Production And Performance Of Gynogenetic Sugarbeet Lines

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ABSTRACT

In vitro techniques were used to obtain haploid and doubled haploid lines of gynogenetic origin in sugarbeet (*Beta vulgaris L.*). Two pathways of regeneration were available for unfertilized ovule culture – embryogenesis and organogenesis. Regenerants were obtained from primary explants of diploid and tetraploid donor plants on MS nutrient medium supplemented with benzylaminopurine and sucrose. Flow cytometry indicated spontaneous polyploidization in all primarily haploid ovule-derived lines that originated from Belarusian cultivars. Mixoploidy that occured in doubled haploids was overcome through micropropagating apices of generative shoots. Performance of sugarbeet plants of ovule origin and hybrids produced with these lines indicated that promising breeding lines could be selected from gynogenetic lines.

Additional Key Words: *Beta vulgaris L.*, breeding, doubled haploid lines, flow cytometry, in vitro gynogenesis, ploidy level, regeneration pathways.

The genetic background of commercial sugarbeet cultivars is narrow. This is due to the narrow base from which sugarbeet originated, the need for disease resistance, and a negative relationship between root yield and sugar concentration (Doney, 1996). Parental lines for sugarbeet hybrids are incomplete inbred populations. The biennial life cycle, cross-pollination, and the polygenic and quantitative inheritance of many important traits slow the production of new cultivars. Biotechnology provides

techniques for preserving and increasing the biodiversity of sugarbeet while providing germplasm not obtainable through classical breeding (e.g. isogenic lines). Haploid lines, arising during culture of generative elements in vitro, produce homozygous plants in a relative short time, compared to inbreeding. Obtaining haploids via gynogenesis by culture of unfertilized ovules and induction of regeneration in these explants is more successful in *Beta* species than production via androgenesis (Rogozinska and Goska, 1982; Hosemans and Bossoutrot, 1983).

This report summarizes seven years of research on gynogenetic sugarbeet lines. The objectives of this project were: to develop the technology for obtaining gynogenetic lines; to assess their karyological status; and to determine the usefulness of ovule-derived lines in applied breeding programs.

MATERIALS AND METHODS

Vegetative plants from diploid cultivars Ganusovskaya 55, Belorusskaya 69 (widely grown in Belarus), Yanash A3, Belotserkovskaya 40, and male-sterile MS 5213, and tetraploid cultivars Verkhnyachskaya 103 and Yaltushkovskaya provided unfertilized ovules. The majority of donor plants were provided by BZOSS (a breeding station near Nesvizh, Belarus). Each gynogenetic line originated from a single ovule. After at least a 7 day cold pretreatment of donor plants at 4° C, ovules 0.3 to 0.6 mm long were removed from surface-sterilized closed floral buds and cultured for 2 weeks in the dark at 31° C, then in the light, 2000 lux, 16 h photoperiod, at 22° C. The induction nutrient medium was comprised of MS salts (Murashige and Skoog, 1962), 1 mg/l benzyladenine, and 60 g/l sucrose, solidified with agar. Shoots were maintained on the medium for sugarbeet micropropagation (MS salts + 0.3 mg/l benzyladenine + 0.5 mg/l gibberellic acid + 20 g/l sucrose + 7 g/l agar) and were rooted on MS medium with 3 to 5 mg/l naphtelene acetic acid (Svirshchevskaya and Bormotov, 1994). Ion-exchange substrates (e.g. Biona 112) were used for overcoming difficulties of adaptation to in vivo conditions (Svirshchevskaya et al., 1995).

Plants of Yanash A3 were treated with colchicine by soaking in vivo roots of regenerated plants in a 0.3% colchicine solution for 24 hours and planted in soil. Seven lines (from 34 attempted) were obtained after re-culturing spontaneously doubled haploid in vitro plants and plants of colchicine-treated haploid gynogenetic lines. Micropropagation through generative shoot apices was utilized for re-culturing.

