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INFLUENCE OF A HEAVY-METAL-POLLUTED ENVIRONMENT ON *VIOLA TRICOLOR* GENOME SIZE AND CHROMOSOME NUMBER

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Intraspecific changes in genome size and chromosome number lead to divergence and species evolution. Heavy metals disturb the cell cycle and cause mutations. Areas contaminated by heavy metals (metalliferous sites) are places where microevolutionary processes accelerate; very often only a few generations are enough for a new genotype to arise. This study, which continues our long-term research on *Viola tricolor* (Violaceae), a species occurring on both metalliferous (Zn, Pb, Cd, Cu) and non-metalliferous soils in Western and Central Europe, is aimed at determining the influence of environments polluted with heavy metals on genome size and karyological variability. The genome size of *V. tricolor* ranged from 3.801 to 4.203 pg, but the differences between metalliferous and non-metalliferous populations were not statistically significant. Altered chromosome numbers were significantly more frequent in material from the polluted sites than from the non-polluted sites (43% versus 28%). Besides the standard chromosome number ($2n = 26$), aneuploid cells with lower ($2n = 18–25$) or higher ($2n = 27, 28$) chromosome numbers were found in plants from both types of site, but polyploid ($2n = 42$) cells were observed only in plants from the metalliferous locality. The lack of correlation between chromosome variability in root meristematic cells and genome size estimated from peduncle cells can be attributed to elimination of somatic mutations in generative meristem, producing chromosome-stable non-meristematic tissues in the peduncle.

Key words: *Viola tricolor*, pseudometallophyte, C-DNA value, chromosome number, aneuploidy, polyploidy.

INTRODUCTION

Genome size and chromosome number have long been regarded as species-specific and constant features, but many studies have reported intraspecific and intrapopulation variability of genome size and karyology (Greilhuber, 1998, 2005, 2008; Ohri, 1998; Małuszyńska and Siwińska, 2004; Doležel et al., 2007). Differences in genome size within a class of organisms, called the C-value paradox, are almost entirely due to differences in the amount of non-protein-coding DNA. In many eukaryotes these non-coding DNA sequences are the necessary outcome of novel cell structures imposing selective forces, meaning that genomes and cell structure co-evolve (Cavalier-Smith, 2005). Due to pronounced intraspecific variation in nuclear DNA content, plant genomes have been referred to as 'fluid', 'dynamic',

and 'in constant flux' (Gregory, 2005). Whether an observed intraspecific variation is real or artifactual can only be clarified using larger sample sizes and/or a superior measurement technique. Real intraspecific variation could result from the differential presence of supernumerary B chromosomes, from polymorphisms in A chromosomes, or genome duplication via autopolyploidy (for examples and references see Gregory, 2005). There are numerous parallels in genome size evolution between plants and animals, including the patterns of DNA content variation among taxa, the cytological, morphological, physiological and evolutionary impacts of genome size, and the mechanisms by which genomes change in size (Beaulieu et al., 2010).

Owing to improved flow cytometry technique, especially nucleus staining procedure, data verification and artifact elimination (Greilhuber, 1998;

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Gregory, 2002, 2005; Murray, 2005), we now have reliable evidence that intraspecific genome size variability can be correlated with altitude, latitude, soil type, rainfall, and mean temperature during flowering (Ohri, 1998; Turpeinen et al., 1999; Šmarda and Bureš 2010). Examples of plant species with flexible genomes (11–28% intraspecific variation) were given by Ohri (1998).

