, Avrora, Babushka, Barin, Baron, Belosnezhka, Beluha, Bezhickii, Bol'shevik, Bolvinskij, Borodjanskij rozovyj, Brat-2, Bravo, Brjanskaja novinka, Brjanskij delikates, Brjanskij krasnyj, Brjanskij nadezhnyj, Brjanskij rannij, Bronnickij, Buket, Chaja, Chajka, Divo, Doncovskij, Druzhnyj, Falenskij, Fermer, Filatovskij, Fioletovyj, Fokinskij, Garant, Gart, Golubizna, Gorizont, Gorjanka, Gornoural'skij, Granat, Gubernator, Hibinskij rannij, Il'inskij, Imandra, Impala, Irbitskij, Iskra, Javar, Jeffekt, Jenergija, Jubilej Zhukova, Jubilejnyj Osetii, Jupiter, Kabardinskij, Kalinka, Kamenskij, Kameraz, Katjusha, Kemerovchanin, Kemerovskij, Kolobok, Kolpashevskij, Komsomolec 20, Korenevskij, Kormilec, Korona, Kortni, Krasavica, Krasnaja gorka, Krasnaja roza, Krasnaja zarja, Krasnoufimskij, Krepysh, Kristall, Kustarevskij, Kuznechanka, Ladozhskij, Lajmdota, Lakomka, Lasunak, Lazar', Lazurit, Lekar', Lider, Ljubava, Ljuks, Lorh, Loshickij, Lugovskoj, Lybid', Manifest, Mats, Matushka, Maugli, Meteor, Moskvoreckij, Murmanskij, Musinskij, Nadezhda, Nakra, Nal'chikskij, Naroch', Nart-1, Narymka, Nauka, Nesterovskij, Nezabudka, Nikulinskij, Odissej, Ognivo, Oktjabrenok, Olimp, Parus, Pobeda, Pogarskij, Prestizh, Pribrezhnyj, Priekul'skij rannij, Prigozhij 2, Pri12 (Primorskij), Priobskij, Prizer, Prolisok, Ramzaj, Rapsodija, Rassvet, Resurs, Rezerv, Rjabinushka, Romashka, Rosinka, Rossijanka, Rumjanka, Rusalka, Rusich, Sambo, Saprykinskij, Sarovskij, Sentjabr', Severjanin, Shaman, Shurminskij 2, Sineva, Sintez, Skarb, Skoroplodnyi, Smena, Sokol'skij, Solnyshko, Start, Svenskij, Svetljachok, Tango, Temp, Teshha, Tomich, Udacha, Ukrainskij rozovyj, Uspeh, Utenok, Varmas, Varsna, Vektar belorusskij, Veselovskij 2-4, Veteran, Virazh, Viza, Vjatka, Volzhskij, Vympel, Zagadka, Zarevo, Zaural'skij, Zdabytak, Zhavoronok, Zhigulevskij, Zhivica, Zhukovskij rannij, Zol'skij, Zvezdochka.

One hundred eighty five varieties of the studied subset had been developed and released by different Russian public institutions, and breeding stations of various geographical locations and in neighboring countries. This subset did not include the 33 varieties bred in LenNIISKh 'Belogorka', since the results of their molecular screening had already been published (Gavrilenko et al., 2018).

The analyzed subset also included five additional genotypes selected earlier for having three gene-specific markers – Rpi-sto1, 1/1′, BLB1F/R (varieties Sudarynja, Evraziya, Baltijskij and breeding clones 1604/16, 1101/10) (Gavrilenko et al., 2018). In the present study these five additional genotypes were involved into sequence analysis and were screened for gene-specific markers covering the other regions of the target gene *RB/Rpi-blb1* = *Rpi-sto1*. These three varieties and two breeding clones originated from the *S. stoloniferum* hybrids, and they all were bred in LenNIISKh 'Belogorka' (Gavrilenko et al., 2018). New perspective breeding clone 3602/28 of the same origin was also involved in molecular screening. In total experimental subset included 191 genotypes (188 varieties and three breeding clones).

The highly late blight resistant genotype of wild species *S. stoloniferum* (seedling from accession PI 205522) with the diagnostic markers of the *Rpi-sto1*, Ry_{sto}, $Ry\text{-}f_{sto}$ genes (Levy et al., 2017) and variety Toluca with the *Rpi-blb2* gene were used as positive controls.

