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Identification of SSR-Markers distinguishing between Apomictic and Sexual plants in the Hybrid progeny of the sorghum line capable for parthenogenesis

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ABSTRACT

The development of lines with an apomictic mode of reproduction in cultivated plant species is one of the important tasks of modern plant biology. Earlier, we reported on the creation of a sorghum line Atc capable of parthenogenesis and autonomous endospermogenesis. In order to further prove the apomictic abilities in the Atc line, we performed genotyping of maternal-type plants observed in the offspring from crosses of the Atc line with the Volzhskoe-4v (V4v) line, and of the endosperm of the kernels from which such plants develop. For the first time, co-dominant SSR markers, *Sb1-10* and *Xtxp320*, have been identified, which make it possible to distinguish between apomictic and hybrid plants in the offspring from crossing the Atc line with the V4v line. It was shown that the endosperm in kernels with apomictic embryos does not contain paternal markers. These data testify in favor of its autonomous origin of endosperm in apomictic kernels and are consistent with the results of cytoembryological studies. The frequency of kernels with apomictic seedlings varied from 14.3% to 20.8%. Cytoembryological analysis showed that the development of parthenogenetic embryos began 3-4 days after the opening of flowers in the absence of pollination. The frequency of ovules with cytologically proven parthenogenesis on day 3 was 12%. The data obtained can be used in research on the identification and cloning of apomixis genes, as well as on creation of sorghum hybrids capable of maintaining a high level of heterosis.

Keywords : apomixis; parthenogenesis; autonomous endosperm; SSR-markers; *Sorghum bicolor*

Development of cultivated crops with apomictic mode of reproduction is one of the most tempting tasks of modern plant biology. It is believed that the implementation of this task will contribute to solving one of the main tasks of plant breeding - fixing heterosis and clonal propagation of the best genotypes, as well as contributing to the understanding of many fundamental processes in the biology of reproductive systems, such as, genetic control of meiosis, development of the female gametophyte, double fertilization, embryo and endospermogenesis (Barcaccia and Albertini 2013, Tavva *et al*. 2015, Fiaz *et al*. 2020).

The complexity of the problem is due to the need to combine in one genotype the ability to form unreduced embryo sacs, parthenogenesis and autonomic endospermogenesis (or the development of pseudogamous endosperm). Over the past years due to intensive study of wild apomictic species a number of candidate genes for different components of apomixis have been identified (Pupilli and Barcaccia 2012, Hand and Koltunow 2014, Brukhin 2017, Brukhin and Baskar 2019, Vijverberg *et al.* 2019, Worthington *et al.* 2019). These studies contribute to the understanding of the molecular mechanisms of apomixis and open prospects for its construction using biotechnological methods, including genomic editing (Xie *et al.* 2019). However, significant progress has not been achieved yet.

There are reports in the literature on the identification of particular elements of apomixis in sorghum - apospory, diplospory, parthenogenesis, autonomous development of endosperm and communications on the discovery of lines in which seed formation occurred on the basis of apomixis (Hanna *et al.* 1970, Rao *et al.* 1978, Murty 1993, Wu *et al.* 1994, Elkonin *et al.* 1995, 2012, Zhang *et al.* 1997, Ping *et al*. 2004, Carman *et al*. 2005). However, some of these reports related to the research of the R-473 line have been criticized because subsequent studies did not confirm the presence of apomixis (Bala Ravi 1993). In addition, from the point of view of some authors, apomixis in diploid plants does not exist in principle (Sokolov *et al.* 2011).

We previously reported the creation of a sorghum line (Atc) capable of aposporous embryo sac formation, parthenogenesis and autonomous endospermogenesis (Belyaeva and Elkonin 2018). The aim of this work was to identify molecular markers, which, in addition to phenotypic markers, would make it possible to reliably distinguish between apomictic and hybrid plants in the offspring from pollinated emasculated panicles of the Atc line with pollen of the Volzhskoe-4v line.