Intraspecific changes in genome size, an object of natural selection, lead to divergence and species evolution. Sites contaminated by heavy metals (metalliferous sites), which are particularly detrimental to plant growth, are places where microevolutionary processes accelerate. Very often only a few generations are enough for a new genotype to arise (Bone and Farres, 2001; Hendry and Kinnison, 2001; Stockwell et al., 2003; Carroll et al., 2007; Medina et al., 2007). Plant species colonizing metalliferous and thus unstable and unpredictable sites have evolved an r-life strategy with the crucial ability to reproduce quickly, owing to fast flowering, seed ripening, and much greater flower and seed yields (Wierzbicka and Rostański, 2002; Grześ, 2007). Such a strategy is correlated with a small genome, as confirmed recently in studies of seventy herbaceous dicot perennial species. Those with large genomes were indeed at a selective disadvantage in extreme environmental conditions (Vidic et al., 2009). If small genome size has adaptive value, metallicolous populations should have smaller genomes than non-metallicolous ones. This subject was recently raised again in the form of the 'large genome constraint hypothesis' put forward by Knight and coauthors (2005), stating that species with too-large genomes are more likely to become extinct in stressful environments because large genomes are inflated with unnecessary junk DNA whose replication burdens the organism. Investigating sixty species along a gradient of heavy metal pollution, Temsch and coauthors (2010) supported that hypothesis, arguing that large nuclei, especially those that are mitotically or meiotically active, receive more hits than small ones and are more prone to heavy DNA damage and elimination.

As heavy metals disturb the cell cycle and cause mutations (Coulaud et al., 1999; Nkongolo et al., 2001; Rayburn and Wetzel, 2002; Sedel'nikova and Pimenov, 2007), they may effect changes in (1) chromosome number (aneuploidy, polyploidy), (2) chromosome structure and (3) ontogeny (endomitosis, endoreduplication) (Jones, 1978; Bayliss, 1980; D'Amato, 1991; Stace, 1991). Those are usually easy to catch with cytological analyses (e.g., Sedel'nikova and Pimenov, 2007) and DNA content measurements (e.g., Rayburn and Wetzel, 2002). Points particularly vulnerable to chromosome breakage are the centromere, secondary constriction and sites with weak chromatin condensation (Jones, 1978;

Lee and Philips, 1988; Coulaud et al., 1999). Inter- and intrapopulation (inter- and intraindividual) variability in chromosome number is a crucial step toward speciation (Raskina et al., 2008).

In some cases, structural chromosome mutations are subtle and are identified by special chromosome staining techniques (e.g., C-banding, FISH). Small chromosome fragments may exhibit different localization even within a single species (Garrido et al., 1994). This is clearly visible with rDNA loci; their rearrangement is a very rapid process driven by unequal recombination and transposon rearrangement (Raskina et al., 2004, 2008).

This study continues our long-term work on the pseudometallophyte pansy *Viola tricolor* L. (Violaceae, sect. *Melanium*; $2n = 26$), a species occurring both on metalliferous (Zn, Pb, Cd, Cu) and on non-metalliferous soils in Western and Central Europe (Dobrzańska, 1955; Ernst et al., 2004; Banášová et al., 2006; Hildebrandt et al., 2006, 2007). Our previous research addressed the influence of soils polluted with heavy metals on the plant antioxidative system (Słomka et al., 2008), plant morphology, reproduction and pollen viability (Słomka et al., 2010c), mycorrhiza colonization (Słomka et al., 2011a) and genetic variation (Słomka et al., 2011b). We found that metallicolous populations exhibit qualitative differences from non-metallicolous ones at the molecular level. Metal-tolerant populations group together in respect of ISSR markers (Słomka et al., 2011b), a pattern which does not correlate with the great morphological variability of this species as measured by quantitative and qualitative characters (Słomka et al., 2011c). Its relatively high genetic polymorphism and gene diversity as compared with other metal-tolerant plant species, especially high in metallicolous populations, give it an advantage in the adverse environments it occupies in dense patches in southern Poland, and are leading to the formation of a new ecotype (Słomka et al., 2011b).

Here we examine the influence of environments polluted with heavy metals on genome size and karyological variability in *Viola tricolor*.

