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GISH Analysis of the Introgression of the B Subgenome Genetic Material of Wild Allotetraploid Species *Solanum stoloniferum* into Backcrossing Progenies with Potato

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Abstract: Wild relatives of cultivated potato are used in breeding to increase the genetic diversity of *Solanum tuberosum* (AAAA genome) varieties. Wild Mexican allotetraploid species *Solanum stoloniferum* (AABB genome) was used in breeding for extreme resistance to viruses and late blight. In this study, genomic in situ hybridization (GISH) was used for visualization of introgression of genetic material of the B subgenome of *S. stoloniferum* into the genome of backcross hybrids. The fertile hexaploid hybrid had 48 chromosomes of the A genome and 24 chromosomes of the B subgenome. Plants of the BC₁ generation were pentaploid having the AAAAB genome constitution and three selected BC₂ hybrids were aneuploid, containing one to six chromosomes of the B subgenome and 48 chromosomes of the A genome. The B subgenome of *S. stoloniferum* was inherited in the backcross generations as single chromosomes and in rare cases as recombinant chromosomes. GISH showed that chromosome pairing in the backcross hybrids was predominantly intragenomic. Most chromosomes of the B subgenome remained as univalents in backcross hybrids. Rare homeologous A/B chromosome pairing was detected in all analyzed hybrids. The obtained data indicate that the B subgenome of *S. stoloniferum* was able to recombine with the A genome.

Keywords: potato; *Solanum tuberosum*; interspecific hybridization; introgressive lines; *Solanum stoloniferum*; homeologous pairing; genomic in situ hybridization (GISH)



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1. Introduction

Transferring genes/Quantitative Trait Loci conferring desirable traits via interspecific hybridization and classical breeding still remains an effective approach in breeding programs aimed at developing new varieties resistant to biotic and abiotic stresses. This is especially relevant for the common potato *Solanum tuberosum* L. Many wild relatives of this cultivated species were used in breeding to increase the genetic diversity of the varieties' gene pool and to introduce alien genes conferring resistance to diseases and pests [1–4].

Wild potato relatives constitute a complex of di-, tri-, tetra-, penta- and hexaploid species of the section *Petota* Dumort of the *Solanum* L. genus having the same basic chromosome number $x = 12$. About 60% of the 107 wild potato species are diploid, and the remaining are polyploid [5]. Genomic in situ hybridization (GISH) is successfully used for the detection of allopolyploidy and discrimination of different subgenomes of wild allopolyploid species [6,7]. Among them, the wild allotetraploid Mexican potato species *Solanum stoloniferum* Schltdl. is known as a source of resistance to late blight [8,9], to potato virus Y (PVY) which can lead to the yield losses on susceptible varieties as high as 80% [1,2,10–13], and is characterized by aphid resistance [14] and by abiotic stress (heat) tolerance [15]. *Solanum stoloniferum* belongs to the secondary potato gene pool [16]. Interspecific hybridization with cultivated potato is complicated by unilateral interspecific

incompatibility (UI) [17,18], differences in endosperm balance numbers (EBNs) [19–23] and male-sterility of interspecific hybrids having cytoplasm of wild species [1,24–26].

GISH analysis supports an AABB genome constitution and hybrid origin of *S. stoloniferum*, with wild Mexican diploid species *S. verrucosum* (or its progenitor) supported as the A subgenome donor and other diploid Mexican species (for example, *S. jamesii* Torrey, *S. bulbocastanum* Dunal) as the donor of B subgenome [6]. GISH confirms the strict allopolyploid nature of *S. stoloniferum*, which is characterized by the only intragenomic chromosome pairing, but not the intergenomic chromosome associations [6].