MATERIAL AND METHODS

Plant material : The lines of grain sorghum (*Sorghum bicolor* (L.) Moench) Atc and Volzhskoe-4v were used in this work.

Atc line was obtained by crossing male-sterile line SK-Mstc with the line KVV-114 and subsequent selection for 15 generations for the ability to formation of aposporous embryo-sacs (apo-ESs) and parthenogenesis (Belyaeva and Elkonin 2018). Volzhskoe-4v line (V4v) is homozygous for a dominant gene *Rs*, controlling anthocyanin pigmentation of the seedlings and was used as genetic marker allowing identification of maternal-type and hybrid plants during selection of Atc line.

Several branches from the central part of Atc panicles were carefully hand-emasculated just before flower opening, and after 3 days were pollinated with the pollen of V4v. Other branches from emasculated panicles were carefully cut-off. The kernels obtained from these crosses were split into two halves: one (without an embryo) was used for genotyping the endosperm, the other (with an embryo) was placed on a MS nutrient medium to obtain plants, which were subsequently planted in the soil and grown in the field for phenotyping.

All the lines and hybrid progenies were grown in experimental field of the Institute of Agriculture of the South-East Region (Saratov, Russia) in 2019 and 2020.

Plant and endosperm genotyping : The modified CTAB method (Shilov *et al.* 2016) was used for isolation of DNA from the endosperm of the kernels obtained in crosses of emasculated panicles of the Atc line with the V4v line, as well as from the leaves of plants obtained from these kernels. Various co-dominant SSR markers were used for genotyping (Table 1). PCR-analysis was performed using the Master Cycler Personal (Eppendorf). The reaction mixture contained 50 ng DNA, 0.01 U/µlSynTaq DNA polymerase (Synthol, Russia), 0.6 pmol of each primer, x1-fold PCR buffer (Synthol, Russia), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Synthol, Russia). The total volume of the reaction mixture was 25 µl. PCR for amplification of *Xtxp320* and *Xtxp321* SSR-markers was performed using the following regime: initial denaturation of 94ºC (4 min); 30 cycles: denaturation for 1 min at 94ºC, annealing for 30 sec at 54ºC (*Xtxp320*) or 55°C (*Xtxt321*) and extension at 72°C for 1 min; the last PCR cycle was followed by a 7-min extension at 72°C (Kong *et al*. 2000). For amplification of other SSR-markers touchdown PCR was performed using the regimes listed in the Table 1. The amplified fragments were fractionated in 3.5% agarose gels in 0.5-fold TAE buffer at 175 V (90 min). A 0.01% aqueous solution of ethidium bromide was used to visualize DNA fragments.

Cytoembryological analysis of female gametophyte: In embryological analysis, branches of sorghum panicles were fixed with ethanol–acetic acid (3:1) for 3 days, washed with 70% ethanol for 3 days, and stored in 96% ethanol. Fixation was performed 3–4 days after the start of flowering, and branches to be fixed were collected from the central part of a panicle. Fixed ovaries were isolated from flowers, washed with distilled water for 20–30 min, treated with 4% ammonium – iron alum at 50ºC for 10 min, washed with distilled water at 50º C for 15−20 min, and stained with 2% acetocarmine at 50ºC for 1.0−1.5 h. ESs were isolated from the stained ovaries with the use of microneedles under a stereomicroscope without squashing and maceration. Isolated ESs were transferred into a drop of glycerol on a slide, and covered by a slip, while their volume structure remained unchanged. For each plant, usually, 25-45 ovaries were studied. Preparations were examined with using Axioscope A1 microscope (Carl Ziess, Germany) under transmitted light (magnification x200, x400). Figures were made by Axio Cam MRc digital camera using AxioVision 4 computer program.