MATERIALS AND METHODS

PLANT MATERIAL

The plants for C-DNA content analysis originated from five sites (two non-metallicolous, three metallicolous). Seeds for chromosome counting originated from three sources (one metallicolous, two non-metallicolous). The material was collected at the same sites as in our previous studies on the influence of soils polluted with heavy metals on *Viola tricolor* (Słomka et al., 2008, 2010, 2011a,b,c). The

metallicolous populations (BH – Bukowno heap, SH – Saturn heap, WH – Warpie heap) are located in the Zn/Pb mining area in Olkusz, Poland. Soils at these sites contain Zn at Pb at levels far exceeding safety norms (Zn 50 ppm, Pb 1 ppm, Cd 0.05 ppm; Greger, 2004). The site most contaminated with Zn is BH (mean 6,725 ppm), and the most Pb-contaminated site is SH (mean 5,004 ppm). These soils have similar pH (~7.0). The non-metallicolous populations (ZM – Zakopane meadow, ZP – Zakopane park) are located in the vicinity of Zakopane on soils with trace amounts of Zn and Pb, except for one in Germany (BGH – Botanical Garden in Hohenheim) from which seeds were received for chromosome analysis. Site ZM has higher loads of the metals (Zn 793 ppm, Pb 468 ppm) than ZP (Zn 83 ppm, Pb 30 ppm), but since the amounts are low as compared with the metallicolous sites, its material is also taken as representing a non-metallicolous (control) population.

C-DNA VALUE

Detailed C-DNA value analyses were preceded by organ selection, due to the presence of mucilaginous substances (Ajalin et al., 2002) in *V. tricolor* leaves which would interfere with measurements. Among several organs tested (leaves, petioles, stems, roots, petals, peduncles, embryos), the most suitable were peduncles. Peduncle fragments 2–3 cm long together with a small piece of *Fagopyrum sagittatum* cv. Kora leaf (internal standard) were chopped with a razor blade in nuclei extraction buffer (Doležel and Gohde, 1995), filtered through 30 µm nylon mesh and stained with propidium iodide using a Partec high-resolution DNA kit according to the manufacturer's instructions. The internal standard, *Fagopyrum sagittatum* cv. Kora (2C DNA = 2.87 pg), was calibrated based on *Lycopersicon esculentum* cv. Stupicke (2C DNA = 1.96 pg) (Doležel et al., 1992). The DNA content of the isolated nuclei in the samples was analyzed with a DAKO Galaxy flow cytometer. Altogether 50 plants (5 populations x 10 plants) were measured. Nuclei isolation, staining and measurements were repeated for 3–5 randomly taken plants/population.

PLANT MATERIAL FOR CHROMOSOME COUNTING

Viola tricolor seeds were germinated on filter paper soaked with distilled water under constant light at room temperature. We applied a treatment to improve their very poor germination frequency. After 8 weeks of cooling at 4°C the seeds were sterilized in commercial bleach diluted with sterilized water (1:3 v/v) for 10 min and rinsed several times in sterilized water, then sown on 1% agar and on moist filter paper and kept in an experimental

chamber under a 16 h photoperiod at 24°C/18°C. This treatment gave up to 80% germination.

SAMPLE PREPARATION

Six-day-old seedlings were incubated in a saturated solution of α -bromonaphthalene for 24 h at 4°C (for orcein staining) or in 0.02 M water solution of 8-hydroxyquinoline for 4 h at room temperature (for DAPI staining). Afterwards they were rinsed three or four times in distilled water. Then the material was fixed in a mixture of glacial acetic acid and 96% ethanol (3:1 v/v) for 24 h. For chromosome counting the material was stained in 2% acetic orcein for 2–3 days or in DAPI. For mitotic stage analysis, root tips were stained with Schiff's reagent without α -bromonaphthalene or 8-hydroxyquinoline pretreatment.

Acetic orcein staining

Fixed seedlings were rinsed in 45% acetic acid several times, then heated to boiling over a flame three times. For slide preparation, dissected root tips with clearly stained ends were squashed in a drop of 45% acetic acid with a cover slip which was then removed with dry ice. The slides were rinsed for 2 s in 96% ethanol, air-dried, mounted in Canada balsam and observed with a light microscope.

DAPI staining

Fixed seedlings were rinsed in 10 mM citric acid – sodium citrate buffer (pH 4.8) and enzymatically digested [20% v/v pectinase (Sigma), 1% (w/v) Calbiochem cellulase, 1% w/v Onozuka (Serva) cellulase] for 2.5 h at 37°C and finally rinsed in citrate buffer for 15 min. Preparations were made from root tips, which were squashed in a drop of 45% acetic acid, dry-iced and air-dried, and stained for 15 min with 1 µg DAPI diluted in 1 ml McIlvaine buffer (pH 7.0). After rinsing in McIlvaine buffer the preparations were mounted in Entellan and observed and photographed with a fluorescence microscope at 360–370 nm.