Since *S. stoloniferum* ($2n = 4x = 48$, AABB genome, EBN = 2) and *S. tuberosum* ($2n = 4x = 48$, AAAA genome, EBN = 4) have different EBN values, involvement of this wild species into breeding is based on the use of artificial polyploids, unreduced gametes [27–30] or using bridge species for crossing with tetraploid *S. tuberosum* [31]. Introgression is achieved using several breeding schemes. Usually, interspecific hybrids are obtained following pollinations of *S. stoloniferum* with pollen of fertile diploid clones of *S. tuberosum* ($2n = 2x = 24$, AA, EBN = 2). The resulting triploid hybrids ($2n = 3x = 36$, AAB) are sterile, and the further breeding cycle is based on their polyploidization, leading to the production of fertile hexaploid hybrids [29–31]. Because of UI, hybrid seeds are usually obtained when wild species are used as a female parent [17,18].

Another way to involve *S. stoloniferum* ($2n = 4x = 48$, EBN = 2) in breeding is the use of selected clones of this wild species which produce unreduced gametes ($2n = 4x = 48$, EBN = 2) and to cross them with potato diploid lines ($2n = 4x = 48$, EBN = 2) [19,31,32] or the use in interspecific crosses of the octaploid clones ($2n = 8x = 96$, EBN = 4) of *S. stoloniferum* and tetraploid potato varieties ($2n = 4x = 48$, EBN = 4) [19,27,28,31]. In exceptional cases, hybrid seeds can be obtained in crosses between the species with different EBN [33,34]. Thus, tetraploid interspecific hybrids (AAAB) were obtained after numerous controlled pollinations in crosses of *S. stoloniferum* ($2n = 4x = 48$, AABB genome, EBN = 2) with tetraploid *S. tuberosum* ($2n = 4x = 48$, AAAA genome, EBN = 4) [20,32,35].

Introgressive lines in different breeding schemes were produced following further pollinations of interspecific hybrids with pollen of fertile varieties or diploid breeding clones. Despite the fact that interspecific hybrids with *S. stoloniferum* are used in potato breeding, the specificity of the introgression of wild genome and the potential for intergenomic recombination are still unclear.

Recently, Yermishin with colleagues realized several breeding schemes to involve *S. stoloniferum* into interspecific crosses both as female and as male parent [23,36,37]. Here, we analyzed these hybrids and their BC progenies to investigate the potential of the introgression of genetic material of the B subgenome of *S. stoloniferum* into the genomes of selected backcross hybrids using GISH and to study the possibility of the homeologous chromosome pairing.

2. Materials and Methods

2.1. Plant Materials

In the previous study of Yermishin with colleagues [23,36], sexual hybrids were produced both with *S. stoloniferum* as a female parent and as a male parent (Figure 1). Plant material in the present study was represented by selected backcross clones derived from two breeding schemes:

- Scheme 'A'—sterile triploid hybrid was obtained in crosses between the *SvSv*-diploid line [diploid *SvSv*-line IGC 08/168.4 (F_2 dihaploid *S. tuberosum* × *S. verrucosum*) [23,36] and *S. stoloniferum*, PI 205522 (Figure 1). After colchicine treatment of this triploid hybrid, a fertile hexaploid hybrid IGC 15.118.3C6/2016 was obtained as the result of chromosome doubling;
- Scheme 'B'—hybrid material was developed in interspecific crosses involved *S. stoloniferum* as a female parent with pollen of the fertile diploid line IGC 10/1.21 (Figure 1).