Statistical methods : To estimate the differences in frequency of elements of apomixis between families, proportion comparisons were performed by Fisher's method used in the case of small samples or samples differing in size (Zaitsev

Table 1 – SSR-markers used in this study

Marker name	Chromosome	Forward primer (5' → 3')	Reverse primer (5' → 3')	Tm (°C)	Reference
SB1539	2	ACATCCGTGAAGCTTCCAAACTCT	ACAGTACTGACCTAGCTGCACCCC	*	Yonemaru *et al.* 2009
Xisep0310[2]	2	TGCCTTGTGCCTTGTTTATCT	GGATCGATGCCTATCTCGTC	**	Billot *et al.* 2013
Xcup32[4]	3	ACTACCACCAGGCACCACTC	GTACTTTTTCCCTGCCCTCC	***	Bucheyeki *et al.* 2009
Sb1-10[2]	4	GTGCCGCTTTGCTCGCA	TGCTATGTTGTTTGCTTCTCCCTTCTC	**	Brown *et al.* 1996
Xtxp321[3]	8	TAACCCAAGCCTGAGCATAAGA	CCCATTCACACATGAGACGAG	55°C	Kong *et al.* 2000
SB5058	9	GAGAATTGGAAGAAAGCCTCGGTT	CAGAGCTCCTAAACGGTCCTCAAA	*	Yonemaru *et al.* 2009
Xtxp320[3]	10	TAAACTAGACCATATACTGCCATGATAA	GTGCAAATAAGGGCTAGAGTGTT	54°C	Kong *et al.* 2000

* Touchdown PCR consisting of 94°C for 5 min; 2 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min; 10 cycles of 94°C for 1 min, 65°C–55°C for 1 min decreasing by 1°C/cycle, and 72°C for 1.5 min; and 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; followed by 72°C for 7 min and 4°C as the holding step.

** Touchdown PCR consisting of 94°C for 4 min initial denaturation; 20cycles of 94° for 30 sec, 65°-56° for 30 sec decreasing by 1°C every 2 cycles, and 72°C for 1 min; and 20 cycles of 94° for 15 sec, 55° for 30 sec, 72°C for 1 min; followed by 72°C for 10 min.

*** Touchdown PCR consisting of initial denaturation for 15 min at 94 °C; 10 cycles of denaturation for 10 seconds at 94 °C, annealing at 61-52° C for 20 seconds decreasing by 1°C/cycle, and extension for 30 seconds at 72 °C; and 30 cycles of denaturation for 10 seconds at 94 °C, annealing at 54 °C for 20 seconds and extension at 72 °C for 30 seconds; followed by final extension of 20 min at 72°C.

1984). In this method, proportions are compared by Fisher's test :

$$F = (\varphi_1 - \varphi_2)^2 \quad [(N_1N_2)/(N_1+N_2)] \geq F_{st}$$

Where F is the Fisher's test, φ 1 and φ 2 are values resulting from a transformation of the proportions into radians, and N_1 and N_2 are the sizes of the samples under study.

RESULTS AND DISCUSSION

The cytoembryological analysis of the Atc line used in the crosses showed that all the studied plants showed the ability to form initial cells of aposporous structures, apo-ESs as well as parthenogenetic embryos (Fig. 1; Table 2). It was found that the development of parthenogenetic embryos began 3-4 days after the opening of flowers in the absence of pollination (Table 3). The frequency of ovules with parthenogenesis on day 3 was 12% (family 5/18). Family 5/18 is the offspring of one plant from family 8/17, in which the frequency of parthenogenesis was 11.3%. In turn, the 8/17 family originated from one of the plants from the 15/14 family, in which the frequency of parthenogenesis was 13.5% (Table 2). Thus, the ability for parthenogenesis is inherited in the Atc sorghum line and manifests itself in different seasons.