Feulgen staining

Seedlings fixed and then washed in distilled water were hydrolyzed in 18% HCl for 0.5 h at room temperature, rinsed several times in distilled water and kept in darkness ~1 h in Schiff's reagent (based on pararosaniline) until purple. After triple rinsing in sulphur water (5 ml 10% Na₂S₂O₅, 5 ml 1 M HCl, 90 ml distilled water) the root meristems were squashed in a drop of 45% acetic acid, dry-iced, air-dried, mounted in Entellan, and observed and photographed with a light microscope.

TABLE 1. DNA 2C-value (pg) of individuals from metallicolous (BH, SH, WH) and non-metallicolous (ZM, ZP) populations of *V. tricolor*

	Soil not polluted with heavy metals		Soil polluted with heavy metals			
	Zakopane Park (ZP)	Zakopane meadow (ZM)	Bukowno heap (BH)	Saturn heap (SH)	Warpie heap (WH)	
Mean	4.016 ^a	4.048 ^a	4.026 ^a	4.081 ^a	4.004 ^a	4.035
S.D.	0.108	0.103	0.106	0.082	0.102	0.100
Minimum	3.845	3.870	3.866	3.912	3.801	
Maximum	4.203	4.193	4.179	4.197	4.152	

Means followed by the same letters do not differ significantly at $p > 0.05$ by ANOVA

TABLE 2. Frequency of standard $2n = 26$ and non-standard (aneuploid, polyploid) chromosome numbers in root meristem cells of *V. tricolor* from non-metallicolous (ZM, BGH) and metallicolous (BH) populations; percent in brackets

Site	No. of cells with standard chromosome number $2n = 26$	No. of cells with chromosome numbers other than $2n = 26^*$
Soil not polluted with heavy metals		
Zakopane meadow (ZM)	23	33
Botanical Garden in Hohenheim (BGH)	62	0
Total	85[72]	33[28]
Soil polluted with heavy metals		
Bukowno (BH)	77[57] ^a	58[43] ^b

*aneuploid (lower or higher than standard $2n = 26$) or polyploid chromosome numbers

Means followed by different letters differ significantly at $0.01 < p < 0.02$ by chi-square test

STATISTICS

The two statistical tests used, ANOVA for inter- and intrapopulation variation in genome size and the chi-square test for comparison of chromosome number frequencies between populations, employed STATISTICA ver. 7.0.

RESULTS

GENOME SIZE

Using peduncles for genome size analyses, we obtained good histograms with acceptable CVs (3.5%–5.5%), lower than from the other tested organs. DNA 2C-value in the metallicolous and non-metallicolous *V. tricolor* populations ranged from 3.801 to 4.203 pg. Intra- and interpopulation variation of genome size was not pronounced in either type of population, and the ranges of intrapopulation variation were similar: 3.845–4.203 pg (ZP) and 3.870–4.193 pg (ZM) in the two populations from non-metalliferous sites, and 3.866–4.179 (BH), 3.912–4.197 pg (SH) and 3.801–4.152 pg (WH) in the three from metalliferous sites. The investigated

populations did not differ in genome size. To our knowledge the mean genome size of 4.035 ± 0.100 pg for *V. tricolor* established in this study is the first such statistic for this species (all data Tab. 1).