Methods

DNA isolation and marker assisted selection (MAS). Genomic DNA was isolated from young leaves of the field grown plants following the modified CTAB method (Gavrilenko et al., 2013). Six gene-specific primers for the *Rpi-sto1*, *RB*, *Rpi-blb1*, and *Rpi-blb2* genes developed by different authors (Table 1) were used in this study. This set included one primer pair 1/1′ specific for the *Rpi-blb1* functional allele (Colton et al., 2006). The location of *RB/Rpi-blb1* = *Rpi-sto1* gene-specific primers is indicated in the schematic diagram (Fig. 1).

We also used STS marker YES3-3A and CAPS marker GP122-406 linked with genes Ry_{sto} and $Ry\text{-}f_{sto}$, respectively. The markers had been earlier validated for MAS of West-

Table 1. Markers of *R* genes and mt-types used in this study

No.	Target resistance gene (chromosome)	Name of the DNA marker	Primer sequences (forward and reverse primer)	Tm, °C	PCR product size, bp	References
			Gene-specific markers for the *Rpi-sto1*, *RB*, *Rpi-blb1*, *Rpi-blb2*			
1	*Rpi-sto1* (VIII)	Rpi-sto1	F: ACCAAGGCCACAAGATTCTC R: CCTGCGGTTCGGTTAATACA	65	890	Zhu et al., 2012
2	*RB* (VIII)	1/1′	F: CACGAGTGCCCTTTTCTGAC R: ACAATTGAATTTTTAGACTT	50	213	Colton et al., 2006
3	*Rpi-blb1* (VIII)	BLB1 F/R	F: AACCTGTATGGCAGTGGCATG R: GTCAGAAAAGGGCACTCGTG	58	821	Wang et al., 2008
4	*Rpi-blb1* (VIII)	517/1519	F: CATTCCAACTAGCCATCTTGG R: TATTCAGATCGAAAGTACAACG	58	651	»
5	*RB/Rpi-blb1* (VIII)	RB-629	F: GAATCAAATTATCCACCCCAACTTTTAAAT R: CAAGTATTGGGAGGACTGAAAGGT	65	629	Pankin et al., 2011
6	*Rpi-blb2* (VI)	Blb2F/R	F: GGACTGGGTAACGACAATCC R: AGCACGAGTTCCCCTAATGC	58	773	Lokossou et al., 2010
			Markers linked to the genes conferring extreme resistance to PVY originating from *S. stoloniferum*			
7	Ry_{sto} (XII)	YES3-3A	F: TAACTCAAGCGGAATAACCC R: AATTCACCTGTTTACATGCTTCTTGTG	55	341	Song, Schwarzfischer, 2008
8	$Ry\text{-}f_{sto}$ (XII)	GP122-406/EcoRV	F: CAATTGGCTCCCGACTATCTACAG R: ACAATTGCACCACCTTCTCTTCAG	52	406	Flis et al., 2005; Valkonen et al., 2008
			Marker used for detection of different mt-types			
9	*rps 10* locus of mtDNA	ALM_4/ALM_5	AAT AAT CTT CCA AGC GGA GAG AAG ACT CGT GAT TCA GGC AAT	55	alpha – 2400, beta – 1600, gamma – "–"	Lössl et al., 2000

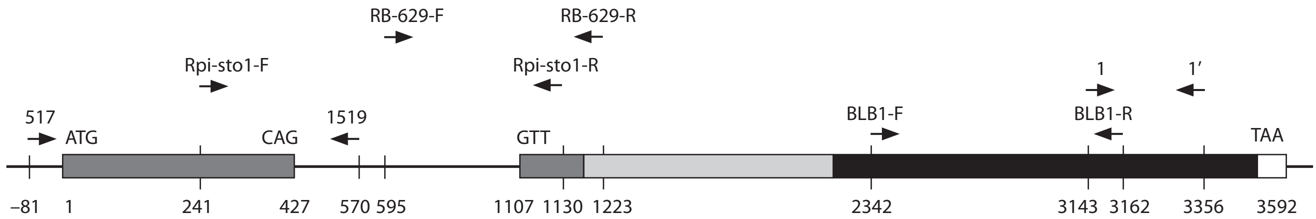

Fig. 1. A structure of the *RB/Rpi-blb1 = Rpi-sto1* gene and location of five gene-specific primers used in this study: Rpi-sto1, BLB1F/R, 1/1′, 517/1519, RB-629.

Bold lines indicate exon 1 (1–427 bp) and exon 2 (1107–3592 bp). Thin lines indicate intron, upstream and downstream regions. Nucleotide numbering begins from the start codon and includes the intron sequence. Regions corresponding to the CC-, NBS- and LRR- domains are highlighted in gray, light gray, and black, accordingly. The arrows show the regions of primer annealing; numbers under the arrows correspond to the position of the first nucleotide on the 5′ end of the primers. The names of primers are indicated above the arrows.