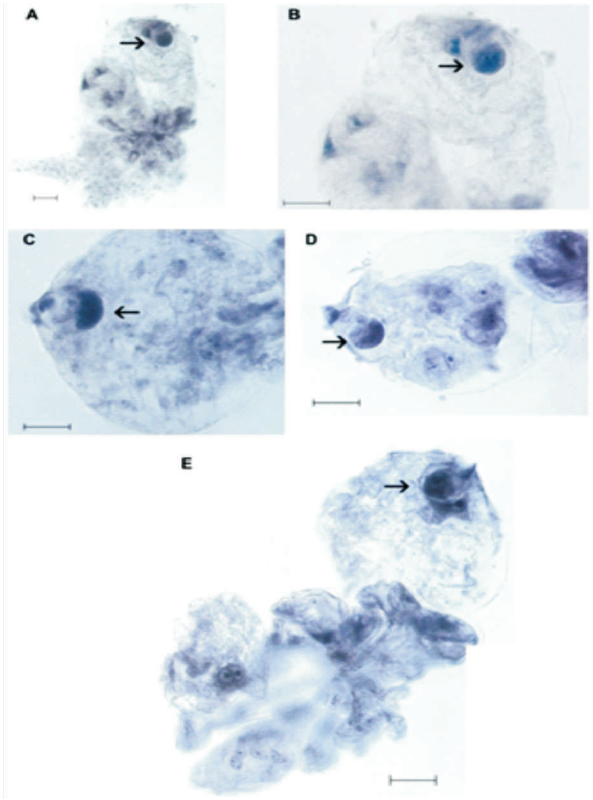

Fig. 1– Parthenogenetic embryos (marked by arrows) in the embryo sacs of sorghum line Atc. A, B – plant #5-7, the same proembryo under different magnifications; C, D – plant #5-8; E - plant #6-13.Scale bar 50 μm.

Table 2 – Manifestation of apomictic potencies in different families of the grain sorghum line Atc

Family	Frequency of ovules with APS[1], %	Frequency of ovules with apoES, %	Frequency of ovules with parthenogenetic pro-embryos, %
5/18	20.0-70.3	0.0-23.2	0.0-12.0
8/17	26.2-50.9	11.9-20.0	0.0-11.3
12/15	25.0-31.8	9.1-12.5	0.0-6.3
15/14	15.4-59.4	7.7-28.1	0.0-13.5

[1]APS – ovules with aposporous initials, aposporous embryo sacs (apoES), and autonomously developed embryos and/or endosperms.

It is noteworthy that the Atc line has a high degree of pollen sterility. The proportion of fertile pollen grains (PGs) was, on average, 1-3% per panicle; the predominated classes were empty PGs (up to 68-69%), PGs with impaired starch accumulation and delamination of the cytoplasm from the PG wall (up to 9-57%), or with pale coloration of starch (up to 31-38.5%). In different panicles of the same plant, and in different layers of the panicle, the ratio of different types of PG varied. At the same time, the high pollen sterility did not correlate with the seed setting, which varied from 40 to 100%.

In the progeny from the crosses of emasculated Atc plants with the V4v, green seedlings lacking the marker gene *Rs* were observed. Their frequency varied from 14.3% to 20.8% (Table 4).

In order to verify the apomictic nature of the plants appeared in the offspring of the crosses Atc × V4v, which had complete phenotypic similarity to the maternal line, genotyping of the offspring with using SSR-markers was undertaken. For this purpose, at the first stage, we have identified the SSR-markers that differentiate parental lines, Atc and V4v. Seven polymorphic co-dominant SSR-markers located in chromosomes 2, 3, 4, 8, 9, 10 were tested (Table 1). These markers were selected on the basis of literature data. When choosing markers, preference was given to those with the highest polymorphism, the difference between fragments of at least 20 bp, and to those which did not exhibit a uniparental inheritance pattern. As a result of the studies, it was found that SSR-markers *Sb1-10* (4 chromosome) and *Xtxp320* (10 chromosome) clearly differentiate the lines Atc and V4v. PCR analysis of DNA isolated from the leaves of green seedlings identified in the crossing Atc × V4v revealed the presence of maternal and absence of paternal alleles of markers *Sb1-10* (Fig. 2). Similar results were obtained with the SSR-marker*Xtxp320* (Fig. 3). At the same time, both alleles were present in red (hybrid) seedlings obtained from the same cross.