CHROMOSOME NUMBERS

Chromosome numbers were determined from 253 root meristem cells of 25 seedlings grown from seeds collected from plants of non-metallicolous populations (118 cells; ZM, BGH) and 27 seedlings germinated from seeds harvested from a metallicolous population (135 cells; BH) (Tab. 2). Specimens from both sites exhibited intra- and interindividual variability of chromosome number. In root meristematic cells of plants from non-metalliferous sites (ZM, BGH), 72% had chromosome number $2n = 26$ (Tab. 2, Fig. 1a), representing the standard chromosome number established for *V. tricolor* from other parts of its distribution area (see, e.g., Bolkovskikh et al., 1969), whereas in individuals from the metallicolous population (BH) the frequency of cells with the standard chromosome number was clearly lower at 57%. Altered chromosome numbers were significantly more frequent in

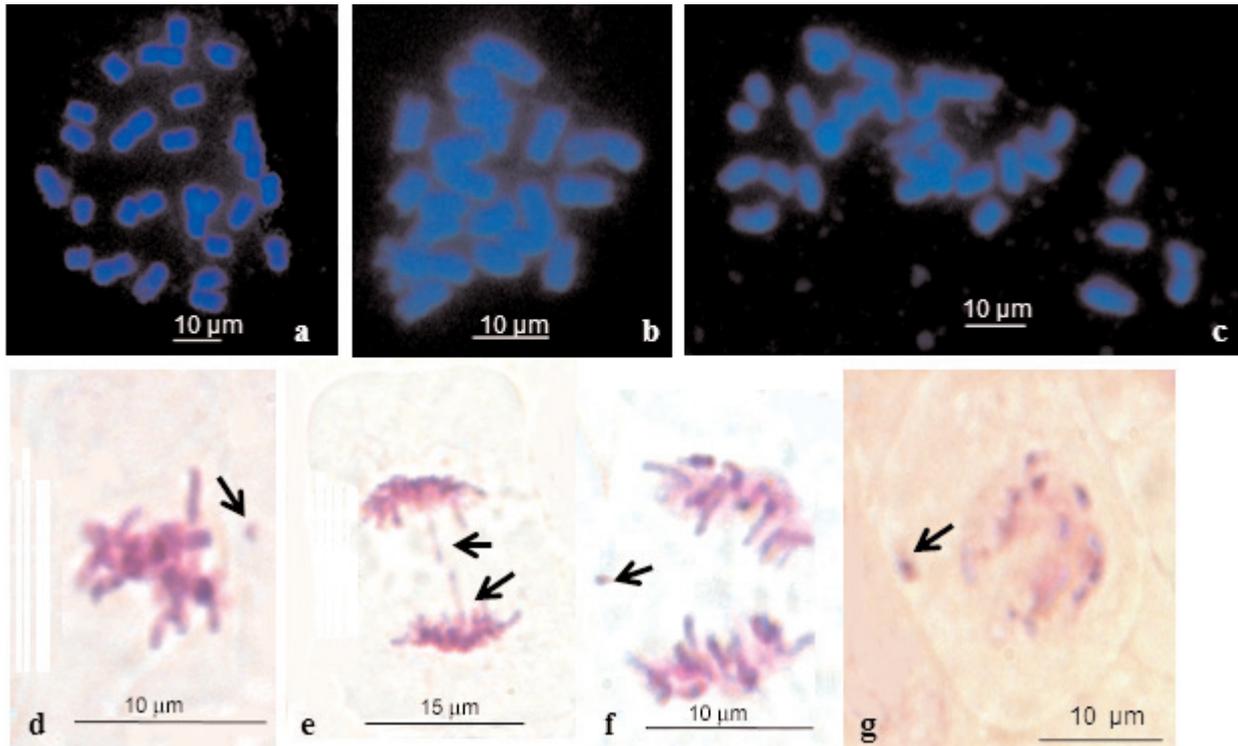


Fig. 1. Chromosome numbers and disturbed mitotic divisions in root meristems of *V. tricolor* from non-metallicolous (ZP; a–e) and metallicolous (f, g) populations. DAPI staining (a–c); Feulgen staining (d–g). (a) Metaphase plate, $2n = 26$, (b, c) Aneuploid metaphase plates with lower ($2n = 18$, b) and higher ($2n = 28$, c) than standard ($2n = 26$) chromosome number, (d) Metaphase with acentric chromosome fragment (arrow) located outside of metaphase plate, (e) Telophase with chromosome bridge (arrows), (f) Early telophase with acentric chromosome fragment (arrow) left between two chromatid groups separated at poles, (g) Cell with chromosome fragment (arrow) located in cytoplasm outside of interphase nucleus.