Suspensions of intact nuclei, prepared by chopping approximately 20 mg of leaf tissue with a razor blade in a glass Petri dish containing 1 ml LB01 buffer made up of 15 mM TRIS, Na_2EDTA, 80 mM KCl, 20 mM

NaCl, 0.5 mM spermine, and 15 mM mercaptoethanol, 0.1% Triton X-100; pH 7.5, were screened for ploidy level (Dolezel et al., 1989). Nuclear DNA was stained in two µg/ml of 4'6-diamidino-2-phenylindole (DAPI) in the same buffer. Released nuclei were filtered through a 50 um nylon mesh. Fluorescence of stained nuclei was determined using a Partec PAS II flow cytometer (Partec GmbH, Münster, Germany). The gain of the instrument was adjusted so that the peak corresponding to G_0/G_1 nuclei isolated from the leaf of in vitro cultured diploid control shoots appeared on channel 100. Ploidy of shoot cells in shoots was determined by the relative position of peaks representing their G_0/G_1 nuclei. In each sample, 2000 to 9000 nuclei were analysed. Histograms of relative DNA content were evaluated using Partec PAS II software and the frequency of cells with 1C and 2C (mean from 5 replications) calculated for each genotype.

Root yield, with and without tops, sugar content, and number of mature leaves of plants of ovule-origin and hybrids with three testers (two male-sterile forms: T1 from KWS, Germany; T2 from MARIBO, Denmark; and a red tester) were evaluated by conventional techniques. In 1996 and 1997, plants were evaluated under field conditions at the Biological Experimental Station of the Institute of Genetics and Cytology, near Minsk. In 1998 and 1999, plants were evaluated in greenhouses at the same station. Statistical analyses were performed using a statistical software package (Anoshenko, 1994).

RESULTS AND DISCUSSION

Gynogenetic shoots appeared from meristematic zones of compact calli, from buds, and from embryoids of ovule origin. Embryogenic structures were observed at least for 2 weeks before organogenesis. Embryoids were transferred to MS medium with reduced sucrose content (20 g/l) and without hormones to obtain well-developed shoots. Subsequently, all shoots were cultured on nutrient medium for sugarbeet micropropagation.

The response of unfertilized ovules on the induction medium is summarized in Table 1. The 200 to 800 unfertilized ovules of each plant made possible the induction of multiple gynogenetic lines originating from one plant, even with the relatively low percent of response (1 to 8 percent). Regeneration included formation of meristems both through organogenesis and embryogenesis. Not all gynogenetic lines were viable, probably because of their haploid origin and long-term (from 3 months to 2 years) exposure to cytokinine containing medium during subculturing.

Chromosome counts (Bormotov et al., 1976) indicated that the majority of diploid donor plants produced haploids that eventually doubled. In the case of Belorusskaya 69 plant 5 and MS 5213 plant 1, ovule-derived

Table 1. Response of unpollinated ovules of different sugarbeet genotypes to cultivation on induction medium (MS salts +1 mg/l BAP +6% sucrose) and primary cytological characteristics of ovule-derived lines

Genotype			Regeneration			
Cultivar	Plant No.	Ploidy level of donor plant	Total number of ovules/ovules with response	Percent	Regeneration mode	Ploidy level of ovule-derived lines in early culture passages
Belorusskaya 69	1	2x	200/4	2.0	O	x+2x
Belorusskaya 69	2	2x	296/9	3.0	O,E	x+2x
Belorusskaya 69	3	2x	240/9	3.8	O	x+2x
Belorusskaya 69	4	2x	240/9	3.8	O,E	x+2x
Belorusskaya 69	5	2x	120/3	2.5	O	2x
Ganusovskaya 55	1	2x	870/10	1.2	O	x+2x
Ganusovskaya 55	2	2x	780/9	1.2	O,E	x+2x

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Abbreviations:

MS – Murashige and Skoog nutrient medium salts (Murashige and Skoog, 1962).
BAP - benzylaminopurine.
O – organogenesis.
E – embryogenesis.

