Final report OTKA K104382

Introgression and molecular cytogenetic identification of wheatgrass (*Agropyron*) chromosomes in cultivated wheat

Species belonging to the genus *Thinopyrum* (formerly *Agropyron*) are known to possess genes conferring resistance to various diseases, such as leaf and stem rusts, barley yellow dwarf virus and *Fusarium* head blight, making these species suitable for improving the disease resistance of wheat. Up till now, several resistance genes have been transferred from perennial *Thinopyrum* species into the cultivated wheat, but majority of these species have not so far been exploited for wheat improvement. Wheat × *Agropyron glael* (*Thinopyrum intermedium* × *Thinopyrum ponticum* synthetic hybrid) intergeneric hybrids were previously produced in Martonvásár in order to transfer its advantageous agronomic traits into wheat.

The main objective of the present project was to select lines with leaf rust resistance among the progenies of the wheat × *A. glael* hybrids and to determine the number of *Agropyron* chromosomes in these plants using molecular cytogenetic techniques.

Results

Selection and molecular cytogenetic analysis of leaf rust and stripe rust resistant lines from the progenies of the Mv9kr1 × A. glael *hybrids*

In 2012, 104 head rows from 34 lines originating from Mv9kr1 (wheat) × *Agropyron glael* hybrids backcrossed once with wheat variety Chinese Spring (CS) and self-pollinated in 8 consecutive generations (BC_1F_8) were sown in the Martonvásár nursery 'Tükrös'. In the 2013 growing season, from 104 rows 91 did not show any symptoms of leaf rust infection, 13 rows were moderately infected (score of 1 on the Stakman 0-4 scale). This segregating population showed high diversity in spike morphology (Fig. 1). Progenies of the wheat-*A. glael* BC_2 and BC_3 generations were susceptible to leaf rust (scors of 3 and 3-4, respectively). Molecular cytogenetic analyses showed that the *Agropyron* chromosomes were completely eliminated from these BC_3 plants.

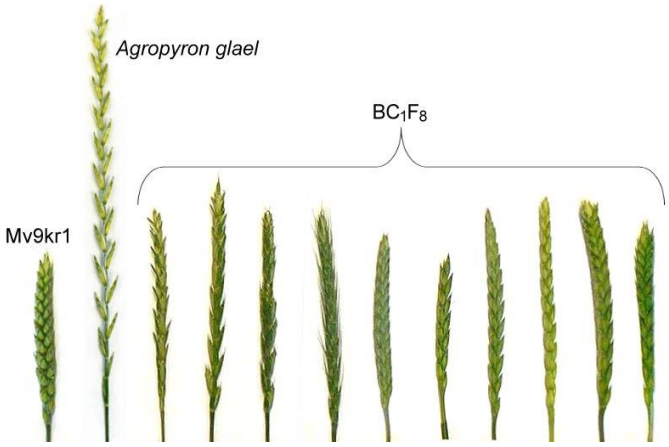

Figure 1 Diverse spike morphology of the $Mv9kr_1$-*A. glael* BC_1F_8 generation.

In 2014, 21 plots (Nos. 194-215) of BC_1F_9 lines selected for leaf rust resistance were sown in 'Tükrös' nursery. In that year, there were serious stripe (yellow) rust epidemics in Hungary. The infection of the highly susceptible wheat parent Mv9kr1 was severe, but BC_1F_9 plants in 9 plots were resistant, and plots Nos. 194, 195 and 196 were morphologically uniform, respectively.

The chromosome number and genome composition of these lines were analysed in somatic metaphase spreads from root tips of 5-20 individual plants by sequential multicolour genomic *in situ* hybridization (mcGISH) and fluorescent *in situ* hybridization (FISH) (Kruppa et al. 2016). Chromosome countings revealed that all the three lines were partial amphiploids. Line 194 has 58 chromosomes. The mcGISH analysis showed that it contained only 20 (instead of 21) pairs of wheat chromosomes. FISH with repetitive DNA probes (Afa-family, pTa71, pSc119.2) detected the

complete absence of the 3D chromosome (3D nullisomy) (Fig. 2). According to the mcGISH and FISH results the genome composition of this line is 14A+14B+12D+8J+8St+2JS^{t}. Line 195 has 56 chromosomes. The chromosome-specific FISH patterns identified two pairs of 4D and no 3D among the 42 wheat chromosomes, so this genotype was identified as a nullitetrasomic line (N3DT4D). The chromosome composition of this partial amphiploid is 14A+14B+14D (N3DT4D)+10 J (including J/St translocations)+4St (Fig. 3a, b). Line 196 has 54 chromosomes. FISH analysis revealed the complete absence of the 3D chromosome (3D nullisomy). This chromosome was substituted by another, which had 3BS as the long arm and an unidentifiable small segment as the short arm (Fig. 3c, d). On the basis of the mcGISH and FISH results the genome composition of line 196 is 14A+ 14B+2 3BS-J translocation+12D+8J+4St.

