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A RELATIONSHIP BETWEEN PLOIDY LEVEL AND THE NUMBER OF CHLOROPLASTS IN STOMATAL GUARD CELLS IN DIPLOID AND AMPHIDIPLOID *Brassica* SPECIES

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Abstract

Doubled haploid lines production through isolated anthers and isolated microspore cultures has been used widely for genetic studies and plant breeding of *Brassica* crops. The ploidy level of microspore derived plants varies, and normally haploid, diploid and mixoploid plants could be obtained in vitro. The determination of ploidy level is essential in doubled haploid pure line production. The determination of ploidy level by counting the number of chloroplast in stomatal guard cells (NCSGC) is less time consuming, laborious and expensive comparing to chromosome counting in root tip cells or mother pollen cells and flow cytometry methods. Several studies have been reported concerning relationship between ploidy level and number of chloroplast in stomatal guard cells of *Brassica rapa*, *B. napus* and *B. oleracea* species, however a small number of genotypes had been analyzed. In our study, the NCSGCs of haploid (n) and diploid ($2n$) Chinese cabbage (*B. rapa* ssp. *pekinensis*), winter oilseed rape (*B. napus* var. *napus*) and haploid, di- and tetraploid white cabbage (*B. oleracea* var. *capitata*) microspore derived plants were estimated, and also the influence of plant growth temperature (6±2 °C и 24±2 °C) and development stage (vegetative or generative) was investigated. High correlation between the ploidy level of microspore-derived plants and NCSGC is found for white cabbage (*Brassica oleracea*, $r = 0.94$), Chinese cabbage (*B. rapa*, $r = 0.90$) and oilseed rape (*B. napus*, $r = 0.94$). The chloroplast average number in stomatal guard cells was very similar among the same ploidy genotypes of Chinese cabbage as well as rapeseed, while the variation of chloroplast number in diploid and tetraploid white cabbage plants was significant. In a range of early, middle and late maturing diploid white cabbage inbred lines there was established the tendency to form more chloroplasts in the early lines (Pl, Sus and others) and less in the late lines (AM2, Sa1, Ges2 and others), with the difference up to 1.7 times, that is comparable to the difference between haploid and diploid plants of Chinese cabbage or rapeseed. The chloroplast number in stomatal guard cells is 4.2-7.8 and 7.9-13.6 for Chinese cabbage (*B. rapa*) haploids and diploids, respectively, 7.5-12.4 and 14.1-20.3 for rapeseed (*B. napus*) amphihaploids and amphidiploid, respectively, and 7.7-9.9, 11.7-17.9 and 18.0-26.5 for white cabbage (*B. oleracea*) haploids, diploids and tetraploids, respectively. No significant influence of vegetative or generative stage of plant development or growth temperature on NCSGC was found.

Keywords: haploid, diploid, white cabbage, Chinese cabbage, stomatal guard cells, number of chloroplasts, ploidy, rapeseed, tetraploid, *Brassica oleracea*, *Brassica napus*, *Brassica rapa*.

The biotechnology of producing pure breeding lines, the doubled haploids, via anthers and isolated microspore cultures is recently widely applicable in genetic studies and breeding cole crops (1-3). Due to this approach, the in vitro generated plants have different ploidy, and, together with doubled haploids, also haploid, tetraploid and mixoploid forms can occur (4). Estimation of ploidy is essential step in doubled haploid production (5, 6).

Various methods different in accuracy, the complexity and cost are used for ploidy estimation. They are counting the number of chromosomes under microscopy of cytological preparations (7); qualitative assay of chromatin content in cell nuclei by flow cytometry (8); analysis of complex indirect signs in plants, namely their morphological features, size of guard cells, the guard cell number per unit leaf area, number of chloroplasts in the guard cells of stomata (NCGCS),

size of pollen grains and the number of pores on its exine, fertility and frequency of seed formation (9).

