Theor Appl Genet (2009) 118:1107–1119
DOI 10.1007/s00122-009-0966-9

ORIGINAL PAPER

A large number of tetraploid *Arabidopsis thaliana* lines, generated by a rapid strategy, reveal high stability of neo-tetraploids during consecutive generations

Zheng Yu · Kristina Haage · Verena E. Streit · Alfons Gierl · Ramón A. Torres Ruiz

Received: 26 February 2008 / Accepted: 6 January 2009 / Published online: 11 February 2009

Abstract *Arabidopsis thaliana* has, in conjunction with *A. arenosa*, developed into a system for the molecular analysis of alloplolyploidy. However, there are very few *Arabidopsis* lines available to study autopolyploidy. In order to investigate polyploidy on a reliable basis, we have optimised conventional methodologies and developed a novel strategy for the rapid generation and identification of polyploids based on trichome branching patterns. The analysis of more than two dozen independently induced *Arabidopsis* lines has led to interesting observations concerning the relationship between cell size and ploidy levels and on the relative stability of tetraploidy in *Arabidopsis* over at least three consecutive generations. The most important finding of this work is that neo-tetraploid lines exhibit considerable stability through all the generations tested. The systematic generation of tetraploid collections through this strategy as well as the lines generated in this work will help to unravel the consequences of polyploidy, particularly tetraploidy, on the genome, on gene expression and on natural diversity in *Arabidopsis*.

Communicated by M. Kearsey.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-0966-9) contains supplementary material, which is available to authorized users.

Z. Yu · K. Haage · V. E. Streit · A. Gierl (✉) ·
R. A. Torres Ruiz (✉)
Lehrstuhl für Genetik, Wissenschaftszentrum Weihenstephan,
Technische Universität München, Am Hochanger 8,
85350 Freising, Germany
e-mail: Ramon.Torres@wzw.tum.de

Present Address:
K. Haage
Lehrstuhl für Genetik, Department Biologie I,
Ludwig-Maximilians-Universität München,
Großhadener Str. 4, 82152 Planegg-Martinsried, Germany

Introduction

Polyploidy is a widespread phenomenon in animals and plants (Comai 2005). Estimates of the polyploid fraction amongst angiosperm species range from 30 to 80%. In fact, possibly all angiosperm species might be so called palaeopolyploids (Otto and Whitton 2000; Bennett 2004 and references therein). Polyploidy, particularly tetraploidy, has several potential advantages because the organisms can resort to a higher number of genes and higher maximum number of allelic variants. This is believed to be advantageous for plant metabolism in terms of elevated rates of synthesis or a higher variability of metabolically relevant compounds (Wolters and Visser 2000; Osborn et al. 2003), which may be one reason why certain tissues of diploid plants increase their genome content endogeneously by endopolyploidisation (Larkins et al. 2001). A further advantage lies in the subfunctionalization of gene copies that take over the task in different tissues (Adams and Wendel 2005a). Plant breeders have taken advantage of polyploidy in order to improve agronomic traits of economically important plants, some of which have been generated by allopolyploidisation of interspecies hybrids or by autopolyploidisation within species (Otto and Whitton 2000; Bennett 2004). Polyploid plants are generally known to exhibit a decrease in fertility and viability due to perturbations in meiotic segregation, mitotic division and/or perturbations in gene expression caused by altered gene dosage and gene

silencing (Comai 2005; Mittelsten-Scheid et al. 2003). It is therefore not clear whether a newly arisen polyploid plant will be evolutionarily successful or not.

An advanced molecular understanding of plant polyploidy will help us to ameliorate or circumvent detrimental effects and contribute to the improvement of plant breeding and agriculture. To this effect, *Arabidopsis thaliana* is increasingly becoming a model system for the molecular analysis of allopolyploidy (Madlung et al. 2002; Wang et al. 2004; Wang et al. 2006 and references therein). Although allopolyploidy is considered much more prevalent in nature, autopolyploids might be much more common than assumed (Soltis and Soltis 2000). Several studies using tetraploid lines obtained with diverse methods have been reported over the years (Redei 1964; Bouharmont 1965; Melaragno et al. 1993; Heslop-Harrison and Maluszynska 1994; Koornneef 1994; Altmann et al. 1994; Weiss and Maluszynska 2000; Mittelsten-Scheid et al. 2003; Santos et al. 2003). However, some of these lines no longer exist, some are unstable and others have undesirable traits due to EMS and X-ray treatment. At present, the stock centers provide six tetraploid, seven (telo)trisomic lines, one aneuploid and two natural tetraploid ecotypes (NASC catalogue http://arabidopsis.info/). Taken together, there are only a few reliable lines available for analysis. Consequently this limits the generalisation of observations when studying autopolyploidy. The identification of the molecular principles of tetraploidy will thus require the generation and analysis of several independent lines (Comai 2005). These will provide a reliable scientific basis when analysing epigenetic changes or exploring the potential of polyploidy in the context of natural diversity. Investigators of allopolyploidy in Brassicas have recognised this point and produced multiple colchicine-induced lines for analysis (Albertin et al. 2006; Lukens et al. 2006; Gaeta et al. 2007).