European PVY resistant varieties (Song, Schwarzfischer, 2008; Valkonen et al., 2008). The different mitochondrial DNA types (mt-types) were identified with the specific primers ALM_4/ ALM_5 developed by A. Lössl et al. (2000) (see Table 1).

The primers were synthesized by Evrogen (Moscow, Russia) (http://evrogen.ru). PCR reactions were performed in a total volume of 20 μl containing 40 ng DNA template, 1 × PCR reaction buffer (Dialat, http://dialat.ru) with 2.5 mM $MgCl_2$, 0.6 mM of each dNTP (Dialat), 0.2 μM of forward/reverse primer and 1 U Taq polymerase (Dialat). PCR-conditions for the *RB/Rpi-blb1* and *Rpi-sto1* gene-specific primer pairs were used as described in the original articles (see Table 1). PCR-conditions for primer pairs YES3-3A and GP122-406/ EcoRV were modified by the use of the touchdown option.

Each PCR reaction was repeated at least three times. In the case of positive results with markers for gene *RB/Rpi-blb1 = Rpi-sto1*, MAS was repeated with independently extracted DNA samples. A reaction mixture with water instead of DNA template was used as a negative control. PCR products were separated by electrophoresis in 2.0 % agarose gels, stained with ethidium bromide and visualized in UV light.

Sequence analysis. The Rpi-sto1- and BLB1 F/R-amplicons from the genotypes selected in MAS were purified with the Cleanup Standard Kit (Eurogen, #BC022, http://evrogen.ru) and sequenced in both directions on 24-capillary 3500xL Genetic Analyzer (Applied Biosystems) using equipment of Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' in ARRIAM. Alignment of nucleotide sequences and their analysis were conducted using software Unipro UGENE version 1.29.0 (Okonechnikov et al., 2012) and BioEdit Version 7.1.9 (Hall, 1999). The obtained sequences were compared against the ones of the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/).

Results

One hundred ninety one genotypes of the analyzed subset were screened using the DNA markers associated with the Ry_{sto}, $Ry\text{-}f_{sto}$, *RB/Rpi-blb1* = *Rpi-sto1* genes and with mt-type gamma originating from *S. stoloniferum*. Based on the screening results this subset was divided into four groups A–D (Table 2).

Group A included 13 varieties all carrying the diagnostic markers GP122-406/EcoRV and YES3-3A_321 tightly linked to the $Ry\text{-}f_{sto}$ and Ry_{sto} genes. According to literature, these varieties are either extremely resistant or resistant to PVY; their pedigree records indicate that they descended from the *S. stoloniferum* hybrids (Simakov et al., 2007; Yashina et al., 2010; Biryukova et al., 2015). All the varieties of group A have mt-type gamma (see Table 2) inherited from *S. stoloniferum* (Lössl et al., 2000). These varieties were characterized by male sterility. None of the diagnostic markers for *RB/Rpi-blb1* = = *Rpi-sto1* and for *Rpi-blb2* were detected in group A.