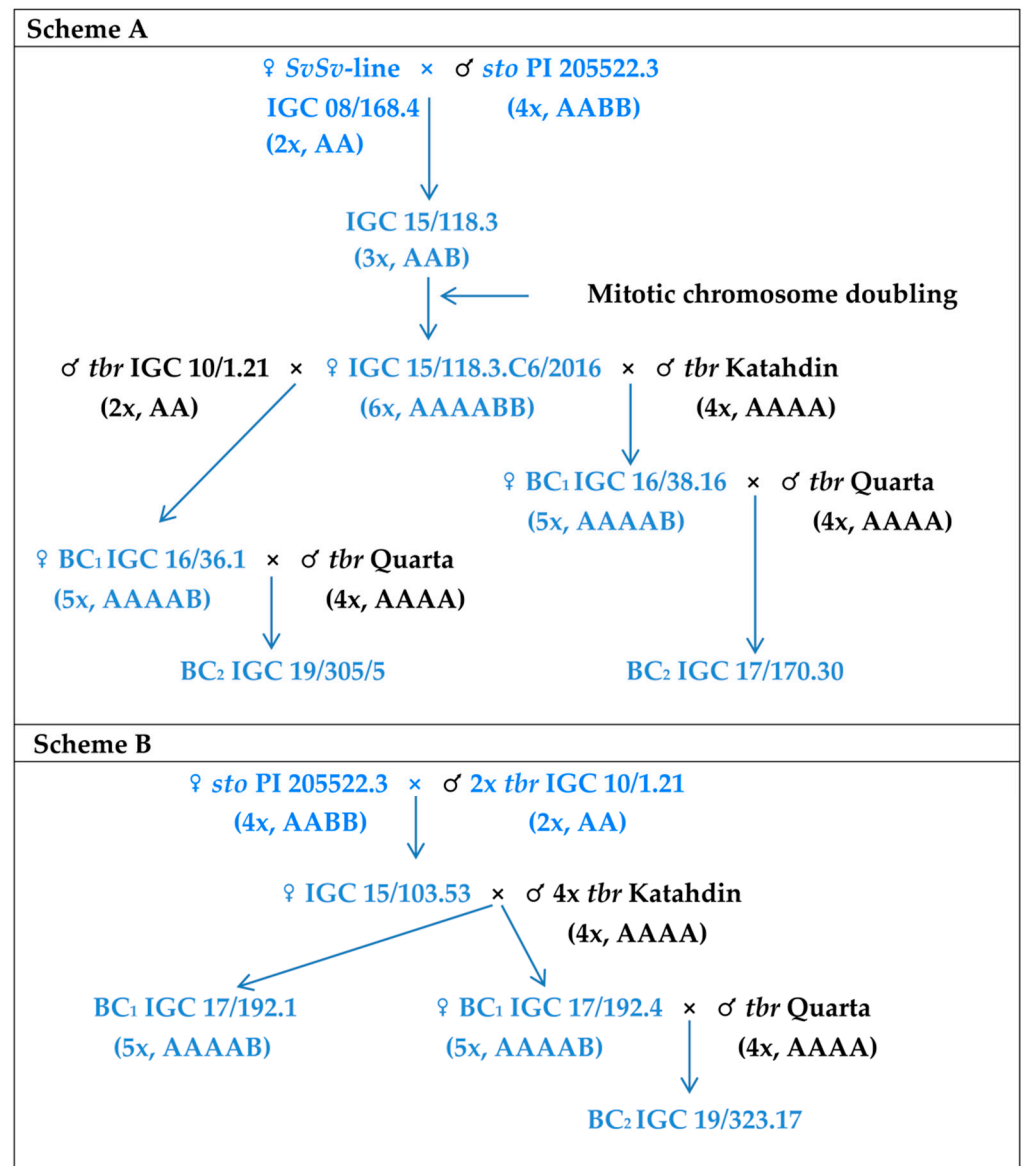


Figure 1. Crosses used for development of the introgressive forms which were analyzed in the present study. Expected genome composition is indicated in brackets. All pollinators are marked by black color, and maternal hybrid forms by blue color.

These two schemes of interspecific crosses, data on confirming hybridity with molecular markers and morphology, results of pathogen resistance tests and data of chromosome counts made by conventional acetocarmine method were described earlier [23,37].

Backcross progenies in both schemes were generated from mature seeds produced after hand emasculations and repeated pollinations of the interspecific hybrids by the pollen of male fertile varieties Katahdin or Quarta (Figure 1).