Table 1 (continued). Response of unpollinated ovules of different sugarbeet genotypes to cultivation on induction medium (MS salts +1 mg/l BAP +6% sucrose) and primary cytological characteristics of ovule-derived lines

Genotype			Regeneration			
Cultivar	Plant No.	Ploidy level of donor plant	Total number of ovules/ovules with response	Percent	Regeneration mode	Ploidy level of ovule-derived lines in early culture passages
Ganusovskaya 55	3	2x	740/21	2.8	O	x+2x
Ganusovskaya 55	4	2x	470/10	2.1	O,E	x+2x
Ganusovskaya 55	5	2x	912/33	3.6	O,E	x+2x,2x
MS 5213	1	2x	160/2	1.3	O	2x
Yanash A 3	4	2x	140/12	8.6	O	x+2x
Yaltushkovskaya	1	4x	400/3	0.8	O	2x
Verkhnyachskaya 103	1	4x	120/1	0.8	O	2x

Abbreviations:

MS – Murashige and Skoog nutrient medium salts (Murashige and Skoog, 1962).

BAP - benzylaminopurine.

O – organogenesis.

E – embryogenesis.

lines were generally diploid. In Ganusovskaya 55 plant 5 both mixoploid (x+2x) and diploid (2x) lines were found. In our experiments mixoploids and diploids were represented by cells with euploid chromosome sets only. It was recognized that ploidy in newly arisen gynogenetic lines needs to be determined in early culture passages (first 2 to 3 months of in vitro cultivation), to avoid mistakes in assessing the homo/heterozygosity of progeny. Intensive polyploidization was also observed in in vivo plants of gynogenetic lines by Bossoutrot and Hosemans (1985) and Van Geyt et al. (1987). Polyploidy was the most common genomic change observed in cultured plant cells. It may originate de novo, e.g., via spindle failure leading to restitution nucleus formation due to prolonged exposure to phytohormones in nutrient media, or may derive from pre-existing polyploid cells in the explant (Morrison and Whitaker, 1988).

The results of the flow cytrometric analysis were displayed in a histogram of relative nuclear DNA content. Ploidy was defined as the number of copies of the chromosome complement. Differentiated plant tissues may contain a high proportion of cells in G_2 phase (i.e. 4C DNA content) or cells that have undergone endoreduplication. A simple DNA content analysis cannot distinguish these cells from polyploid cells (Dolezel, 1997). Because of this, the interpretation was based on the frequency of cells with 1C and 2C DNA content (4C DNA data are not shown) . The relative DNA content measurements in cultured leaves of ovule-derived lines are summarized in Table 2. Each line represents the gynogenetic progeny of a single plant maintained for various lengths in culture.

Four gynogenetic lines regenerated from Belotserkovskaya 40 and three from Yanash A3 were originally haploid and became mixoploid after spontaneous and/or colchicine-induced polyploidization. Ploidy level was determined by microscope chromosome counting. Plants of these mixoploid gynogenetic lines propagated with apical meristems of generative shoots (second in vitro cycle), gave rise to lines 1 to 7. Flow cytometric analysis revealed diploid cells in different cell cycle phases and the absence of haploid cells. This result indicated that re-cultivation of mixoploid (x + 2x) sugarbeet plants can be used for haploid elimination. Re-culturing seems to be essential to avoid the poor agronomic performance characteristic of plants containing haploid tissues. The frequency of cells with 2C DNA content in leaves within seven re-cultured gynogenetic lines (60 to 70%) was close to that observed in diploid controls (data not shown). Only two lines (8 and 33), from Verkhnyachskaya 103 and Yaltushkovskaya, were regenerated in vitro from ovule culture of tetraploid donor plants. As expected, their leaf cells had 2C DNA and lines consequently were classified as dihaploids.

Table 2. Flow cytometric characteristics of sugarbeet gynogenetic lines cultured in vitro.