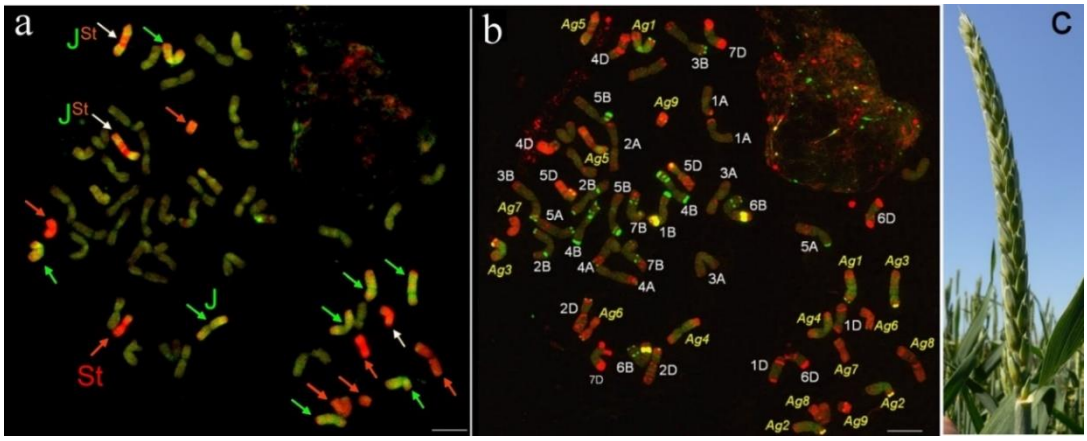

Figure 2 a Genomic *in situ* hybridization (mcGISH) on mitotic chromosomes of the partial amphiploid line 194 (58 chromosomes) derived from the Mv9kr1 × *A. glael* cross using J (*Th. bessarabicum*, green) and St (*Ps. spicata*, red) genomic DNA probes. Wheat chromosomes are unlabelled (brown). Alien chromosomes are indicated with arrowheads. JSt chromosomes are green with red signals at the pericentromeric region. **b** The fluorescent *in situ* hybridization (FISH) pattern on the same cell of line 194 using Afa-family (red), pSc119.2 (green) and pTa71 (yellow) repetitive DNA probes. *A. glael* chromosomes are numbered in yellow, not based on homology, while the wheat chromosomes are numbered in white. **c** Spike of the line 194. Scale bars = 10 μm.