the population from the metalliferous site (BH, 43%) than in the non-metallicolous populations (ZM and BGH, 28%; $\chi^2_{0.05;1} = 6.2$; $0.01 < P < 0.02$). Due to difficulties in obtaining well-spread metaphase plates, not all analyzed metaphases were good enough to count the chromosomes precisely; we could estimate only whether the chromosome number was higher or lower than $2n = 26$ in a particular cell, without giving the exact count (Tab. 2). Detailed counts were based on 123 selected metaphase plates (56 from ZM and 67 from BH); besides standard chromosome numbers ($2n = 26$), we found aneuploid cells with lower ($2n = 18$ –25) or higher ($2n = 27$, 28) chromosome numbers in material from both sites (Tab. 3, Fig. 1b, c), whereas polyploid ($2n = 42$) cells were found only in material from the polluted locality (Tab. 3).

Disturbances in mitotic division, observed in material not treated with chemicals destroying the spindle, explained the occurrence of altered chromosome numbers. In metaphases (Fig. 1d) and in telophases (Fig. 1f) we observed single vagrant chromosome fragments. Additionally, ana-telophase

bridges were formed by dicentric chromosomes (Fig. 1e). In interphases these chromosome fragments formed micronuclei (Fig. 1g).

DISCUSSION

Heavy metal genotoxicity and heavy metal effects on cell cycle duration have been investigated frequently in plants, using pot and in vitro experiments. Via generation of reactive oxygen species in plant cells, heavy metals disturb mitosis, leading to the occurrence of anaphase bridges, chromosome stickiness, micronuclei formation and other aberrations (Steinkellner et al., 1998).

There are no genome size data for members of the Violaceae except for *Viola riviniana* and *V. anagae* in the famous C-DNA database of the Royal Botanic Gardens at Kew (<http://data.kew.org/cvalues/>). Recently it was established for the third species, *Viola hirta* (Temsch et al. 2010).

In this study we measured genome size in *Viola tricolor* in order to test the hypothesis (Price et al.,

TABLE 3. Chromosome numbers in root meristem cells of *V. tricolor* from non-metallicolous (ZM) and metallicolous (BH) populations; percent in brackets

Chromosome numbers	No. of cells	
	Zakopane meadow (ZM)	Bukowno heap (BH)
<hr/>		
$2n < 26$		
18	3	2
19	1	0
20	0	4
22	3	3
23	2	0
24	12	11
25	4	12
	25[45]	32[48]
<hr/>		
$2n = 26$	23[41]	22[33]
<hr/>		
$2n > 26$		
27	3	7
28	5	3
42	0	3
	8[14]	13[19]
<hr/>		
Total	56	67
<hr/>		

1981; Price, 1988) that individuals with smaller genomes should occur in habitats with adverse environmental conditions. We expected to find differences in genome size between plants from non-metallicolous and metallicolous populations, but in fact there were no statistically significant differences between these two population types. Individuals from all five investigated populations (two non-metallicolous and three metallicolous) had genomes similar in size, corresponding to results on *Lotus peregrinus* (Pavliček et al., 2008) and *Cyclamen persicum* (Gasmanová et al., 2007) but contrary to results on *Hordeum spontaneum* (Kalendar et al., 2000), all of which were growing under extremely differing environmental conditions in Evolution Canyon (Israel).

Chromosome number databases (see Bolkhovskikh et al., 1969; Verlaque and Espeut, 2007; Góralski et al., 2009) indicate that *Viola tricolor* is karyologically uniform, with somatic chromosome number $2n = 26$. Data from Krahulcová and coauthors (1996) and Lausi and Cusma Velari (1986) give sparse information about intraspecific karyological variability in *V. tricolor*: the former occasionally observed aneuploid cytotypes $2n = 25$ and $2n = 27$ in the Czech Republic; the latter found changes in absolute chromosome size, centromeric symmetry and relative size symmetry, but not in