It is noteworthy that the paternal alleles were also absent in the DNA isolated from the endosperm of the kernels from

Table 3 – Manifestation of apomictic potencies at different stages after beginning of flowering in the line Atc (5/18 family)

Plant No.	Number of day safter flowering	Numbero fo vules	Frequency of ovules with APS, %	Frequency of ovules with apoES, %	Frequency of ovules with parthenogenetic pro-embryos, %
5-7	0	35	11.4	2.9	0.0
	3	50	20.0	0.0	12.0**
5-1	0	28	32.1	3.6	0.0
	3	55	45.5	10.9	3.6
5-12	0	20	5.0	0.0	0.0
	3	20	30.0*	8.8	0.0
58-5	0	25	16.0	4.0	0.0
	3	61	21.3	6.6	3.2
58-11	0	20	35.0	0.0	0.0
	3	61	57.4	13.1*	3.3
58-9	0	25	20.0	0.0	0.0
	3	60	30.0	5.0	5.0
7-8	0	22	9.1	0.0	0.0
	3	34	41.2**	8.8*	0.0
Total	0	175	17.7	1.7	0.0
	3	341	35.5***	7.3**	4.4***

*p>95%, **p>99%, ***p>99.9% incomparisonwiththebeginningoffloweringaccording to F-criterion

Table 4 – The frequency of apomictic plants in the progeny of crosses of Atc × Volzhskoe-4v

Plant no.	Number of seeds		Number of plants	
	Total	non-germinated	Hybrid	Apomictic
7-6/19	42	21	16	6 (14.3%)
18-2/19	5	—	5	—
8-5	24	9	10	5 (20.8%)

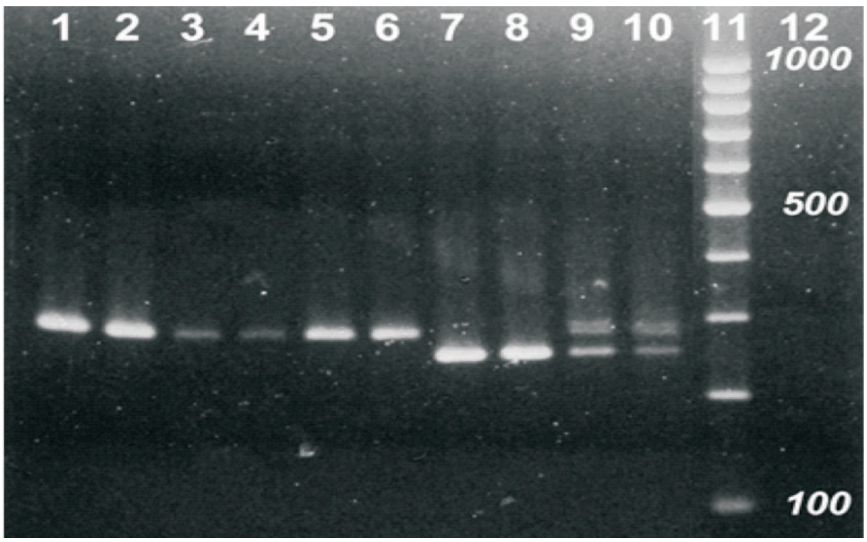

Fig. 2- PCR analysis with primers to the SSR marker Sb1-10.1, 2 – DNA from the leaves of plants of the sorghum line Atc, green maternal-type seedlings (3, 5) from the offspring of the emasculated panicle of the line Atc, pollinated with pollen from the line Volzhskoe-4v, the paternal line Volzhskoe-4v (7, 8), endosperm of kernels, from which maternal-type seedlings were obtained (4, 6), and hybrid seedlings (9, 10);11 - markers of the length of DNA fragments; 12 - negative control (no DNA). Tracks 9, 10 clearly show the co-dominant nature of the marker.