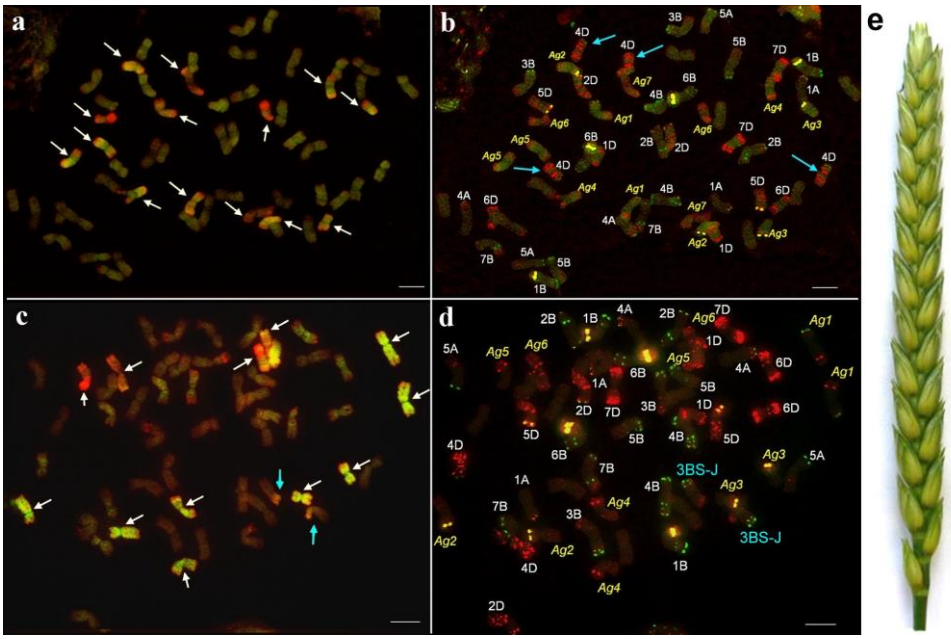

Figure 3 McGISH on mitotic chromosomes of the partial amphiploid lines 195 (56 chromosomes, **a**) and 196 (54 chromosomes, **c**) derived from the Mv9kr1 × *A. glael* cross using J (*Th. bessarabicum*, green) and St (*Ps. spicata*, red) genomic DNA probes. Wheat chromosomes are unlabelled (brown). Alien chromosomes are indicated with arrowheads. The FISH pattern on the same cell of lines 195 (**b**) and 196 (**d**) using Afa-family (red), pSc119.2 (green) and pTa71 (yellow) repetitive DNA probes. *A. glael* chromosomes are numbered in yellow, not based on homology, while the wheat chromosomes are numbered in white. Four 4D chromosomes present in line 195 (**b**) are marked with blue arrowheads. Translocations between the 3BS wheat and an unidentified *A. glael* chromosome arm are marked with blue (**c, d**). **e** Spike of the line 195. Scale bars = 10 μm.

From Table 1 summarising morphological characteristics, it is clearly seen that the partial amphiploids, besides their leaf rust and stripe rust resistance (Fig. 4), have disadvantageous traits, too, owing partly to the spring wheat variety Chinese Spring and partly to the *A. glael* parent. Lines 194 and 195 are late-flowering and significantly higher then the wheat parent Mv9kr1, while the line 196 is also late-flowering and dwarf. Furthermore, despite the higher number of spikelets per main spike, the fertility of these lines is lower than that of Mv9kr1.

Fig. 4 a Symptoms of spontaneous leaf rust infection on a leaf of the susceptible wheat genotype Mv9kr1. **b** Leaves of leaf rust-resistant Mv9kr1-*A. glael* partial amphiploid line 195. **c** Stripe rust infection on leaves of the susceptible wheat genotypes Mv9kr1, and (**d**) healthy leaves of partial amphiploid line 194. 'Tükrös' nursery, Martonvásár, 2014.

Table 1 Morphological traits of Mv9kr1-*A. glael* partial amphiploid lines (194, 195 and 196) grown in the field compared with the wheat parent Mv9kr1 (2014, 2015 Tükrös nursery, 2015 Breeder's nursery, Lászlópuszta)

Year and location	Genotype	Fertility (seeds/ spikelet)	Plant height (cm)	Tillering (spikes/ plant)	Length of main spike (cm)	Spikelets/ main spike	Seeds/main spike
2014 Tükrös nursery	Mv9kr1	1.6 ±0.1	99.0 ±3.7	8.6 ±2.2	10.2 ±0.63	22.0 ±1.1	34.4 ±1.9
	L.194	1.8 ±0.3	100.6 ±6.8	5.9 ±1.9*	12.4±1.17*	25.4 ±1.4*	44.6 ±10*
	L.195	no data	no data	no data	no data	no data	no data
	L.196	1.5 ±0.5	64.0 ±6.8*	6.2 ±2.2	13.2 ±0.95*	24.0 ±0.6*	36.4 ±14.2
2015 Tükrös nursery	Mv9kr1	2.5 ±0.3	68.9 ±2.3	5.6 ±2.2	8.4 ±0.41	19.9±15	50.1 ±4.3
	L.194	2.1 ±0.5*	100.0 ±6.4*	5.5 ±2.0	10.1 ±0.88*	23.2 ±2.4*	48.8 ±13.0
	L.195	1.8 ±0.5*	92.8 ±8.9*	4.5 ±1.6	12.0±13*	21.7 ±1.7*	39.1 ±11.0
	L.196	1.1 ±0.4*	57.2 ±2.9*	5.0 ±2.0	11.6 ±1.65*	20.7 ±23	22.1 ±2.1*
2015 Breeder's nursery	Mv9kr1	2.7 ±0.2	75.3 ±4.6	5.2 ±0.8	9.1 ±0.77	19.6 ±25	53.8 ±8.4
	L.194	2.4 ±0.6	100.4 ±4.8*	9.1 ±2.9*	10.3 ±0.97*	22.7 ±1.9*	54.8 ±14.2
	L.195	1.9 ±0.3*	100.1 ±5.2*	5.9 ±2.1	13.2 ±1.27*	25.3 ±25*	48.2 ±11.0
	L.196	1.8 ±0.3*	69.6 ±4.0*	7.8 ±3.2*	11.3 ±1.18*	22.6 ±15*	40.0 ±8.4*1