Counting the number of chromosomes in the mitotic cells of root meristems is laborious and time-consuming, as the *Brassica* chromosomes are small, and the number of metaphase plates depends on root growth. The counting can not be performed for a large number of plants, therefore, it remains a laboratory method (5). Flow cytometry seems to be one of the most effective, accurate and convenient. Easy methods for preparation allow scanning few hundred samples daily, besides, a minimal amount of leaf tissue is enough. Nevertheless, it application is limited by high price of the device, resulting in high cost per analysis (10). Phenotypic identification on distinguishing features of haploid plants, e.g. male sterility, the smaller size of the vegetative organs, narrow leaves, etc., is uncomfortable and long because the plants must be cultivated for a few months to reach flowering (11). NCGCS estimation is easy to perform, cheap and used in practical plant breeding for a long time.

In anther derived winter rape (*B. napus*) plants the varying number of chloroplasts, 12.0-14.0 in haploids and 19.5-20.9 in diploids, was detected in stomatal guard cells (12). In *B. campestris* ssp. *pekinensis* the reported NCGCS values were 2-4 for haploids, 4-6 for diploids and 8-10 for tetraploids (5). Another study found that the number of chloroplasts in a pair of stomatal guard cells in Chinese cabbage varies from 6.1 to 8.6 in haploids, from 10.1 to 12.7 in diploids and from 15.9 to 17.8 in tetraploids (13).

In the report of S.J.C. Dias the NCGCS values in haploid, diploid and tetraploid *B. oleracea* ssp. were 6-9, 10-15 and 20-25, respectively (11). In experiments carried out by S. Yuan et al. (14), in *B. oleracea* var. *capitata*, *B. oleracea* var. *italica* and *B. oleracea* var. *alboglabra* derived from isolated microspore the NCGCS were less than 10 for haploids (at an average value of 6.96 to 7.67 per plant), 11-15 for diploids (at an average value of 12.36 to 13.89 per plant), and more than 15 for polyploids (at an average value of 16.96 to 17.61 in a triploid plant and 22.61 to 24.97 in a tetraploid plant). In the experiment the accuracy of the method for determining the ploidy reached 93.93 % and did not depend on the growth conditions, in particular cultivation in the greenhouse or cold nursery (14).

An average numbers of chloroplasts in stomatal guard cells in *B. oleracea* var. are very similar in top, middle and bottom part of a leaf, and in 3^{d}, 5th and 7th true leaves of the same ploidy plants (14). Also no significant differences were noted in NCGCS within a single plant and between regenerated plants of the same ploidy in *B. campestris* ssp. *pekinensis* indicating stability of this trait independently of plant age, nevertheless, the stages of plant development when the study was conducted are not specified (5).

In our study, the NCSGCs of haploid (n) and diploid ($2n$) Chinese cabbage (*B. rapa* ssp. *pekinensis*), winter oilseed rape (*B. napus* var. *napus*) and haploid, di- and tetraploid white cabbage (*B. oleracea* var. *capitata*) microspore derived plants were estimated, and also the influence of plant growth temperature (6±2 °C и 24±2 °C) and development stage (vegetative or generative) was investigated.

Technique. We studied the members of three *Brassica* species, i.e. the *Brassica rapa* ssp. *pekinensis* populations of haploid and diploid regenerants MEDH, (MChE)DH, XMDH, Xa642DH, MlchDH, Kit1-3DH, TPV36DH (a total of 219 plants), derived from isolated microspore culture; the winter oilseed rape *B. napus* var. *napus* Severyanin variety (originated by V.R. Vil'yams All-Russian Research Institute of Forage), breeding lines Gal1, Lim1 and RS23 (N.N. Timofeev Breeding Station) and haploid and diploid regenerants SevDH, GalDH,

LimDH and RS23DH derived via isolated microspore culture of the abovementioned accessions; white cabbage *B. oleracea* var. *capitata* populations of haploid, diploid and tetraploid regenerants FarDH, SurDH, EtDH, ParDH, NazDH (a total of 100 plants), derived through isolated microspore culture, and 22 inbred lines of white cabbage (genetic collection of N.N. Timofeev Breeding Station) including early maturing lines (80-100 days; Pl, Sus1, Et1, Sf, Sh5a, Dpp2, Dt), middle maturing lines (110-140 days, Ak3, Meg1, B25, Uf1, S110) and late maturing lines (150-180 days, Fl4, Pr3, Vb4, Xt5, Pm4, Fu44, Bu1, AM2, Sa1, Ges2.