Although methods for the induction of tetraploids in *Arabidopsis* and other plants are known, the scarcity of available lines highlights the tedious nature of generating polyploids. The bottleneck lies not so much in the induction of polyploidy, as in the rapid identification and assessment of new lines. We have scanned a number of cellular traits that can act as markers for ploidy levels. This study presents a rapid and reliable procedure, based on the evaluation of trichome morphology, for the systematic establishment of collections of polyploid *Arabidopsis* lines. Our analysis is limited to the assessment of correct total chromosome numbers. However, in this respect it demonstrates a surprisingly high stability of tetraploidy in the lines generated and provides new data on the relationship between ploidy and cell size.

Materials and methods

Plant material and growth conditions

We obtained the following *Arabidopsis* ecotypes from the *Arabidopsis* stock centers *Arabidopsis* Biological Resource Center (ABRC) and Nottingham *Arabidopsis* Stock Center (NASC): Bor-1 (CS22590), Br-0 (CS22628), Bur-0 (CS22656), C24 (CS22620), CIBC-5 (CS22602), Cvi-0 (CS22614), Col-0 (N1092), Ct-1 (CS22639), Eden-1(CS22572), Kas-2 (CS22638), Kas-2 (N1264), L*er*-0 (NW20), L*er*-1 (CS22618), Mt-0 (CS22642), Nd-0 (N1390), Nd-1 (CS22619), Oy-0 (CS22658), Ra-0 (CS22632), Tamm2 (CS22604), Wa-1 (N1587, a natural tetraploid), Ws-2 (CS22659), Yo-0 (CS22624), selected RI-lines (see Table 1 and Supplementary Table 2) from the Col-0xL*er*-0 Recombinant Inbred Population set (N1899, Lister and Dean 1993) and the tetraploid lines N141, N3900, N3247, N3151, N3238, N3432.

Further lines were kindly provided by the following colleagues: ecotypes Mt-0 and Oy-0 by Thomas Debener (University of Hannover; these lines are distinguished from the same ecotypes from NASC/ABRC by the suffix TD and were used in our initial work), the diploid and tetraploid Zürich lines (Zü, Zü4x) and the repeatedly assessed tetraploid Col-line (N3432) by Ortrun Mittelsten-Scheid (GMI Wien), the tetraploid Wilna ecotype by Jolanta Maluszynska (University Silesia, Poland), the transgenic lines *CYCAt1*:CDB:GUS by D. Celenza (via M.-T. Hauser, University BOKU Wien) and $DR5_{rev}$::GFP by J. Friml (University Ghent).

Seeds were sown on soil (mixture of one third quartz sand and two thirds peaty mould) and grown under constant light (80–100 µmol photons/m^2 s), 40% relative humidity and 18°C in a Heareus (HEMZ 20/240/S) walk-in growth chamber.

Conversion of diploid into tetraploid plants

The first step in the generation and identification of polyploid plants is induction with colchicine. Colchicine concentrations are critical and had to be tested separately for every ecotype (Fig. 1). We also tested several procedures. A number of polyploid plants were generated by sterile submersion for 3–4 h in 0.5% colchicine solution, followed by an interim sterile culture on ½ Murashige-Skoog medium (ca. 1 week) and subsequently planted on soil. In a second treatment (modified after Santos et al. 2003), a drop of colchicine solution (~15 µl) was placed on the apex of young seedlings with less than five primary leaves (one-drop method). We tested several concentrations in combination with different numbers of treatments and found that a single treatment was sufficient for the generation

Table 1 Efficiency of colchicine treatments in L*er*-0 (NW20) and Col-0 (N1092) as estimated from "polyploid" sectors