*Significantly different from Mv9kr1 wheat at the P = 0.05 level

In order to reduce the number of *Thinopyrum* and Chinese Spring chromosomes of the partial amphiploids, crossing programs were carried out with Mv9kr1 and the modern, high-yielding Mv Karizma, a facultative Martonvásár wheat cultivar which is moderately susceptible to leaf rust. As the flowering period of the spring-sown Mv Karizma overlapped with that of the amphiploids, crossing could easily be carried out. In 2013 and 2014, a total of 3301 flowers were pollinated with Mv9kr1, and 2632 flowers with Mv Karizma. From these crosses 502 and 667 seeds were produced, respectively, and sown in the Breeder's ('Prebreed') nursery. Plants showing resistance to diseases were selected. Head rows were sown from them in 11 small plots in 2015. In four plots (Nos. 10, 11, 12 and 13/Prebreed/2016) no leaf rust infection could be observed, and in two plots (Nos. 13 and 14) very little stripe rust infection appeared in 2016. Seeds from heads of individual lines harvested from these plots were planted again in 9 small 6-row and 12-row plots. Significant segregation of progeny plants was observed in most of the plots in 2017, except for plot No. 53 (Fig. 5) originating from plot No. 12/Prebreed/2016. Unfortunately, in the 2016/2017 growing season there were no leaf rust and stripe rust epidemics, consequently there was no possibility of selection for resistance to these diseases. From plots No. 53 and No. 58, we were able to select lines having excellent morphological characteristics (big ears, good tillering capacity and fertility, appropriate plant height, and broad leaves) from which ears were harvested for the next sowing and for cytological analyses. A large number of seeds were produced which can be used for analysing the nutritional parameters next autumn.

Figure 5 **a** Ear of the wheat cultivar Mv Karizma. **b** Plot No. 53/2017. **c** Ears of the non-segregating wheat line originating from a cross between Mv9kr1-*A. glael* partial amphiploids and the wheat cultivar Mv Karizma (Breeder's nursery, 2017)

Chromosome number and genome composition of the Mv9kr1 × *A. glael* hybrid progenies produced between 2014-2016 is summarised in Table 2.

Table 2 Chromosome number and genome composition of the Mv9kr1 × *A. glael* hybrid progenies deteremined by using mcGISH (2014-2016)

Combination	No. of seeds studied	Total chromosome number		Wheat/ *Thinopyntm* translocations (No. of plants)	Origin of *Th.* chromosomes	No. of *Th.* chromosomes is unidentifiable (No. of plants)
		48-52	<48			
Mv9krl×*A. glael*/ CS//Mv9krl	33	18	10	21	St genome	5
Mv9kr1×*A. glael* /CS//Mv Karizma/3/Mv Karizma	66	44	22	28	St genome	24
Mv9krl×*A. glael* /CS//Mv9krl	60	0	60	14	St genome	1
Mv9krl×*A. glael* /CS//Mv9krl	50	0	50	0	J genome	17
Mv9krl/*A .glael* /CS//Mv Karizma/3/Mv Karizma	60	0	60	32	St genome	24
	269			**95**		**71**

The chromosome number of the studied plants ranged beetween 42-51. The number of the *Thinopyrum* chromosomes in these plants is shown in Table 3. Most of the plants contained still a high number of *Thinopyrum* chromosomes, but there were plants which had only one or two *Thinopyrum* chromosomes besides the wheat genome. In the next generation several plants contained wheat-*Thinopyrum* translocations, mostly centric fusions (Table 4).

Table 3 Number of *Thinopyrum* chromosomes in plants of the Mv9kr1 × *A. glael*/CS//Mv9kr1 backcross progenies detected using mcGISH.