Seven introgressive forms were used to prepare mitotic and meiotic chromosome spreads for GISH including: an F₁ (chromosome doubling) hybrid 15/118.3; three BC₁ clones (IGC 16/38.16, IGC 17/192.1, IGC 17/192.4); three BC₂ clones (IGC 17/170.30, IGC 19/305.5, IGC 19/323.17). Backcross hybrids selected for the present study had normal growth and morphology, good pollen fertility, as well as resistance to late blight and to PVY [23,36,37].

2.2. Genomic In Situ Hybridization of the Introgressive Forms

Plant material for GISH assays was grown in a greenhouse. Root tips and flower buds were used to determine the genome composition of the hybrids. Root tips were fixed in

Carnoy's solution (an ethanol–glacial acetic acid = 3:1) after pretreatment in water with ice for 24 h and further stored at -20°C for chromosome counts. The flower buds were fixed directly in Carnoy's solution and then stored at -20°C for meiotic analysis in pollen mother cells (PMC). Chromosome slides were prepared after the enzymatic treatment of anthers and root meristems. The enzyme solution contained 4% cellulase (1.14 U/mg) and 1% pectolyase (0.94 U/mg), the time of incubation was 65–120 min.

Genomic DNA was isolated from green leaves according to the protocol of Bernatzky and Tanksley [38]. For GISH, DNA was isolated from the leaves of the wild A genome diploid species *S. verrucosum* (PI 545745) and the B genome diploid species *S. jamesii* (PI 458424).

DNA of both species was labeled using Nick-Translation Digoxigenin-NT Labeling Kit or BIO-NT Labeling Kit (Jena Bioscience (Jena, Germany), PP-310-DIGX and PP-310-BIO16). FITS anti-DIG conjugate (FAB fragments) (Roche 11207741910) and Rhodamine conjugated avidin Red TM-X (Thermo Fisher Scientific (Waltham, MA, USA) S6366) were used in a two-color GISH. Differentially labeled DNA of the A and B genomic species was used in the hybridization mix. GISH was performed according to the standard techniques [39] with slight modifications [6].

An AxioImager M2 epifluorescence microscope with an AxioCamMRm camera and AxioVision Rel 4.8 software was used to analyze the slides and create and process images. In addition, Adobe Photoshop 6.0 was used for image processing.

3. Results

3.1. Genomic In Situ Hybridization of the Introgression Forms

Chromosomes of the A subgenome of *S. stoloniferum* and the A genome of *S. tuberosum* are indistinguishable in GISH analysis of the interspecific hybrids. The B subgenome of *S. stoloniferum* was identified using *S. jamesii* genomic DNA. Table 1 presents the content of the B subgenome of *S. stoloniferum* in analyzed hybrid material.

Table 1. Genomic constitution of the backcross hybrids of cultivated potato with *S. stoloniferum*.

№	Hybrid Genotype	Progeny	Chromosome Number, 2n	Genome Composition— Number of Chromosomes of the	
				Genome A	Subgenome B
Scheme A					
1	IGC 15/118.3. C6.2016	F ₁ (chromosome doubling)	72	48	24
2	IGC 16/38.16	BC ₁	60	48 (one of them with B genome fragment)	12 (one of them—with A genome fragment)
3	IGC 17/170.30	BC ₂	53	48	5 (one of them—with A genome fragment)
4	IGC 19/305.5	BC ₂	54	48	6 (two of them—with A genome fragments)
Scheme B					
5	IGC 17/192.1	BC ₁	60	48	12
6	IGC 17/192.4	BC ₁	60	48	12 (one of them—with A genome fragment)
7	IGC 19/323.17	BC ₂	49	48	1

Fertile hexaploid hybrid IGC 15.118.3C6/2016 derived from the chromosome doubling of the primary F₁ sterile triploid hybrid had, as expected, AAAABB genome composition with 48 chromosomes of the A genome and 24 chromosomes of the B subgenome (Figure 2a).