Table 2. Molecular screening results in a subset of 191 accessions

No.	Name of variety	Presence (1) or absence (0) of the diagnostic markers associated with the genetic material introgressed from *S. stoloniferum*							
		$Ry\text{-}f_{sto}$	Ry_{sto}	*RB/Rpi-blb1/Rpi-sto1*					mt-type
		GP122-406/EcoRV	YES3-3A	Rpi–sto1	BLB1F/R	1/1′	517/1519	RB-629	ALM4/5
	Group A: varieties with PVY – resistance genes markers and the mt-type gamma inherited from *S .stoloniferum*								
1	Brjanskij krasnyj	1	1	0	0	0	0	0	gamma
2	Il'inskij	1	1	0	0	0	0	0	gamma
3	Jubilej Zhukova	1	1	0	0	0	0	0	gamma
4	Kolobok	1	1	0	0	0	0	0	gamma
5	Korona	1	1	0	0	0	0	0	gamma
6	Meteor	1	1	0	0	0	0	0	gamma
7	Moskvoreckij	1	1	0	0	0	0	0	gamma
8	Nakra	1	1	0	0	0	0	0	gamma
9	Olimp	1	1	0	0	0	0	0	gamma
10	Pogarskij	1	1	0	0	0	0	0	gamma
11	Resurs	1	1	0	0	0	0	0	gamma
12	Sokol'skij	1	1	0	0	0	0	0	gamma
13	Vektar belorusskij	1	1	0	0	0	0	0	gamma
	Group B: varieties with mt-type gamma with no diagnostic *R* genes markers								
1	Brjanskaja novinka	0	0	0	0	0	0	0	gamma
2	Fokinskij	0	0	0	0	0	0	0	gamma
3	Odissej	0	0	0	0	0	0	0	gamma
4	Zdabytak	0	0	0	0	0	0	0	gamma
	Group C: varieties and breeding clones with the gene-specific markers of *RB/Rpi-blb1* = *Rpi-sto1*								
1	Avrora	0	0	1	1	1	1	1	beta
2	Ognivo	0	0	1	1	1	1	1	beta
3	Baltijskij	0*	0*	1*	1*	1*	1	1	beta*
4	Evraziya	0*	0*	1*	1*	1*	1	1	gamma*
5	Sudarynja	1*	1*	1*	1*	1*	1	1	gamma*
6	Breeding clone 3602/28	0	0	1	1	1	1	1	gamma
7	Breeding clone 1604/16	0*	0*	1*	1*	1*	1	1	gamma*
8	Breeding clone 1101/10	0*	0*	1*	1*	1*	1	1	alpha*
	Group D: varieties with no DNA markers associated either with *R* genes or the mt-type introgressed from *S. stoloniferum*.								
1-166	The rest 166 of 188 varieties of the studied subset	0	0	0	0	0	0	0	49.4 % – alpha, 50.6 % – beta
	Control accession								
	S. stoloniferum PI 205522	1	1	1	1	1	1	1	gamma

* Taken from (Gavrilenko et al., 2018).

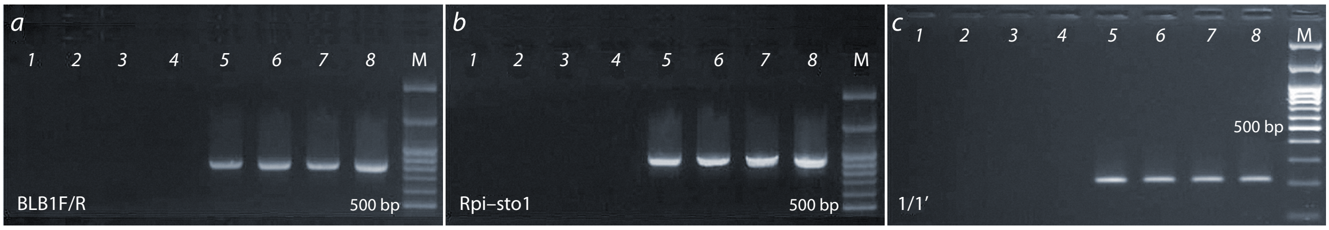

Fig. 2. PCR amplification of 821-bp, 890-bp, 213-bp fragments using gene-specific primers: BLB1F/R (*a*); Rpi-sto1 (*b*); 1/1′ (*c*).

Varieties and breeding clones: *1* – Il'inskij; *2* – Meteor; *3* – Nakra; *4* – Pogarskij; *5* – Avrora; *6* – 3602/28; *7* – Ognivo; *8* – sto, PI 205522. M – molecular marker 100 bp + 1.5 Kb + 3 Kb DNA Ladder.

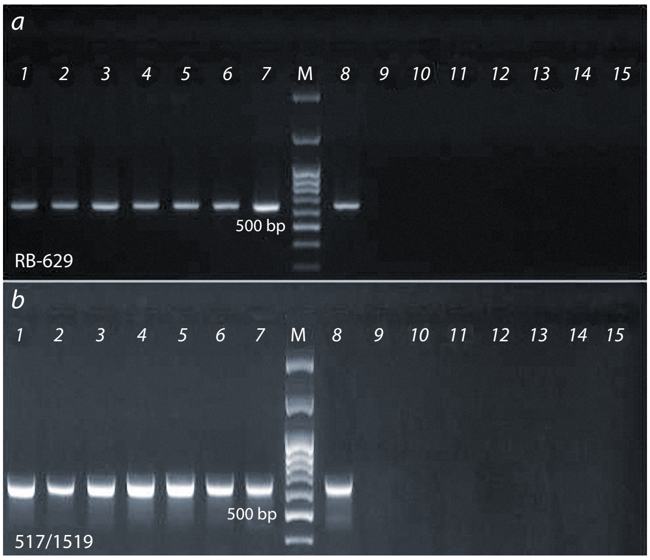

Fig. 3. PCR amplification of 629-bp and 651-bp fragments using RB-629 (*a*) and 517/1519 (*b*) gene-specific primers.