Line characteristics						
Line No.	Cultivar	Origin	Age of culture in months	Regeneration mode	Percentage of cells with 1C DNA	Percentage of cells with 2C DNA
1	Belotserkovskaya 40	SDH/RC - 1	6	organogenesis	0.0	61.7
2	Belotserkovskaya 40	SDH/RC - 2	6	organogenesis	0.0	76.4
3	Belotserkovskaya 40	SDH/RC - 3	6	organogenesis	0.0	64.8
4	Belotserkovskaya 40	SDH/RC - 4	6	organogenesis	0.0	69.8
5	Yanash A3	CDH/RC - 1	6	organogenesis	0.0	73.2
6	Yanash A3	SDH/RC - 2	6	organogenesis	0.0	60.8
7	Yanash A3	SDH/RC - 3	6	organogenesis	0.0	57.1
8	Verkhnyachskaya 103	DiH/RC - 1	6	organogenesis	0.0	59.8
9	Belorusskaya 69	2 a	12	organogenesis	47.1	43.7

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Abbreviations: Line origin:

DiH - dihaploid (regenerant from 4x - donor plant).
CDH - colchicine- induced doubled haploid.
SDH - spontaneously in vitro doubled haploid.
RC - re-cultivation using apices of generative shoots.
1 a, ..., 2 b,... - numbers of plants (1,2,...) and different ovules (a, b, ...).

Table 2 (continued). Flow cytometric characteristics of sugarbeet gynogenetic lines cultured in vitro.

Line characteristics					Percentage of cells with 1C DNA	Percentage of cells with 2C DNA
Line No.	Cultivar	Origin	Age of culture in months	Regeneration mode		
10	Belorusskaya 69	2 b	12	organogenesis	18.6	54.4
11	Belorusskaya 69	2 c	12	organogenesis	42.0	45.0
12	Belorusskaya 69	2 d	12	organogenesis	29.5	51.8
13	Belorusskaya 69	2 e	12	embryogenesis	60.0	30.9
14	Belorusskaya 69	3 a	12	organogenesis	31.8	55.6
15	Belorusskaya 69	3 b	12	organogenesis	64.3	35.7
16	Belorusskaya 69	3 c	12	organogenesis	59.0	37.0
17	Belorusskaya 69	3 d	12	organogenesis	55.5	44.5
18	Belorusskaya 69	3 e	12	organogenesis	33.6	49.2

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Abbreviations: Line origin:

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Table 2 (continued). Flow cytometric characteristics of sugarbeet gynogenetic lines cultured in vitro.

Line characteristics						
Line No.	Cultivar	Origin	Age of culture in months	Regeneration mode	Percentage of cells with 1C DNA	Percentage of cells with 2C DNA
19	Ganusovskaya 55	4 a	6	organogenesis	44.9	45.8
20	Ganusovskaya 55	4 b	6	organogenesis	31.7	49.0
21	Ganusovskaya 55	4 c	6	organogenesis	58.5	37.2
22	Ganusovskaya 55	4 d	6	organogenesis	42.1	46.8
23	Ganusovskaya 55	5 a	6	organogenesis	0.0	51.1
24	Ganusovskaya 55	5 b	6	organogenesis	31.1	56.5
25	Ganusovskaya 55	5 c	6	organogenesis	0.0	60.5
26	Ganusovskaya 55	5 d	6	organogenesis	0.0	36.9
27	Ganusovskaya 55	5 e	6	organogenesis	35.3	54.0

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Abbreviations: Line origin:

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CDH - colchicine- induced doubled haploid.
SDH - spontaneously in vitro doubled haploid.
RC - re-cultivation using apices of generative shoots.
1 a, ..., 2 b,... - numbers of plants (1,2,...) and different ovules (a, b, ...).

Table 2 (continued). Flow cytometric characteristics of sugarbeet gynogenetic lines cultured in vitro.

Line characteristics						
Line No.	Cultivar	Origin	Age of culture in months	Regeneration mode	Percentage of cells with 1C DNA	Percentage of cells with 2C DNA
28	Ganusovskaya 55	5 f	6	organogenesis	0.0	60.8
29	Yanash A 3	4 a	3	organogenesis	52.9	47.1
30	Yanash A 3	4 b	3	organogenesis	49.0	43.0
31	Yanash A 3	4 c	3	organogenesis	49.6	42.4
32	Yanash A 3	4 d	3	organogenesis	54.7	39.7
33	Yaltushkovskaya	DiH 1a	15	organogenesis	0.0	65.6
34	MS 5213	1 a	6	organogenesis	0.0	64.0
	$LSD_{0.05}$				6.4	5.4
	$LSD_{0.01}$				8.5	7.2

Abbreviations: Line origin:
DiH - dihaploid (regenerant from 4x - donor plant).
CDH - colchicine- induced doubled haploid.
SDH - spontaneously in vitro doubled haploid.
RC - re-cultivation using apices of generative shoots.
1 a, ..., 2 b,... - numbers of plants (1,2,...) and different ovules (a, b, ...).