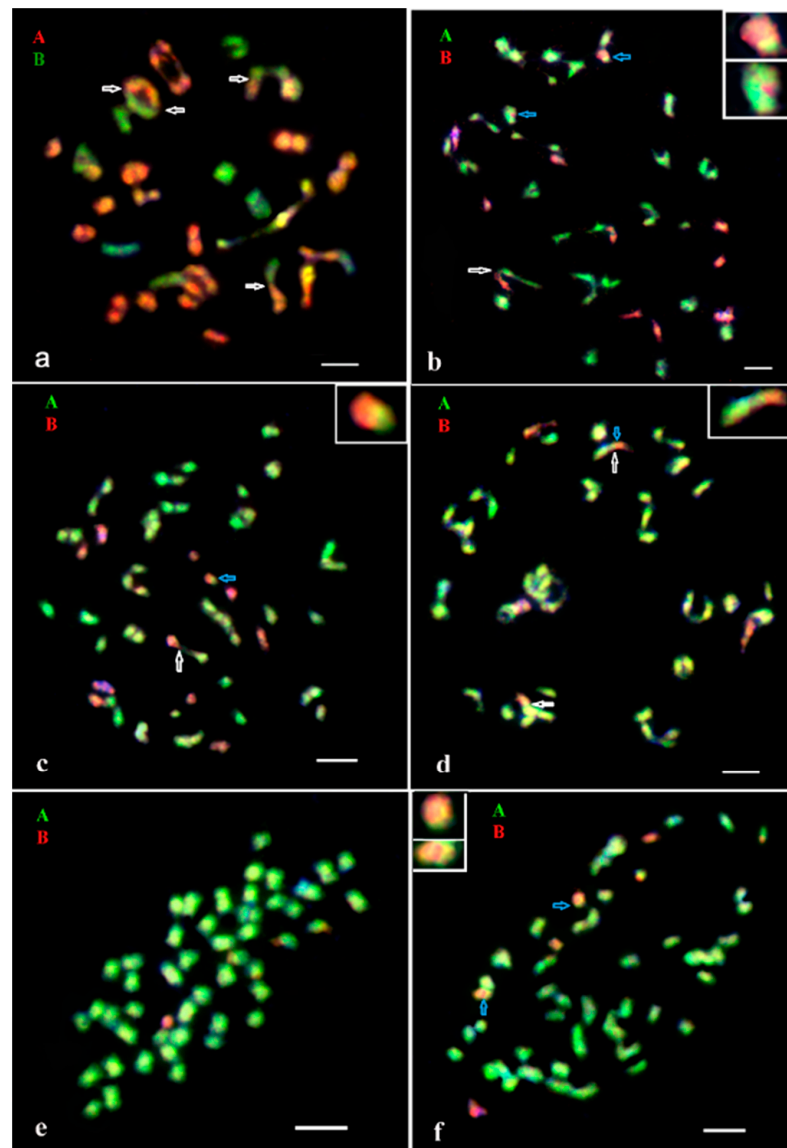


Figure 2. GISH analysis of chromosome content and homeologous pairing in hybrids of different generations. (a) Diakinesis in PMC of F₁ (chromosome doubling) hybrid IGC 15/118.3.C6.2016: 48 chromosomes of the A genome and 24 chromosomes of the B subgenome of *S. stoloniferum*, three intergenomic chromosome associations were identified (2 IV A/A/B/B and 1 II A/B). (b) Diakinesis in PMC of BC₁ hybrid IGC 16/38.16: 48 chromosomes of the A genome (one of them with the B subgenome introgression) and 12 chromosomes of the B subgenome (one of them with the A genome introgression), A/A/B trivalent is detected. (c) Diakinesis in PMC of BC₁ hybrid IGC 17/192.4: 48 chromosomes of the A genome and 12 chromosomes of the B subgenome (one of them with the A genome introgression), A/B rod bivalent is observed. (d) Diakinesis in PMC of BC₂ hybrid IGC 17/170.30: 48 chromosomes of the A genome and five chromosomes of the B subgenome (one of them with the A genome introgression), two A/B rod bivalents are observed. (e) Somatic chromosomes of BC₂ hybrid IGC 19/323.17 hybrid: 48 chromosomes of the A genome and one chromosome of the B subgenome of *S. stoloniferum*. (f) Somatic chromosomes of BC₂ hybrid IGC 19/305.5: 48 chromosomes of the A genome and six chromosomes of the B subgenome (one of them with the A genome introgression). The color of the fluorescence of the B subgenome (labeled DNA probe of *S. jamesii*) and the A genome (DNA probe of *S. verrucosum*) corresponds to the color of the letters above the images. Blue arrows show recombinant chromosomes. Homeologous pairing between the A genome chromosomes and the B subgenome chromosomes are indicated by white arrows. Scale bar = 5 μ m.

GISH revealed the AAAAB constitution of the BC₁ hybrid plants. All three BC₁ hybrids were pentaploid ($2n = 60$) having 48 chromosomes of the A genome and 12 of the B subgenome of *S. stoloniferum* (Table 1, Figure 2b,c).

Three selected BC₂ hybrids were aneuploids as expected (Table 1), indicating the loss of the B subgenome chromosomes in the backcross progeny. According to the GISH results, these BC₂ hybrids have the chromosome counts of $2n = 48A + 1B$ (IGC 19/323.17) (Figure 2e); $2n = 48A + 5B$ (IGC 17/170.30) (Figure 2d); $2n = 48A + 6B$ (IGC 19/305.5) (Figure 2f).