	No. of *Thinopyrum* chromosomes in wheat detected with mcGISH													
	1	2	3	4	5	6	7	8	9	10	0	not germinated	no root tips	no data	*total number of plants*
No. of plants	5	13	7	15	15	18	15	7	4	2	5	16	11	28	*161*

Table 4 Chromosome number of the Mv9kr1 × *A. glael*/CS//Mv9kr1 selfed progenies containing wheat-*Thinopyrum* translocations.

	Chromosome number of the plants									
	46	47	48	49	50	51	52	not germinated	no data	*total number of plants*
No. of plants with translocations/ No. of plants studied	2/2	3/5	14/14	2/2	1/1	-	1/1	4	4	*33*

Twenty backcross derivatives from the leaf rust resistant amphiploids selected in the field in 2015 were screened using GISH. One plant contained two *Thinopyrum* chromosomes, which might be a chromosome pair. Six plants contained a wheat-*Thinopyrum* translocation, but all of them carried other *Thinopyrum* chromosomes, too, beside the translocation. A wheat-*A. glael* substitution line (40 wheat + 2 J) was also identified in 2015. This line was sown in two plots in Tükrös nursery in October of 2015. In 2016 there was a strong leaf rust infection and the substitution line was seriously infected. Thus, it was concluded that the introgressed J chromosome did not carry any leaf rust resistant genes.

Identification of a disomic wheat-Agropyron glael *terminal translocation originating from a leaf rust resistant plant*

Among the detected addition, substitution and translocation (mainly centric fusion) lines a genotype containing a terminal translocation was found. This line derived from the combination Mv9kr1 × *A. glael*/CS//Mv Karizma/3/Mv Karizma. The identification of the chromosome involved in the translocation was carried out using specific repetitive DNA probes (pSc119.2, Afa family and pTa71) and the translocated chromosome was identified as 6DL.6DS-St terminal translocation carrying an unidentifiable St chromosome segment. Two plants carrying the homozygous translocation were selected using GISH from the F_3 generation (Fig. 6). The inheritance of the terminal translocation was observed by selfing the plants, and 19 homozygous plants were found among 40 analyzed individuals.

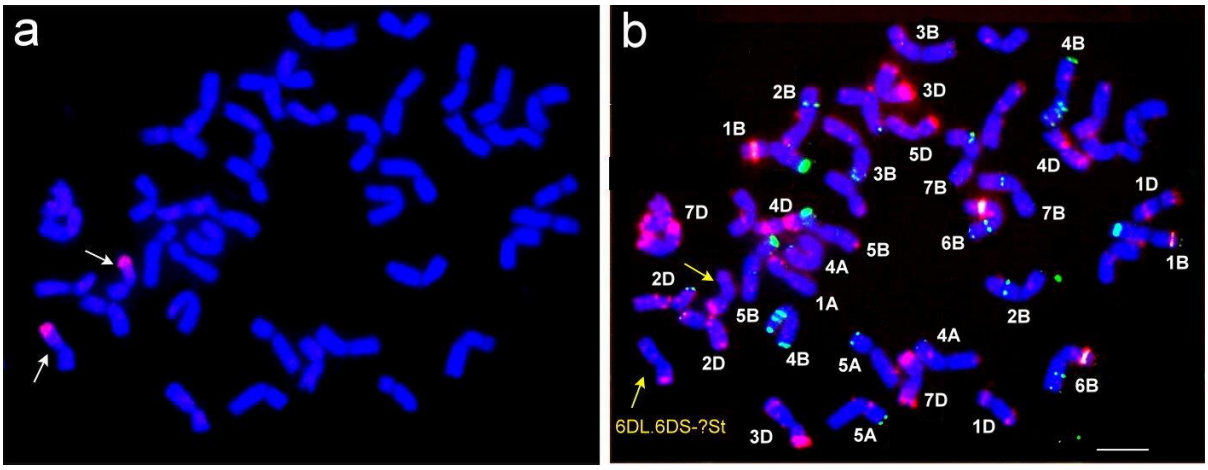

Figure 6 GISH (**a**) and FISH (**b**) identification using wheat-specific repetitive DNA probes of the homozygous terminal translocation originating from the Mv9kr1 × *A. glael*/CS//Mv Karizma/3/Mv Karizma combination. pSc119.2, Afa family and pTa71 signals are green, red and yellow, respectively. This genotype contains 42 chromosomes (20 pairs of wheat and 1 pair of 6DL.6DS-St wheat/*Thinopyrum* translocation chromosome. Scale bar = 10µm.