Varieties and breeding clones: *1* – Avrora; *2* – 3602/28; *3* – Baltijskij; *4* – Evraziya; *5* – 1604/16; *6* – Ognivo; *7* – Sudarynja; *8* – sto, PI 205522; *9* – Il'inskij; *10* – Meteor; *11* – Nakra; *12* – Pogarskij; *13* – Golubizna; *14* – Zhigulevskij; *15* – Veteran. M – molecular marker 100 bp + 1.5 Kb + 3 Kb DNA Ladder.

Group B included four varieties (Brjanskaja novinka, Fokinskij, Odissej, Zdabytak) that had no *R*-gene diagnostic markers (see Table 2). At the same time, all these varieties had the sterile mt-type gamma which derived from *S. stoloniferum* (Lössl et al., 2000). According to the pedigree records only one variety of group B – Brjanskaja novinka – had originated from the *S. stoloniferum* hybrids.

As group C we marked the genotypes with diagnostic fragments generated by five gene-specific markers of the *RB/Rpi-blb1 = Rpi-sto1*. Within the screened subset these markers were found in two varieties: Avrora, Ognivo and in breeding clone 3602/28 (Fig. 2 and 3; Table 2). The breeding material from LenNIISKh 'Belogorka' selected earlier for the presence of three gene-specific markers (Rpi-sto1, BLB1F/R and 1/1′) (Gavrilenko et al., 2018) also was MAS-positive: two additional markers 517/1519 and RB-629 covering different regions of the target gene *RB/Rpi-blb1 = Rpi-sto1* were detected in varieties Baltijskij, Evraziya, Sudarynja (see Fig. 3) and in breeding clones 1101/10 and 1604/16. As a result, group C included eight genotypes (five varieties and three breeding clones) which all were MAS-positive for the five gene-specific markers (BLB1F/R, 1/1′, 517/1519, RB-629, Rpi-sto1) of *RB/Rpi-blb1 = Rpi-sto1* homologues (see Table 2).

The *Rpi-blb2* diagnostic marker was detected in control variety Toluca but was not found in the analyzed subset including the control accession of *S. stoloniferum* PI 205522.

Group D included the most (166 or 86.9 %) varieties of the analyzed subset, which were MAS-negative for all markers associated with *R* genes from *S. stoloniferum* (see Table 2). Genotypes with mt-type gamma were also not found in this group. Eighty two varieties (49.4 %) of this group possessed mt-type alpha and 84 (50.6 %) – mt-type beta (see Table 2). It should be mentioned that several accessions of group D had the *S. stoloniferum*-hybrids in their pedigree records (see the Material part), e. g. there were seven cultivars extremely resistant to PVY (Brjanskij rannij, Effekt, Golubizna, Zhigulevskij, Ramzaj, Skoroplodnyi, Veteran) which had originated from self-fertile hybrid F_2Bn of *S. stoloniferum* (Simakov et al., 2007; Yashina, 2010).

Seven varieties from the analyzed subset had been previously screened for marker GP122_564 of the *Ry-f*$_{sto}$ gene – (Pavlyuchuk et al., 2013) and eight varieties – for YES3-3A marker of the *Ry*$_{sto}$ gene (Biryukova et al., 2015); in the both cases, material from patent holder institutions had been used. The results obtained at present study fully confirmed the data for these 15 varieties.

All the genotypes of group C, each carrying five gene-specific markers for the *RB/Rpi-blb1 = Rpi-sto1*, were selected for further sequence analysis.

Sequence analysis

The bands amplified by the Rpi-sto1 primer pairs were purified and sequenced from the eight genotypes of group C: five varieties (Avrora, Baltijskij, Evraziya, Sudarynja, Ognivo), three breeding clones (1101/10, 1604/16, 3602/28) and control genotype *S. stoloniferum* PI205522. The nucleotide sequences data obtained from the partial CC-region amplified with the Rpi-sto1 primer pairs were identical in all the nine genotypes (Fig. 4).