One-two A/B recombinant chromosomes were observed in four of the six analyzed backcross hybrids (Table 1, Figure 2b–d,f). The GISH signal from the A genome probe was located at the terminal parts of the recombinant chromosomes of the B subgenome. In one hybrid (BC₁ IGC 16/38.16) the B subgenome fragment was detected in the terminal part of one A genome chromosome (Table 1, Figure 2d). It is also possible that some of the introgressed fragments were out of range of the GISH resolution.

Detection of the A/B recombinant chromosomes in two BC₁ (IGC 16/38.16, IGC 17/192.4) and in two BC₂ (IGC 17/170.30, IGC 19/305.5) genotypes indicates that homoeologous recombination occurred in earlier generations (F₁ and in BC₁ plants) (Table 1, Figure 2a–c). To confirm the occurrence of intergenomic recombination, GISH analysis of meiosis was further performed for corresponding F₁ and BC₁ plants.

3.2. Chromosome Pairing in Interspecific Hybrids of Different Generations

Meiotic chromosome pairing at diakinesis was studied in three backcross hybrids and in F₁ (chromosome doubling) hybrid (Table 2, Figure 2a–d).

GISH showed that chromosome pairing was predominantly intragenomic, representing the A genome chromosome associations with the mean number of about 20 per cell presented mainly by A/A bivalents (Table 2, Figure 2a–d). The number of the A genome multivalents in all analyzed genotypes was extremely low.

The most chromosomes of the B subgenome of *S. stoloniferum* were paired in the hexaploid F₁ (chromosome doubling) hybrid (AAAABB genome) as expected. Whereas in the BC₁ and BC₂ hybrids, the most chromosomes of the B subgenome of *S. stoloniferum* were represented by univalents with the exception of a few chromosomes of the B genome included in various intergenomic associations (Table 2). For example, the pentaploid BC₁ hybrids (AAAAB genome) had more than nine univalents of the B subgenome on average per PMC (Table 2).

The homoeologous pairing between chromosomes of A and B genomes was seen at diakinesis relatively seldom, nevertheless, it was detected in all analyzed genotypes (Table 2, Figure 2a–d).