During a spontaneous powdery mildew infection we observed that wheat parental genotypes of the 6DL.6DS-St translocation line were susceptible to the disease, while the leaves of the terminal translocation were asymptomatic (Fig. 7).

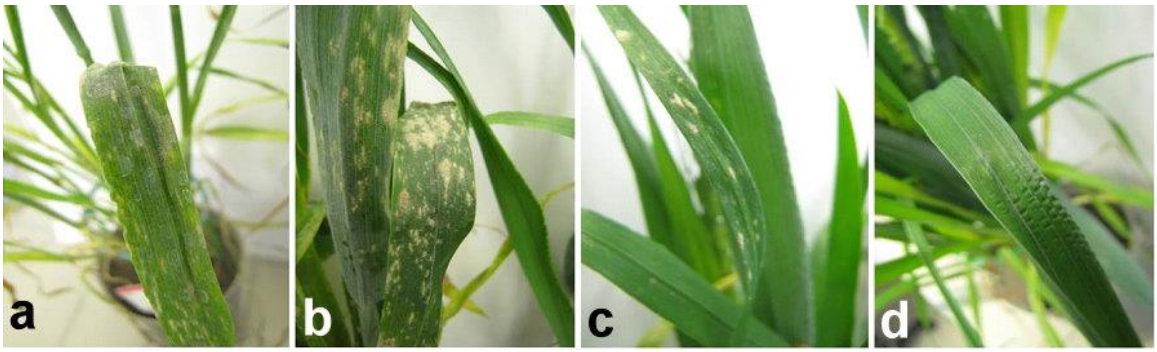

Figure 7 Spontaneous powdery mildew infection on the leaves of control wheat genotypes Chinese Spring (**a**), Mv9kr1 (**b**) and Mv Karizma (**c**), and resistant leaves of the 6DL.6DS-St wheat/*A. glael* translocation line grown in phytotron chamber (2016).

Molecular marker analysis of disease resistant lines

Two STS markers (STSLr19130 and J09-STS) and a SCAR marker with Lr29F18-Lr29R18 primers (http://maswheat.ucdavis.edu/ protocols/Lr29/) were used to reveal the presence or absence of *Thinopyrum*-derived leaf rust resistance genes *Lr19*, *Lr24* and *Lr29*, respectively, in the partial amphiploid lines. The STSLr19130 marker gave PCR products of the expected 130 bp fragment size in the positive control wheat line S09-1027 and in the wheatgrasses *Th. intermedium*, *Th. ponticum* and *A. glael*. The primer pairs failed to amplify any fragments in the wheat parents Mv9kr1 and Chinese Spring and in the partial amphiploid lines, signalling the absence of *Lr19*. The J09-STS marker, which has complete linkage with *Lr24*, amplified the 310 bp fragment in the positive control wheat line TC24, in *Th. intermedium*, *Th. ponticum* and *A. glael*, and in the partial amphiploid lines 195 and 196. Line 194 showed no band intensity (Fig. 7). With the help of the Lr29-linked Lr29F18-Lr29R18 primers, PCR products were identified in the TC29 positive control, *Th. intermedium* and *Th. ponticum*, while these primers gave no amplification products in *A. glael*, the wheat parents Mv9kr1 and Chinese Spring or the partial amphiploid lines.

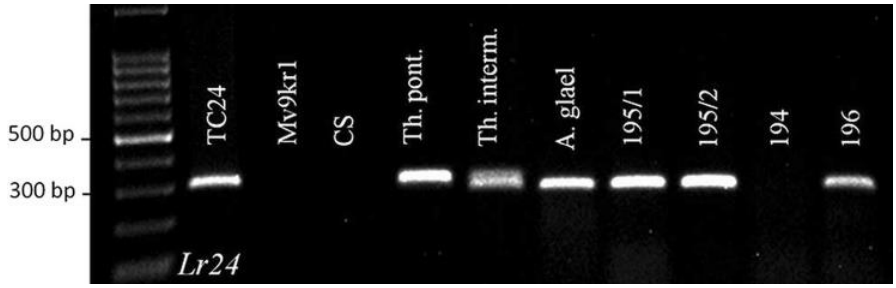

Figure 8 Agarose gel electrophoresis patterns of the J09-STS (*Lr24*) marker. The following DNA templates were used: positive control wheat line TC24, wheat genotype Mv9kr1, wheat cultivar Chinese Spring (CS), *Th. ponticum*, *Th. intermedium*, *A. glael*, Mv9kr1-*A. glael* partial amphiploid lines 195 (two samples), 194 and 196. A 100-bp DNA-ladder was used to estimate molecular weight.