The influence of temperature on NCSGCs was assessed using the diploid Chinese cabbage *B. rapa* (F_1 hybrids Hydra and Nezhnost), the white cabbage *B. oleracea* (F_1 hybrids SB-3 and Valentina) originated by N.N. Timofeev Breeding Station, and the amphidiploid winter oilseed rape *B. napus* (Severyanin variety). Three plants of each sample were grown in greenhouse at 24±2 °C and in climatic chamber at 6±2 °C according to standard agrotechnology. NCSGCs were counted after 75 days of cultivation.

To elucidate variability of NCSGCs due to the stage of plant development, the Chinese cabbage inbred line Xa642 and white cabbage line Bu1b originated by N.N. Timofeev Breeding Station, and also winter oilseed rape variety Severyanin were studied. NCSGCs were counted in 20-day seedlings with 4-5 true leaves and in the leaves of flowering shoots after vernalization for the time necessary for the respective culture (the flowering stage).

The regenerated plants derived from isolated microspores, after their adaptation, and the plants derived from seeds were grown at controlled conditions in greenhouse at 24-26 °C/20-22 °C (day/night) in spring and summer according to common procedure. The sowing was carried out into 64-cell containers, 5×5 cm per cell, and 25-30 day seedlings were further transferred into plastic 8-liter pots. Milled peat Klasmann TS-1 («Klasmann-Deilmann GmbH», Germany) with mineral fertilizers (N — 100-120 mg/l, P_2O_5 — 120-220 mg/l, K_2O — 140-240 mg/l, 14:16:18) served as the substrate. Watering and mineral fertilizing were held if necessary.

Chloroplasts were counted in leaves as described (15) with slight modifications. The leaf segments were rinsed with running tap water to remove waxy layer and dust, then, with forceps, an epidermal layer was manually removed from the underside of a leaf and placed on a microscope slide into drop of 1 % $AgNO_3$ with cover glass. The preparations were viewed under a microscope (Axioskop 40, Carl Zeiss, Germany). In each sample chloroplasts were counted in 10 pairs of stomatal guard cells at magnification ×400. The chromosomes were counted in meiotic anthers cells of young flower buds. The specimens were collected in the morning and fixed in 3:1 96 % ethanol to glacial acetic acid fixative. Permanent cytological preparations were made by spreading method (16) with some modifications. Before preparing permanent slides, the buds with cell division were chosen. The stage of cell cycle and meiotic phase for each bud was classified by preparing anther squashes with drop of acetocarmine. The buds with anaphase I and II cells were used for preparing permanent slides.

Fixed specimens were rinsed by running tap water for 15 min, then 5-10 removed anthers were placed into 1.5 ml Eeppendorf tubes containing of *Aspergillus niger* pectinase (13.5 U/ml) and *Trichoderma reesi* cellulase (80.0 U/ml) (Serva, Germany) in citric buffer (pH 4.8) and incubated for 50-70 min at 37 °C in water bath. With pipette, anthers were gently removed, placed on a glass slide, carefully crushed with a dissecting needle in a drop of 60 % acetic acid and waited for 1 min. The obtained suspension was traced around by fixative with followed addition of 1-2 drops of fixative into the center of it. Preparations were

rinsed in 96 % ethanol, dried, stained in 1 % Giemsa's solution in phosphate buffer (pH 6.9-7.0) for 10-15 min, then rinsed in distilled water and air dried. The preparations were observed using Axioskop 40 immersion system (Carl Zeiss, Germany). Chromosomes were counted in 10-15 metaphase and/or anaphase plates at magnification ×630.

Correlation coefficients, the reliability of differences and the confidence interval were calculated using Microsoft Excel 2010 on the base of *t*-Student's distribution at 0.05 level of significance.

Results. We estimated an average number of chloroplasts in stomatal guard cells in Chinese cabbage *B. rapa* and winter rape *B. napus* haploid and doubled haploid regenerants derived through isolated microspore culture. In each of 7 tested populations of Chinese cabbage and 4 population of rape, there were two groups. In Chinese cabbage, an average NCSGC values in these groups were 5.50 and 9.95, respectively (Table 1, 2), and in rape they were 9.85 and 17.03, respectively (see Table 2, 3).