Intergenomic pairing was revealed in the hexaploid F₁ (chromosome doubling) hybrid with an average frequency of 0.82 per PMC (0.41 A/B bivalents, 0.18 A/A/B trivalents, 0.12 A/A/B/B quadrivalents) (Table 2, Figure 2a).

In the pentaploid BC₁ hybrids intergenomic pairing was observed with an average frequency 2.7 per cell (2.36 A/B bivalents, 0.14 A/A/B trivalents and 0.05 A/A/A/B quadrivalents) (Table 2, Figure 2b,c). The average frequency of homoeologous pairing in the BC₂ hybrid was 1.08 per cell (0.96 A/B bivalents and 0.04 A/A/A/B quadrivalents) (Table 2, Figure 2d). Most homeologous A/B bivalents were rod; ring A/B bivalents were observed in single meiocytes.

Table 2. Mean chromosome pairing at diakinesis in selected hybrid genotypes analyzed by GISH.

Hybrid Genotype	No. of PMC Studied	Average Chromosome Number and Type of Chromosomal Associations per PMC									A/B Associations on Average per Cell	
		I *		II **		III ***			IV ****			
		A	B	AA	BB	AB	AAA	AAB	AAAA	AAAB		AABB
Scheme A												
F ₁ (chromosome doubling) IGC 15/118.3. C6.2016 (6x, 48A + 24B)	17	2.00 (0–4; 5)	1.88 (0–5)	22.41 (20–24)	10.71 (9–12)	0.41 (0–2)	0	0.18 (0; 1)	0.06 (0; 1)	0	0.12 (0; 2)	0.82 (0–2; 4)
BC ₁ — IGC 16/38.16 (5x, 48A + 12B)	29	4.10 (0–6; 8; 10)	9.52 (5; 8–12)	20.24 (16; 18–24)	0	2.40 (0–4; 6)	0	0.14 (0; 1)	0.21 (0–2)	0.03 (0; 1)	0	2.90 (0–4; 6; 9)
BC ₂ —IGC 17/170.30 (48A + 5 B)	25	2.52 (1–4)	4.00 (3–5)	21.56 (19–23)	0	0.96 (0–2)	0	0	0.28 (0; 1)	0.04 (0; 1)	0	1.08 (0–3)
Scheme B												
BC ₁ — IGC 17/192.4 (5x, 48A + 12B)	14	3.93 (2–4; 6; 7)	9.62 (8–11)	20.71 (19–22)	0	2.21 (1–4)	0.07 (0; 1)	0.14 (0; 1)	0	0.07 (0; 1)	0	2.50 (1–4)

I *—univalents; II **—bivalents; III ***—trivalents; IV ****—quadrivalents. Bold type indicates the average frequency of homeologous A/B chromosome associations.

4. Discussion

The use of *S. stoloniferum* to improve potato varieties dates back more than 60 years [1]. This wild species was utilized in breeding mainly for transferring extreme resistance to potato virus Y. The genes *Ry_{sto}* and *Ry_{f_{sto}}* conferring PVY resistance were introgressed by classical breeding from *S. stoloniferum* into potato germplasm, which was further used to produce some PVY-resistant European varieties [12,40–43]. These genes were mapped on chromosome XII, and *Ry_{sto}* was recently isolated from the potato dihaploid clone [44].

Another gene *Rpi-sto1* conferring broad-spectrum resistance to late blight, which was mapped on chromosome VIII [8], was also introduced via conventional breeding into several varieties [45–47]. *Rpi-sto1* is the functional *Rb/Rpi-blb1* homologue which was identified in diploid ancestor species of *S. stoloniferum* [8,48,49]—the B-genome species of the series *Bulbocastana*, *Pinnatisecta* (according to system of Hawkes, 1990 [50]) and in the A genome wild species *S. verrucosum*. However, it is still unknown which of the subgenomes of *S. stoloniferum* contributes functional alleles of the *Ry_{sto}*, *Ry_{f_{sto}}*, *Rpi-sto1* into potato varieties. Furthermore, the potential of intergenomic recombination in interspecific hybrids and their progenies is not fully understood.