These results suggested that the partial amphiploids might carry different *Lr* genes. The *Lr24* gene was detected in lines 195 and 196, but line 194 lacking this gene was also resistant to leaf rust.

In the case of the 6DL.6DS-St terminal translocation line, the presence of the Y38SCAR982 telomere-specific marker linked to the *Lr38* leaf rust resistance gene was detected. As the Mv9kr1 wheat parent is susceptible to leaf rust, it was concluded that the resistance of the three partial amphiploids and the terminal translocation line originated from *A. glael*.

*Pm21*and *PmL962* are powdery mildew resistance genes of *Thinopyrum* origin. The SCAR marker with primers Pm21D and Pm21E, and the EST-STS marker with primers BE443737F and BE443737R were tested on the 6DL.6DS-St translocation line to prove or disapprove the presence of these genes. The SCAR marker amplified the expected PCR fragments in the control wheat genotype (Nannong 02Y23) and in *Th. intermedium*, and the EST-STS marker produced the expected PCR amplicons in the control wheat genotype and in *Th. elongatum* and *Th. intermedium*. Non of them justified the presence of known powdery mildew resistance genes in the terminal translocation line. The susceptibility of the wheat parental genotypes denotes by all means the *A. glael* origin of the powdery mildew resistance of this line.

Drought tolerance studies

The drought tolerance of a Mv9kr1-*A. glael* partial amphiploid (line 242/2013, chromosome number 54) was analysed at the Department of Plant Sciences and Biotechnology (Pannon University, Georgicon Faculty, Keszthely). The drought tolerance was studied in glasshouse in sandtubes, a special method to analyse root development. Roots play decisive role in adaptation to low water supply. The method applied in this experiment made it possible to study the root system till the depth of 75 cm, as the roots can be removed from the tubes without any damage, thus development of the roots can be studied at different depth.

Twenty seeds were germinated from the partial amphiploid line and from the Mv9kr1 wheat line, respectively, and the seedlings were planted into tubes (length 75 cm, diameter 10 cm) filled up with

sand. The control plants were irrigated till 60% of the water capacity of the soil in the field. The stress treatment was started at booting stage, plants were irrigated till 30% of the water capacity of the soil. The dry weight of shoots, and that of the roots in two soil layers (0-30 cm and 30-75 cm) of the Mv9kr1-*A. glael* amphiploid and the wheat parent Mv9kr1 were measured in drought treatment and in control conditions.

The total root weight of the Mv9kr1-*A. glael* partial amphiploid was significantly higher than that of the wheat parental line Mv9kr1 in each treatment, and in both soil layers. The root weight of the amphiploid was 36% higher than that of the Mv9kr1 line in the upper soil layer (0-30 cm). The difference between the root weight of the amphiploid and the wheat parent was much bigger in the deeper soil layer (30-75 cm), the root weight of the amphiploid was 2.3 times higher than that of the wheat parent (2.310 g and 1.007 g, respectively) in the control treatment. The root weight reduced as a result of the water defficiency, but the level of reduction was genotype dependent. The root weight of the Mv9kr1 line was reduced by 30% in the soil layer of 0-30 cm, and similar reduction was observed in the amphiploids (32%). Significant difference was found in the reduction of the root weight in the deeper layers: the root weight of Mv9 kr1 line was reduced by 6% in the soil level of 30-75 cm, but it was reduced by 48% of the Mv9 kr1-*A. glael* partial amphiploid. In spite of the higher level of reduction, the root weight of the amphiploid was still 31% higher than that of the wheat line after the drought stress. There was a 15% reduction in the shoot weight of the Mv9kr1 wheat parent as an effect of water deficiency, but the shoot weight of the amphiploid has not changed, thus the root/shoot ratio was 0.359 and 0.307, respectively.

Our results prove unequivocally that genetic materials originating from Mv9kr1-*A.glael* hybrids are valuable basic materials for wheat improvement. It is expected that, in the near future, we will be able to select disease resistant and/or drought tolerant wheat lines with appropriate morphological traits containing introgressed *A. glael* chromosomes or chromosome segments.