1. Average number of chloroplasts in a pair of stomatal guard cell (ANCSGC) **in haploid and diploid regenerants of Chinese cabbage (*Brassica rapa*), derived through isolated microspore culture**

Population	Haploids			Diploids			Diploids to haploids ratio
	plant number	ANCSGC		plant number	ANCSGC		
		$X \pm x^{1,2}$	limits		$X \pm x^{1,2}$	limits	
MEDH	16	5.29±0.46[a]	4.2-6.9	47	9.71±0.30[a]	7.9-12.0	1.84
(MChe)DH	20	5.42±0.45[a]	4.3-7.8	21	9.51±0.44[a]	7.9-11.6	1.75
XMDH	24	5.28±0.30[a]	4.3-7.4	25	9.66±0.24[a]	8.5-10.8	1.83
Xa642DH	6	5.16±0.27[a]	4.5-5.8	6	9.05±0.50[ac]	8.4-11.1	1.75
MlchDH	9	6.23±0.41[b]	4.8-7.7	19	11.29±0.42[b]	8.6-13.6	1.81
Kit1-3DH	8	5.76±0.57[ab]	4.9-6.8	6	10.55±1.35[ab]	8.4-12.2	1.83
TPB36DH	2	4.90±1.27[ab]	4.8-5.0	10	8.45±0.41[c]	7.9-9.5	1.72
Total	85	5.50±0.17	4.2-7.8	134	9.95±0.20	7.9-13.6	1.81

C o m m e n t s. 1 — confidence interval according to *t*-Student's distribution at 0.05 level of significance; 2 — values marked with the same letters (a, b, c) do not differ at P ≤ 0.05 according to *t*-Student's test; 3 — seed progeny of initial donor plant used in isolated microspore culture.

2. Average number of chloroplasts in a pair of stomatal guard cells in *Brassica* plants of different ploidy

Crop	Species	Haploids	Diloids	Tetraploids
Chinese cabbage	*B. rapa*	5.50[a]	9.95[b]	–
Rape	*B. napus*	9.85[a]	17.03[b]	–
White cabbage	*B. oleracea*	8.53[a]	13.46[b]	21.28[c]

C o m m e n t s. Values marked with the same letters (a, b, c) do not differ at P ≤ 0.05 according to *t*-Student's test. Dashes mean the data were not obtained.

3. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in haploid and diploid rape *Brassica napus***

Population	Haploids			Diploids			Diploids to haploids ratio
	plant number	ANCSGC		plant number	ANCSGC		
		$X \pm x^{1,2}$	limits		$X \pm x^{1,2}$	limits	
RS23[3]	–	–	–	3	17,97±0,76[a]	17,7-18,3	–
RS23DH	37	9.55±0.35[a]	7.5-11.9	12	16.48±0.88[a]	14.5-18.4	1.73
Severyanin	–	–	–	3	16.27±1.46[a]	15.6-16.7	–
SevDH	19	10.19±0.44[b]	8.5-11.8	5	17.94±2.49[a]	14.9-20.3	1.76
Gal1[3]	–	–	–	3	16.23±1.86[a]	15.5-17.0	–
GalDH	23	9.95±0.31[ab]	8.7-11.5	6	17.58±1.29[a]	16.0-19.4	1.77
Lim1[3]	–	–	–	2	17.40±1.05[a]	17.1-17.7	–
LimDH	28	9.95±0.40[ab]	8.0-12.4	5	16.36±2.65[a]	14.1-19.7	1.64
Total	107	9.85±0.19	7.5-12.4	39	17.03±0.46	14.1-20.3	1.73

C o m m e n t s. 1 — confidence interval according to *t*-Student's distribution at 0.05 level of significance; 2 — values marked with the same letters (a, b, c) do not differ at P ≤ 0.05 according to *t*-Student's test; 3 — seed progeny of initial donor plant used in isolated microspore culture. Dashes mean the data were not obtained.