Few studies were reported in the literature regarding the possibilities of homeologous pairing in interspecific hybrids between *S. stoloniferum* and the A genome potato species. In conventional cytological meiotic analysis (acetocarmine or acetorcein chromosome staining) multivalents were observed—about two trivalents per meiocyte in triploid hybrids [29,51,52], and rare quadrivalents were detected at metaphase I of tetraploid hybrids [31,32,37]. These results could indicate the possibility of intergenomic pairing.

In recent decades GISH was successfully used to study alien introgression and homeologous pairing in somatic hybrids between potato, *S. tuberosum* and distantly related non-tuber bearing species belonging to other sections of the genus *Solanum*: *Etuberosum* [53–58], *Lycopersicum* [59,60] and the *Solanum nigrum* complex—also known as *Solanum* L. section *Solanum* [61]. In these distant hybrids, the parental genomes were easily discriminated by GISH and alien chromatin introgression was distinguishable in backcross progenies of somatic hybrids (Reviews: [62–65]). Within the section *Petota* to which cultivated potato belongs, GISH was able to discriminate parental genomes in somatic hybrids between *S. tuberosum* (AAAA) and wild diploid species *S. bulbocastanum* (BB) from the tertiary gene pool [66,67]. In these studies, GISH helped to detect alien chromosome transmission and introgressions in backcross progenies. However, distinguishing chromatin using GISH for more closely related potato species within the section *Petota* is more difficult or impossible. Thus, parental genomes could not be discriminated through GISH in hybrids between diploid cultivated potato and South American wild diploid species *S. commersonii* [68], which belongs to the tertiary gene pool of potato [69].

Detection of homeologous chromosome pairing in the present study suggests meiotic recombination as the possible mechanism of alien fragment introgressions in interspecific hybrids of potato with *S. stoloniferum* and their backcross progenies. To our knowledge, the present research is the first GISH study of the introgression into potato genome A of the chromosomes/chromosomal segments of the subgenome B of *S. stoloniferum* which belongs to the secondary gene pool. Our results provide evidence that homeologous recombination between chromosomes of the B subgenome of *S. stoloniferum* and the A genome might occur. In four of the seven analyzed hybrids, intergenomic recombinant chromosomes were observed. Small terminal regions have been recombined into one or two chromosomes of A or B genomes. Besides hybrids carrying A/B recombinant chromosomes, one BC₂ hybrid with a single additional alien chromosome was distinguished; this indicates potential for receiving addition lines.

Our results improve the understanding of the introgression of *S. stoloniferum* genetic material into the potato genome and can help to plan introgression breeding to contribute adaptive traits, including resistance to aphids and tolerance to abiotic stresses from this wild allotetraploid species into the varieties' gene pool.

In future, it will be of value to observe the transmission of the B subgenome chromosomes and stability of recombinant chromosomes in introgressive lines over several cross generations. We plan to focus our research on GISH and FISH with Chromosome-Specific Cytogenetic DNA Markers (CSCDMs). This approach helped to identify individual chromosomes in interspecific hybrids of *Solanum* species [55,58,70] as well as in a wide hybrids of other crops having small chromosomes [71,72].

5. Conclusions

New data about the potential of homeologous pairing and intergenomic recombination between chromosomes of the subgenome B of wild Mexican species *S. stoloniferum* and chromosomes of the A genome of potato were obtained using the GISH method. Genomic in situ hybridization allows visualization of the chromosomes/chromosomal segments of the B subgenome of *S. stoloniferum* into the backcross progenies. Chromosomes of the B subgenome of *S. stoloniferum* undergo the process of chromosome elimination during backcrosses with cultivated potato. Recombinant A/B chromosomes were revealed in backcross hybrids. Detection of intergenomic pairing suggests meiotic recombination as the possible mechanism of alien segment introgressions.

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