Almost all gynogenetic lines obtained from diploid donor plants were mixoploid (x+2x). Being originally haploid, these lines underwent spontaneous chromosome doubling (from x to 2x). Among the progeny of plant 5 of Ganusovskaya 55, four lines had diploid cells only, while two other lines had both haploid and diploid cells. The male-sterile line MS 5213 produced a gynogenetic line with an unreduced (2x) ploidy level. The occurance of diploid ovule-derived lines from a diploid donor could be explained by regeneration from an unreduced gamete or regeneration from somatic tissue surrounding the embryo sac that is capable of morphogenesis.

Under the in vitro conditions, the frequency of polyploidization was dependent upon genetic background. The relationship between the haploid : diploid cell ratio and the length of culture was not simple. Cells with 1C DNA content within the progeny of the plant no. 2 of Belorusskaya 69 ranged from 18.5 to 60.0% (the last line was characterized by embryogenic origin) and those of plant no. 3 had 31.8 to 64.3% 1C DNA cells (age of in vitro culture was the same for these lines). Progeny of two plants of Ganusovskaya 55 (4 and 5), had from 0 to 58.5% haploid cells. Young cultures of gynogenetic progeny of plant 4 of Yanash A3 (3 months of subculturing) had the expected high (close to 50%) frequency of haploid cells. Such karyological heterogeneity is consistent with observations of gametoclonal variants of triticale, wheat, rice, and other species (Kunakh, 1995).

Plants produced from unfertilized ovules had well-developed leaves and roots. Root yield and sugar content of regenerants of Verkhnjachskaya 103 (DiH), Yanash A3 (SDH, CDH, CDH/RC), and Belotserkovskaya 40 (CDH, SDH, SDH/RC) differed. Spontaneously doubled haploids produced higher root weights and sugar yield, than diploids of the same origin produced with colchicine in 1996. In some cases these differences were statistically significant. Root yield, sugar content and sugar yield per root of all gynogenetic lines were compared with corresponding diploid heterozygous controls. Three lines that produced plants exceeding the diploid control for yield and sugar concentration (Verkhnjachskaya 103 DiH, Yanash A3 CDH, Belotserkovskaya 40 SDH) are of interest to breeders and indicate the utility of gynogenesis (Svirshchevskaya and Borzyak, 1998).

Plants of these three lines derived through ovule culture were placed under isolators together with plants of two male-sterile lines from KWS and MARIBO and a fertile red tester to produce testcross hybrids. Three hundred and twenty hybrid plants were grown in 1997 at the Biological Experimental Station, Institute of Genetics and Cytology (Minsk). Root weight with and without tops, number of mature and young leaves, other

characteristics of leaf development, sugar content, and sugar yield per root were observed (Table 3). A dihaploid line, induced in ovule culture of tetraploid Verkhnjachskaya 103 had higher values for all investigated traits, compared to hybrids. Its performance also significantly exceeded that of diploid cultivar-standards ($P<0.05$).

In a group of lines and hybrids with Belotserkovskaya 40, Bts 40 SDH/RC (which is probably not fully homozygous after a long period of culture and re-culture,) was better than its heterozygous control; hybrid T1 x Bts SDH was significantly better than both parents.

In a group of lines and hybrids developed from Yanash A3, the best performance with both male-sterile testers was obtained with gynogenetic line Yanash A3 CDH/RC and Yanash SDH (Svirshchevskaya et al., 1997).