A comparison of complex morphological traits in regenerated plants (i.e.

the thickness of the stem, leaf size, size and fertility/sterility of flowers) indicated their haploid and diploid characters in different groups, being also confirmed by cytological analysis of pollen mother cells in several typical plants from each group (Fig. 1).

Both in Chinese cabbage and rape, the lines of the same ploidy, except for one or two populations, were rather homogenous according to NCSGC. That allows to suggest no significant effect of the genotype to NCSGC within tested accessions and probably subspecies. An absence of significant differences between diploid rape regenerants derived from microspore culture and the plants from seeds of initial donor plants should also be noted.

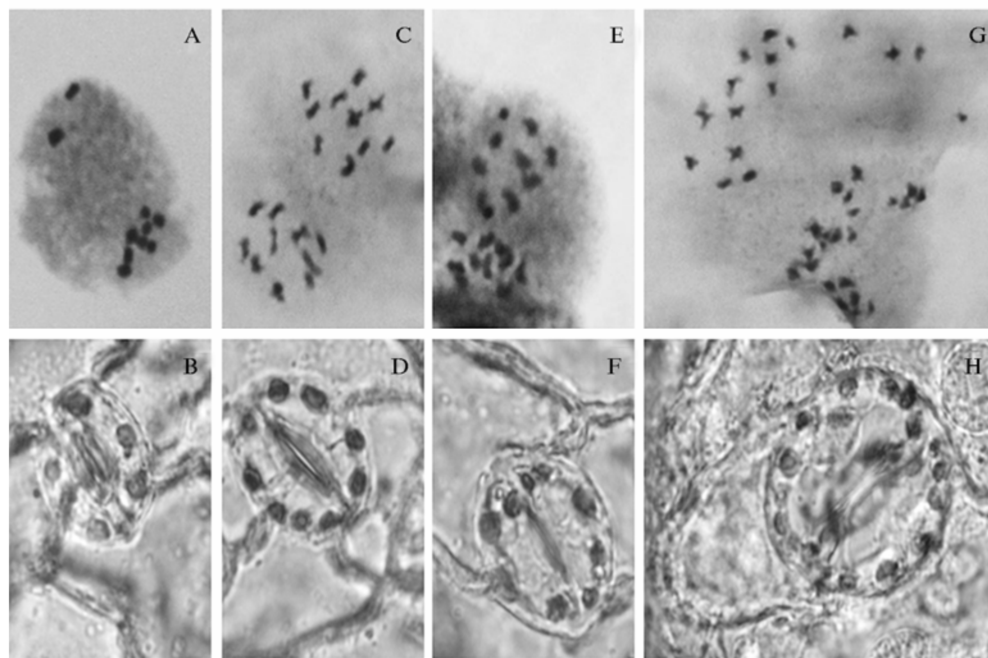

Fig. 1. Chromosomes in dividing pollen mother cells (anaphase I) (A-D) **and stomatal guard cells with chloroplasts** (E-H) **in *Brassica* plants of different ploidy:** A, B — haploids (n = 10, the number of chloroplasts in a pair of stomatal guard cells/NCSGC = 5), C, D — diploids ($2n$ = 20, NCSGC = 9) of Chinese cabbage (*B. rapa*); E, F — amphidiploids (n = 19, NCSGC = 9), G, H — amphidiploids ($2n$ = 38, NCSGC = 19) of rape (*B. napus*). Silver staining, magnification ×630.

Minimum and maximum average NCSGC values in a pair of stomatal guard cells in Chinese cabbage plants were 4.2 and 7.8 in haploids and 7.9 and 13.6 in diploids, respectively. In rapes, these values were 7.5 and 12.4 in haploids and 14.1 and 20.3 in diploids, respectively. In the absence of overlapping NCSGC maximum values in haploids and minimum values in diploids, in Chinese cabbage and rape the NCSGC in diploids was 1.7-1.8 times higher than in haploids. The Pearson's correlation coefficient (r) for NCSGC and the chromosome number (ploidy) was 0.90±0.03 in 219 haploid and diploid Chinese cabbage plants and 0.94±0.03 in 146 rape plants.