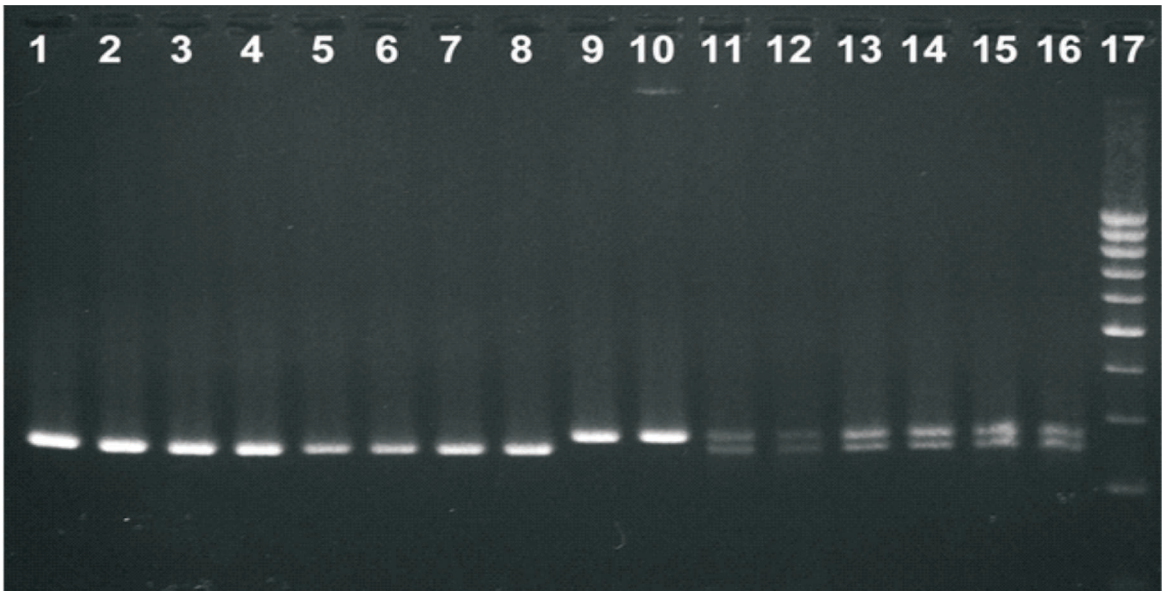

Fig. 3– PCR analysis with primers to SSR marker *Xtxp320*. 1-4 – DNA from the leaves of the Atc plants (1-4), green maternal-type seedlings (5, 7) from the offspring of the cross Atc × Volzhskoe-4v, the paternal line Volzhskoe-4v (9, 10), endosperm of the kernels, from which maternal-type seedlings were obtained (6, 8), leaves of hybrid seedlings (11, 12), and endosperm of the kernels, from which they were obtained (13-16) (each in duplicate); 17 - markers of the length of DNA fragments.Tracks 11-16 clearly show the co-dominant nature of the marker.

which the green seedlings were obtained, while both the maternal and the paternal alleles were present in the endosperm of the kernels from which the hybrid plants developed. These data confirm the autonomous nature of endosperm in apomictic kernels of the Atc line and is clearly consistent with our earlier cyto-embryological studies (Belyaeva and Elkonin 2018). The results of genotyping of seedlings using PCR analysis were fully confirmed by phenotypic differences between plants carrying only maternal alleles of markers *Sb1-10* and *Xtxp320*, and maternal and paternal alleles of these markers.

CONCLUSION

In this study, we, for the first time, identified SSR-markers, *Sb1-10* and *Xtxp320*, differentiating the sorghum line Atc, which has the ability to parthenogenesis and autonomous endospermogenesis, and the Volzhskoe-4v. Using these markers, the presence of apomixis in the Atc was confirmed; the endosperm in kernels with apomictic embryos also does not contain paternal markers, which indicates in favor of its autonomous origin. These data are fully consistent with the results of cytoembryological studies. It was found that the development of parthenogenetic embryos began 3-4 days after the opening of flowers in the absence of fertilization. The frequency of ovules with cytologically proven parthenogenesis on day 3 reached 12%. The data obtained can be used in research on the identification and cloning of apomixis genes, as well as on the creation of sorghum hybrids capable of maintaining a high level of heterosis.

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