The identified variant of the nucleotide sequence of Rpi-sto1-fragments was not found in the GenBank database, but the sequences had 99 % similarity with the corresponding region of the two *R* gene sequences in the database: (1) *Rpi-sto1* of *S. stoloniferum* (EU884421) and (2) *Rpi-blb1* of *S. bulbocastanum* (AY426259.1). Both reference sequences correspond to the functional alleles (van der Vossen et al., 2003; Vleeshouwers et al., 2008). In comparison with corresponding region in reference sequence EU884421, three single-nucleotide po-

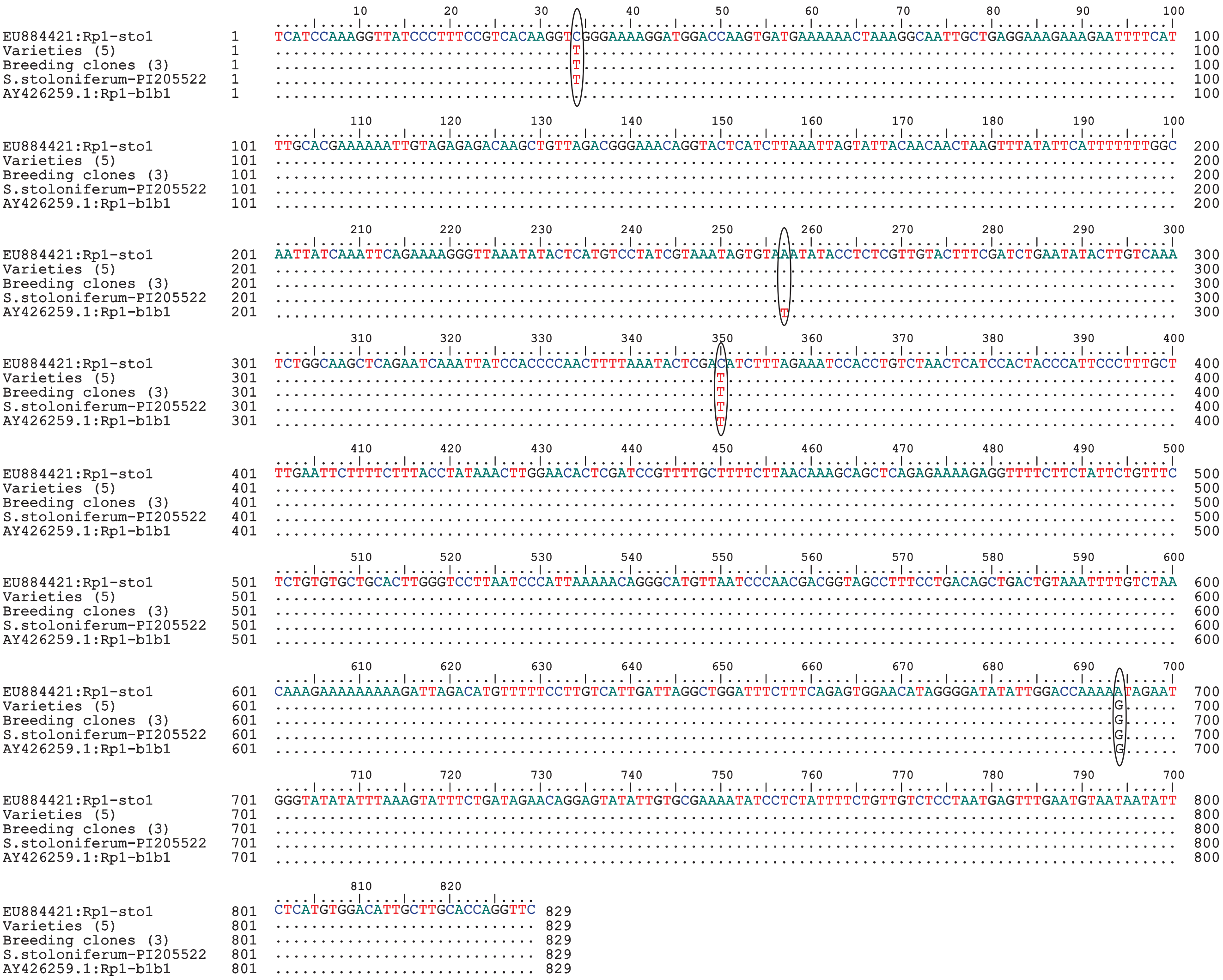

Fig. 4. Alignment of the Rpi-sto1-amplicon sequences from the eight genotypes of group C to the corresponding region of reference sequences (AY426259.1 and EU884421).

Varieties (5): Avrora, Baltijskij, Evraziya, Ognivo, Sudarynja; breeding clones (3):1101/10, 1604/16, 3602/28. The ovals indicate the SNP-positions which correspond to the sites 315, 538, 631, 975 in the reference sequences.