chromosome number. In our study we found a wide range of chromosome numbers ($2n = 18-42$) in root meristematic cells of plants from metallicolous and from non-metallicolous populations. Aneuploid cells with lower or higher chromosome numbers than the standard $2n = 26$ were the result of disturbed mitosis (confirmed by observations of mitotic division disturbances) and probably also structural chromosome mutations. The contribution of B chromosomes cannot be excluded, as they are difficult to identify in the analyzed plates, because five species in the genus *Viola* have 2–10 supernumerary chromosomes which could give additional chromosomes in the karyotype (Jones and Rees, 1982). The altered chromosome numbers we counted, including mixoploidy but also showing intra- and inter-population karyological variability, suggest that this species is karyologically unstable. This may be explained by the relatively young age of sect. *Melanium* (pansies) (Erben, 1996; Yockteng et al., 2003 and lit. cited therein). Assuming the basic chromosome number in sect. *Melanium* to be $x = 11$ (Erben, 1996), $2n = 26$ in *V. tricolor* would be the result of increasing dysploidy leading to an increase from 22 to 26 chromosomes. The polyploid origin of *V. tricolor*, combined with hybridization, has also been proposed based on a lower basic chromosome number ($x = 5$ or $x = 7$) in *Viola* (Yockteng et al., 2003). Regardless of the two evolutionary pathways, the disturbed male and female meiosis, some abnormalities in female and male gametophyte development (Słomka et al., 2010, 2011c), the variability of chromosome numbers and disturbances in mitosis (this paper) in plants from non-metallicolous sites suggest that this species is still in the process of stabilization. In adverse environmental conditions, plant species with a relatively unstable karyotype may be favored by their ability to change rapidly (Coulaud et al., 1999; Nkongolo et al., 2001; Sedel'nikova and Pimenov, 2007). Many data suggest that such unstable species are at an advantage in adverse heterogeneous environmental conditions (Degenhardt et al., 2005; Seehausen et al., 2008). In several plant species with flexible genomes growing in the presence of heavy metals (e.g., *Larix sibirica*, *Armeria maritima*, *Deschampsia cespitosa*) the frequency of different aberrations (e.g., links between nonhomologous chromosomes, vagrant chromosomes, extra constrictions, fragile chromosomes) was markedly higher than in plants from non-contaminated sites (Coulaud et al., 1999; Nkongolo et al., 2001; Sedel'nikova and Pimenov, 2007).

The big question raised by our results is why the great cytological variability we found in root meristematic cells is not reflected in genome size variability as measured in peduncle cells. There are three possible explanations: (1) the quantitative

changes in DNA content are relatively small, below the detection limit of flow cytometry equipment, or the changes are only qualitative; (2) generative meristem cells (generative shoots), giving rise to the flower including the peduncle, are more stable than root meristem cells (vegetative shoots); or (3) somatic mutations in generative meristematic cells are eliminated. As for these possibilities, it is well known that plants have the potential for somatic mutations to accumulate during ontogenesis, particularly plants with longer generation times, because they do not sequester a germ line early in development as animals do (Klekowski et al., 1985; Klekowski and Godfrey, 1989; Klekowski, 1998); an increased number of somatic mutations could influence genome size, resulting in intraindividual and intraspecific genome size variation. However, such genome size variation is not commonly observed in plants (Doležel and Bartoš, 2005; Greilhuber, 2005, 2008), suggesting that although plants have the potential to accumulate somatic mutations in different tissues it may not be a significant factor contributing to genome size differences. Even if mutations in somatic tissues occur, there are known mechanisms for removing them from meristematic cells, as described for woody angiosperms (e.g., Mellerowicz et al., 2001; Petit and Hampe, 2006).

In this work, an environment polluted with heavy metals did not influence genome size in *V. tricolor*. The genome downsizing we expected to see in plants colonizing waste heaps was not observed. Variability of chromosome numbers occurred with higher frequency in root meristematic cells of specimens from polluted sites. The lack of correlation between chromosome variability in root meristematic cells and genome size estimated in peduncle cells probably was due to elimination of somatic mutations in generative meristem, leading to chromosome-stable non-meristematic tissues in the peduncle, or else it can be explained by greater cytologically stability in generative than in vegetative meristem.

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