Based on NCSGC values analysis, each population of FarDH, ParDH and NazDH white cabbage (*B. oleracea*) regenerated plants was divided into three groups, and two groups were identified in each of SurDH and EtDH populations (Table 4). By means of cytological analysis of dividing cells within a bud and the pollen fertility, the ploidy was identified for all the groups. In the haploid, diploid and tetraploid groups the NCSGC was the lowest (8.53), inter-

mediate (13.46), and the highest (21.28), respectively (Fig. 2).

In FarDH, ParDH and NazDH regenerants the haloid group was rather homogeneous on NCSGCs which varied slightly (7.7-9.9), while in the diploids and tetraploids a very wide range of variation was observed, 11.7-17.9 and 18.0-26.5, respectively, leading to essential differences between the populations. Moreover, there were few plants with significantly higher NCSGCs comparing to the tetraploids. Such plants usually were tetraploid with one or two additional chromosomes (data not shown).

4. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in haploid, diploid and tetraploid white cabbage (*Brassica oleracea*) regenerants derived through isolated microspore culture**

Population	Haploids			Diploids			Tetraploids		
	plant number	ANCSGC		plant number	ANCSGC		plant number	ANCSGC	
		$X \pm x^{1,2}$	limits		$X \pm x^{1,2}$	limits		$X \pm x^{1,2}$	limits
FarDH	6	8.27 ± 0.63^{a}	7.7-8.9	36	12.76 ± 0.24^{a}	11.7-14.2	17	20.31 ± 0.66^{a}	18.0-22.7
SurDH	–	–	–	9	12.72 ± 0.37^{a}	11.7-13.4	5	20.70 ± 2.59^{ac}	19.0-23.3
EtDH	–	–	–	5	15.36 ± 0.56^{b}	13.0-16.8	2	22.60 ± 0.93^{bc}	21.6-23.6
ParDH	3	9.30 ± 0.53^{a}	8.5-9.9	5	15.24 ± 0.44^{b}	13.4-16.3	2	23.60 ± 2.44^{bc}	20.7-26.5
NazDH	1	7.80 ± 0.30^{a}	7-8	6	15.65 ± 0.72^{b}	14.5-17.9	3	25.30 ± 0.48^{b}	24.9-26.1
Total	10	8.53 ± 0.56	7.7-9.9	61	13.46 ± 0.38	11.7-17.9	29	21.28 ± 0.87	18.0-26.5

Comments. 1 — confidence interval according to *t*-Student's distribution at 0.05 level of significance; 2 — values marked with the same letters (a, b, c) do not differ at $P \leq 0.05$ according to *t*-Student's test; 3 — seed progeny of initial donor plant used in isolated microspore culture. Dashes mean the data were not obtained.