A dihaploid line (220 plants) had both root yield and sugar content similar to that of its hybrids and Verkhnjachskaya 103, in 1998. Bts 40 SDH was inferior to its diploid heterozygous counterpart and other standard cultivars. Hybrids including this gynogenetic line yielded more than the original line in root weight, with tops and without tops;

Hybrid T2 x Bts 40 SDH was superior to T2 x Bts 40 CDH, based upon relative yields. In particular, for root weight with tops the values were 576.7 compared with 299.0 grams; for number of mature leaves, 19.2 compared with 11.4 ($P<0.01$), consistant with results of previous years. Yanash A3 CDH/RC performed well in comparison with cultivar-standards (453.0 gram for root plus top weight compared to 205.0 for Ganusovskaya 55, a diploid standard; sugar content - 16.7 and 16.5%; root weight 153.0 and 78.0 gram, number of mature leaves – 18.9 and 9.9 respectively). Thus, performance of ovule-derived lines and their hybrids from 1996 through 1998 indicated the expediency of using Bts 40 SDH/RC, Yanash A3 CDH/RC and DiH Verkhnjachskaya 103 as breeding lines.

Table 3. Productivity traits of sugarbeet gynogenetic lines and their hybrids with testers, 1997.

Genotype	Root weight with tops	Root weight	Number of mature leaves	Sugar content
	—g—	—g—		—%—
Lines:				
Bts 40 SDH	205.7	110.9	10.2	17.9
Bts 40 SDH/RC	670.8	377.7	18.6	19.4
Yanash A3 SDH	223.6	128.2	11.7	18.9
Yanash A3 CDH	204.0	103.0	9.8	18.5
Yanash A3 CDH/RC	542.2	315.8	14.9	19.3
Ver 103 DiH	507.0	239.0	20.2	17.8

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Abbreviations:
Bts 40 - Belotserkovskaya 40
Ver 103 -Verkhnyachskaya 103
Rt - Red tester
T1,T2 - testers with male sterility
CDH - colchicine-induced doubled haploid
SDH - spontaneously doubled haploid
DiH - dihaploid (from 4x-donor)
RC -re-cultured in vitro plant

Table 3 (continued). Productivity traits of sugarbeet gynogenetic lines and their hybrids with testers, 1997.

Genotype	Root weight with tops	Root weight	Number of mature leaves	Sugar content
	—g—	—g—		—%—
Hybrids:				
T1 x Bts 40 SDH	526.1	226.9	17.9	17.2
T2 x Bts SDH	331.9	188.1	10.3	19.6
T1 x Bts 40 CDH	186.0	92.0	10.2	18.9
T2 x Bts 40 CDH	301.3	167.0	13.7	19.2
Bts 40 SDH x Rt	159.1	79.1	6.6	16.0
T1 x Yanash A3 SDH	420.0	235.0	12.5	18.4
T2 x Yanash A3 SDH	357.1	212.4	8.4	19.4
Yanash A3 CDH x Rt	253.3	122.7	8.3	15.8
T1 x Ver 103 DiH	234.0	118.0	15.1	17.5
T2 x Ver 103 DiH	303.1	120.8	14.5	17.0
Ver 103 DiH x Rt	172.7	91.8	7.5	17.2

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Abbreviations:
Bts 40 - Belotserkovskaya 40
Ver 103 -Verkhnyachskaya 103
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T1,T2 - testers with male sterility
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RC -re-cultured in vitro plant

Table 3 (continued). Productivity traits of sugarbeet gynogenetic lines and their hybrids with testers, 1997.

Genotype	Root weight with tops	Root weight	Number of mature leaves	Sugar content
	—g—	—g—		—%—
Controls (2x):				
Bts 40	451.7	204.2	16.8	18.4
Ver 103	646.4	328.5	17.0	18.9
T1 (KWS)	218.6	131.4	12.9	17.3
Red tester	100.0	55.0	8.8	13.2
Belorusskaya 69	238.1	128.1	12.9	17.5
Ganusovskaya 55	162.9	87.9	12.2	18.1
$LSD_{0.05}$	180.9	104.7	2.9	0.7
$LSD_{0.01}$	237.7	137.6	3.8	0.9

Abbreviations:
Bts 40 - Belotserkovskaya 40
Ver 103 -Verkhnyachskaya 103
Rt - Red tester
T1,T2 - testers with male sterility
CDH - colchicine-induced doubled haploid
SDH - spontaneously doubled haploid
DiH - dihaploid (from 4x-donor)
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