Table 3. Single-nucleotide polymorphisms in the Rpi-sto1-amplicon sequences from eight MAS-positive genotypes of group C compared to the corresponding region of reference sequences *Rpi-sto1* (AY426259.1) and *Rpi-blb1* (EU884421)

Reference sequences (GenBank #)			8 genotypes selected in MAS: 5 varieties (Avrora, Baltijskij, Evraziya, Ognivo, Sudarynja), 3 breeding clones (1101/10, 1604/16, 3602/28) and control – *S. stoloniferum* PI 205522
SNP position in sequence AY426259.1	*Rpi-blb1* (AY426259.1)	*Rpi-sto1* (EU884421)	
315	C	C	T
538	T	A	A
631	T	C	T
975	G	A	G

lymorphisms were detected in the analyzed genotypes: one C → T – in the first exon at site 315 and two SNPs – in the intron part at sites: 631 (C → T) and 975 (A → G) (Table 3, Fig. 4).

Comparison with *S. bulbocastanum* reference sequence AY426259.1 revealed the same 1-bp substitution in the coding region at site 315 and one SNP – in the intron at site 538 (see Fig. 4, Table 3). Single nucleotide change at position 315 in the first exon resulted in synonymous codon substitution (GTC → GTT) that did not alter the encoded amino acid valine.

The amplicons generated by primer pair BLB1F/R from the partial LRR region were sequenced from control genotype *S. stoloniferum* PI 205522, varieties Avrora, Ognivo, and breeding clone 3602/28. The sequences of BLB1F/R amplicons gave identity score 100 % to a corresponding partial LRR region in both reference sequences EU884421 and AY426259.1 and they were identical to the BLB1F/R fragment sequences from varieties Baltijskij, Evraziya, Sudarynja and breeding clones 1101/10, 1604/16 which had been analyzed earlier (Gavrilenko et al., 2018).

Thus, all the genotypes of group C had the same variant of the *RB/Rpi-blb1 = Rpi-sto1*-like sequences to be identical to the corresponding haplotype of *S. stoloniferum* PI 205522.

The sequences of the Rpi-sto1- and Rpi-blb1-PCR fragments were submitted to GenBank and are available under the accession numbers: MH518315, MH062177 (cv. Sudarynja); MH518316, MH062178 (cv. Evraziya); MH521008 (cv. Baltijskij); MH844527 (*S.stoloniferum* PI 205522) and MH844526 (Avrora).

Discussion

Wild Mexican species *S. stoloniferum* is an important source of *R* genes for extreme resistance to PVY and for durable resistance against late blight as well as for unfavorable abiotic stresses, but the interspecific hybrids with this species are often male sterile that complicate conventional breeding (Ross, 1986; Ortiz, 1998). According to literature, many Western European varieties created by breeders in Germany, Holland and Poland through introgressive hybridization with *S. stoloniferum*, carry Ry_{sto} and/or $Ry\text{-}f_{sto}$ genes conferring extreme resistance to PVY (Flis et al., 2005; Song, Schwarzfischer, 2008). Varieties with the Ry_{sto} gene show male sterility associated with mt-type gamma. This is due to the fact that these European varieties originated from a few accessions of *S. stoloniferum* which had been used in initial interspecific crosses as a female parent. The varieties and breeding clones developed from such interspecific hybrids inherited both the valuable Ry_{sto} gene and male sterile mt-type gamma (Lössl et al., 2000; Song, Schwarzfischer, 2008; Sanetomo, Gebhardt, 2015). Western European varieties have never been screened for the presence of *Rpi* genes, since the efforts of breeders were aimed at selection of PVY resistant material. An exception is our recent work in which a number of extremely resistant to PVY German varieties (Forelle, Kuba, Kuras, Maxi, Bettina, Amado, Solara) carrying Ry_{sto} and mt-type gamma (Song, Schwarzfischer, 2008) were screened for the Rpi-sto1 and BLB1F/R markers of the *RB/Rpi-blb1 = Rpi-sto1* gene, and none of these varieties were MAS-positive (Gavrilenko et al., 2018).