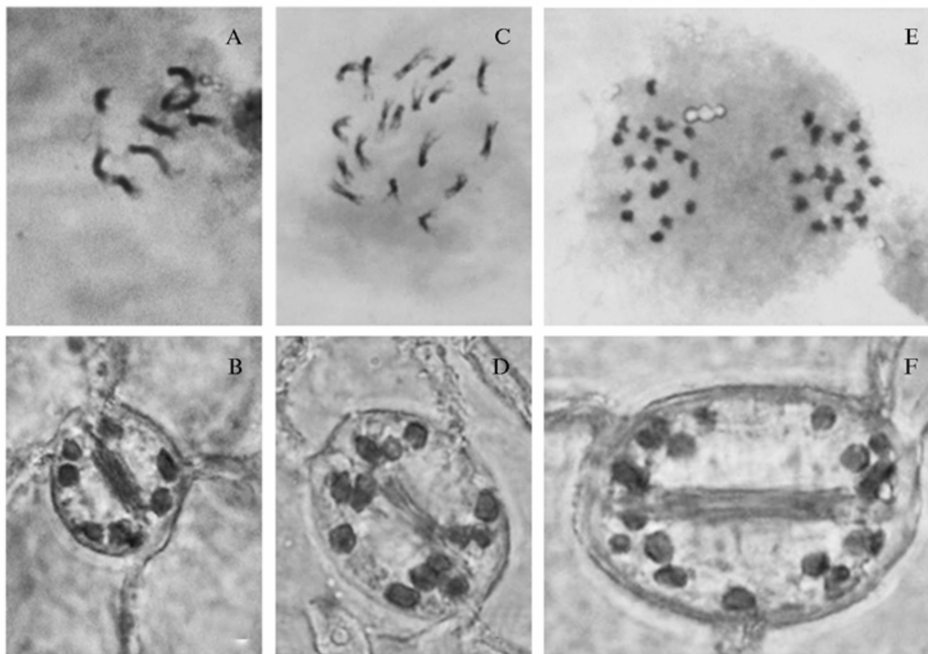

Fig. 2. Chromosomes in dividing pollen mother cells (metaphase I) (A-C) **and stomatal guard cells with chloroplasts** (D-F) **in white cabbage (*Brassica oleracea*) plants of different ploidy:** A, B — haploids (n = 9, the number of chloroplasts in a pair of stomatal guard cells/NCSGC = 8); C, D — diploids ($2n$ = 18, NCSGC = 13); E, F — tetraploids ($4n$ = 36, NCSGC = 20). Silver staining, magnification ×630.

In 22 inbred diploid lines of white cabbage with different maturing time the NCSGCs varied widely, from 11.1 to 19.2, depending on a genotype at the genotype-specific maximum to minimum average NCSGCs ratio of 1.73 (Fig. 3). Such difference is comparable to the average NCSGCs ratios in the diploid and haploid Chinese cabbage or rape plants. In early, middle and late maturing lines the NCSGCs were 15.7-19.2, 14.4-16.3 and 11.1-14.7, respectively.

Thus, we first noted a trend towards the formation of a larger number of stomatal guard cell chloroplasts in the white cabbage early maturing lines comparing to late maturing lines, probably due to biological peculiarities of their growth and development not taken into consideration by the other investigators (15) when dividing into groups with respect to ploidy.

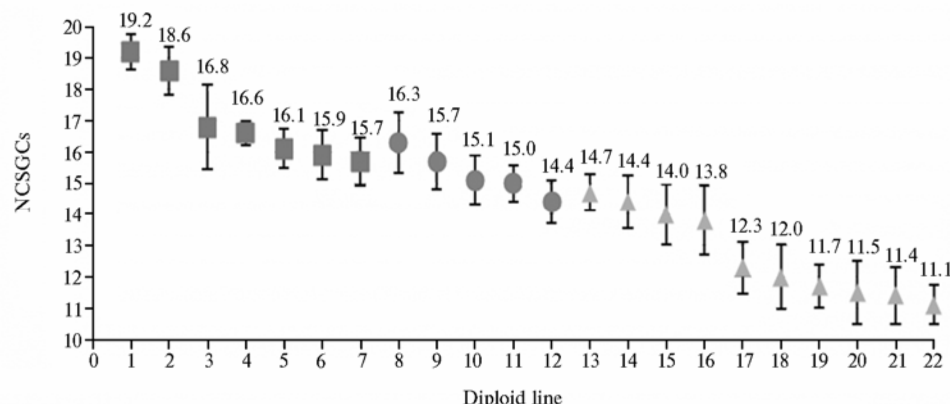

Fig. 3. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in diploid** ($2n$) **white cabbage (*Brssica oleracea*) lines with different maturing:** 1 — Pl, 2 — Sus1, 3 — Et1, 4 — Sf, 5 — Sh5a, 6 — Dpp2, 7 — Dt (■, early maturing, 8-100 days); 8 — Ak3, 9 — Meg1, 10 — B25, 11— Uf1, 12 — S110 (●, middle maturing, 110-140 days); 13 — Fp4, 14 — Pr3, 15 — Vb4, 16 — Xt5, 17 — Pm4, 18 — Fu44, 19 — Bu1, 20 — AM2, 21 — Sa1, 22 — Ges2 (▲, late maturing, 150-180 days). The confidence intervals are shown according to t-Student's test at P ≤ 0.05.