Similar results have been obtained at the present study with PVY resistant domestic varieties from group A carrying simultaneously the markers of the Ry_{sto} and $Ry\text{-}f_{sto}$ genes. Eleven of the thirteen varieties were bred in VNIIKH (Moscow region) based on the common sources derived from the Hungarian *S. stoloniferum* hybrid exhibiting extreme resistance to PVY (Simakov et al., 2007; Yashina, 2010). The hybrid maternally transferred its mt-type gamma to the breeding progenies. Cultivar Nakra from the group A had in its pedigree German variety Bison carrying the Ry_{sto} gene and mt-type gamma as its female parent (Song, Schwarzfischer, 2008). The varieties of group A did not have the diagnostic markers of the *RB/Rpi-blb1 = Rpi-sto1* conferring broad-spectrum late blight resistance. It was obvious that in a breeding process aimed at the selection of PVY resistant genotypes with Ry_{sto} and/or $Ry\text{-}f_{sto}$ genes (both localized on chromosome XII), the other alien *S. stoloniferum* chromosomes (for example, VIII and VI, in which the *RB/Rpi-blb1 = Rpi-sto1* and *Rpi-blb2* genes were mapped as well) would be lost.

The objective of creating varieties with high field resistance to late blight has been a priority for breeders from the north-western part of Russia, because in this region the weather conditions – moderate temperatures and high humidity – contribute to late blight development and often lead to epiphitoties. Seven of the eight selected in the MAS genotypes of group C having gene-specific markers for *RB/Rpi-blb1 = = Rpi-sto1* were developed by breeders from the North-Western region of Russia – LenNIISKh 'Belogorka' (Sudarynja, Evrazia, Baltijskij, 1101/10, 1604/16, 3602/28) and from the Vsevolozhskaya breeding station (Avrora). The patent holder of variety Ognivo is the Falenskaja breeding station located in the central-eastern part of European Russia.

The selected varieties and breeding clones from LenNIISKh 'Belogorka' grown without fungicide applications have been tested in the field trials for several years including epiphytotic seasons. The high level of foliar resistance to late blight was reported for Sudarynja, Baltijskij (Gavrilenko et al., 2018) and for 3602/28 (Evdokimova, not published). The Medium to low levels of field resistance was registered for Evrazia, Avrora and Ognivo (Simakov et al., 2009). At the same time all the genotypes of group C (Avrora, Baltijskij, Evraziya, Sudarynja, Ognivo, 1101/10, 1604/16, 3602/28) had an identical variant of the *RB/Rpi-blb1 = Rpi-sto1*-like sequences with 99 % similarity to the corresponding regions of the *Rpi-sto1* and the *Rpi-blb1*genes from the Genbank database. These six genotypes from LenNIISKh 'Belogorka' had similar origin – they all derived from the same hybrid 8889/3 (*S. demissum-S. stoloniferum-S. andigenum*) (Gavrilenko et al., 2018). The pedigree of variety Avrora is unknown as well as the pedigree of Ognivo hybrids. The differences in the level of their late blight resistance can be influenced by the number of copies of resistance gene(s) or by gene interaction and genetic background. Further research is required to study the late blight resistance types in the genotypes from group C.

The undoubted success of breeders has been creation of the male fertile breeding material derived from the *S. stoloniferum* hybrids. The selected varieties and breeding clones of group C bred in LenNIISKH 'Belogorka' had originated from the same male fertile hybrid 8889/3. This hybrid was an effective pollinator and it had been always used in crosses as a male parent (Gavrilenko et al., 2018). Various mt-types in the descendants of hybrid 8889/3 were determined by the different female parents used in crosses. As a result, within the selected breeding material of group C there were male fertile genotypes: cultivar Baltijskij (mt-type beta), breeding clone 1101/10 (mt-type alpha) and genotypes with mt-type gamma exhibiting male sterility (Evraziya, Sudarynja, 1604/16). Varieties Aurora and Ognivo selected in the present study both had mt-type beta, similar to cultivar Baltiysky from LenNIISKh 'Belogorka' (see Table 2). Varieties Avrora and Baltiysky are known as effective pollinators. Recently N. Zoteyeva et al. (2017) have selected male fertile *S. stoloniferum* hybrids with mt-type alpha and shown the possibility of pyramiding *R* genes from different wild Mexican species in breeding material.

As it has been mentioned before two conventionally bred varieties (Toluca and Bionica) possess the *Rpi-blb2* gene introgressed from *S. bulbocastanum* to common potato for a 46-year period (Haverkort et al., 2009). Additionally, the *Rpi-blb2* homolog has been detected in Hungarian cultivar White Lady (Hajianfar et al., 2016). The present paper represents a first report of finding the *RB/Rpi-blb1* = *Rpi-sto1*-like sequences in conventional bred varieties. With further investigation of late blight resistant types, co-segregation and expression analysis, the selected breeding material of group C might be used for gene pyramiding through traditional breeding methodologies.

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Conflict of interest

The authors declare no conflict of interest.

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