Because of NCSGCs dependence on maturing and the wide range of NCSGCs variation found in the regenerants with the same ploidy, there are some difficulties in setting precise variation limits that could be reliably used under estimation of white cabbage plant ploidy. Herewith, the essential differences in NCSGCs between haploid, diploid and tetraploid white cabbage (see Table 2) and the high correlation between NCSGCs and the level of ploidy ($r = 0.94 \pm 0.03$) indicate the possibility for accurate differentiation of the regenerants derived from microspore culture in genotypes not studied earlier, providing that diploid donor plant is used as a control.

Success in using NCSGC as an index depends on its stability during plant growth and development and at different conditions. We estimated the influence of temperature on NCSGC in diploid Chinese cabbage (*B. rapa*), white cabbage (*B. oleracea*) and amphidiploid rape (*B. napus*) (Table 5). The NCSGC variability in the plants of different age was also studied (Table 6).

5. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in dioloid and amphidiploid *Brassica* species depending on the growth temperature**

Population, variety	Species	Temperature, °C	
		6	22
F_1 Hidra	*B. rapa*	9.97[a]	10.30[a]
F_1 Nezhnost'	*B. rapa*	10.45[a]	11.00[a]
F_1 SB-3	*B. oleracea*	12.00[a]	12.23[a]
F_1 Valentina	*B. oleracea*	12.45[a]	12.20[a]
Severyanin	*B. napus*	17.50[a]	17.45[a]

C o m m e n t s. Values in the line marked with the same letters (a) do not differ at P ≤ 0.05 according to t-Student's test.

In all three studied *Brassica* species, the obtained data confirmed stability of NCSGCs slightly differing during ontogenesis at the phase of 4-5 true leaves and at the beginning of flowering. The growth temperature also did not affect significantly the NCSGCs in five genotypes of these *Brassica* species. It should be

noted that the obtained results were the same as in doubled haploid regenerants of these diploid species.

6. Average number of chloroplasts in a pair of stomatal guard cells (ANCSGC) **in diploid and amphidiploids *Brassica* species depending on the stage of plant development**

Population, variety	Species	Stage	
		4-5 true leaves	flowering
Xa642	*B. rapa*	10.75[a]	10.5[a]
Bu16	*B. oleracea*	11.70[a]	12.0[a]
Severyanin	*B. napus*	16.90[a]	17.0[a]

C o m m e n t s. Values in the line marked with the same letters (a) do not differ at P ≤ 0.05 according to *t*-Student's test.

Hence, the essential differences in NCSGCs between haploid and doubled haploid Chinese cabbage, white cabbage and rape allow accurately differentiate the plants according to their ploidy. Nevertheless, it is incorrect to state that the NCSGCs discovered in this investigation are absolute and unchangeable for these species. For instance, despite of almost complete conformity of an average NCSGC to that observed in *B. rapa* (5), there some inconsistencies with other data for *B. rapa* (13), *B. napus* (12) and *B. oleracea* (14), probably due to more diversity of genotypes we used in our investigation and because of some biological peculiarities of plants, such as the length of growing period, or some external factors.

So, an average NCSGCs (number of chloroplasts in stomatal guard cells) are 4.2-7.8 and 7.9-13.6 in haploid and diploid Chinese cabbage (*Brassica rapa*), respectively, 7.5-12.4 and 14.1-20.3 in amphihaploid and amphidiploid rape (*B. napus*), respectively, and 7.7-9.9, 11.7-17.9 and 18.0-26.5 in haploid, diploid and tetraploid white cabbage (*B. oleracea*), respectively. In Chinese cabbage and rape the NCSGC is not genotype specific, since in the plants with the same ploidy the same NCSGCs are observed independently of the crop variety. In white cabbage, the NCSGC depends on the length of growing period, and there is a trend towards NCSGC increase in the early-maturing lines and decrease in late-maturing lines. In diploid early-maturing inbred lines the NCSGC is 1.7 times higher than in late-maturing lines, that is comparable to the differences between haploid and diploid Chinese cabbage or rape plants. Temperature and plant development do not affect significantly the expression and stability of NCSGCs. According to our data, the chloroplast counting in stomatal guard cells is a reliable method to estimate the ploidy level in *B. rapa*, *B. napus*, and, with some assumption, in *B. oleracea*, thus being a routine procedure applicable in the analysis of regenerated plants derived through isolated microspore culture, or in other plant material.

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