Ecotype/stock-no., line designation	Colchicine conc. (%)/ no. of treatments	No. of seedlings	Survived seedlings (% of treated seedlings)	Seedlings with polyploid sectors (% of survived seedlings)
L*er*-0/NW20	0.5/4 h submerged	142	1 (0.7)	0 (0)
Col-0/N1092	0.5/4 h submerged	140	17 (12)	7 (41)
L*er*-0/NW20	0.5/9[a]	18	1 (5.5)	0 (0)
Col-0/DR5[c]	0.5/3[b]	11	9 (82)	6 (67)
Col/CYCAT1[d]	0.5/3[b]	11	7 (64)	5 (71)
Col-0/DR5[c]	0.1/3[b]	10	9 (90)	5 (56)
Col/CYCAT1[d]	0.1/3[b]	11	11 (100)	4 (36)
RI-lines group I[e]	0.5/3[b]	128	29 (22.6)	21 (72)
RI-lines group II[f]	0.1/1	242	151 (62.4)	128 (84.8)
Col-0/N1092	0.5/1	30	25 (83.3)	10 (40)
L*er*-0/NW20	0.5/1	150	36 (24)	7 (19.4)
L*er*-0/NW20	0.1/1	200	53 (26.5)	11 (20.8)
L*er*-0/NW20	0.05/1	40	12 (30)	3 (25)

[a] Application during 2 weeks on 9 days Tuesday–Friday and Monday–Friday

[b] Application during 1 week on 3 days Monday, Wednesday and Friday

[c] Full designation of the transgene: $DR5_{rev}$::GFP (Friml et al. 2003)

[d] Full designation of the transgene CYCAT1:CDB:GUS (Hauser and Bauer 2000)

[e] RI-lines from the Lister and Dean ColxL*er*-RI-population (NASC, Lister and Dean 1993): RI-13, -35, -191, -238, -263, -295, -302, -303, -332, -358, -367, -370 and -377 (see also supplementary Table 2)

[f] RI-lines from the Lister and Dean ColxL*er*-RI-population (NASC, Lister and Dean 1993): RI-13, -37, -115, -190, -191, -194, -217, -231, -232, -238, -242, -245, -263, -267, -283, -288, -303 and -367 (see also supplementary Table 2)

of tetra-/polyploid sectors. For "sensitive" ecotypes we reduced solutions to 0.1 or 0.05% (Table 1, Supplementary Table 1). The evaluation of cellular size, in particular trichomes, as explained in the text, enabled us to identify polyploid versus non-polyploid sectors on a treated plant. This allowed us to harvest plants producing polyploid seeds selectively, which in turn alleviated the identification of the desired tetraploids in the second round of analysis. In our hands, this treatment was the most efficient procedure and led predominantly to tetraploid plants. We did not harvest specific inflorescences and aimed to harvest polyploid plants (or sectors), which often also included diploid sectors. This selection led to progeny of mixed ploidies in the following generation. We therefore selected single plants from the first generation onwards.

Estimation of cell size and statistical analyses

We measured trichome cell size indirectly by counting the number of trichome branches on rosette leaves. For each ecotype and ploidy level, we classified the trichomes on the third leaf of five to ten plants (between 20 and 110 trichomes per plant). In some cases, we evaluated additional data sets from counts from the fifth leaf. For the analysis of stomatal cells, we did not perform epidermal lifts but took images instead. This enabled us to measure the stomatal size and to evaluate chloroplasts numbers. Thus, rosette leaves were fixed (30% EtOH, 5% acetic acid, 3.7% formaldehyde, 0.01% Triton X-100) overnight at 4°C and passed through an ethanol series (40, 50, 60, 70%; each step for ca. 45 min) at room temperature and stored in 70% at 4°C until use. Prior to analysis, the ethanol series was reversed down to 10% ethanol and the epidermis was analysed microscopically. Images of 30 randomly selected adaxial stomata (three plants, ten stomata each) were taken and cut out from the printed images (leaving out the stomatal opening). Their weights were used to estimate the actual surface area in μm^2 with the aid of a calibration curve.

For statistical analyses we used the GraphPad-PRISM® statistical software (version 4.0). All data sets were tested for normal (= Gaussian) distribution and where necessary transformed ($\exp x$, $\sin x$) to obtain normality. This allowed us to perform parametric tests of trichome and stomatal size data, which included *t* tests (and *F* tests) for comparisons of two data sets or ANOVA (one-way, two-way) and appropriate post-tests (Bonferroni, Tukey) for multiple comparisons, respectively (two-tailed analyses). The data for chloroplast number could not be adequately transformed to yield a Gaussian distribution. In this case we applied non-parametric tests (Mann–Whitney for comparison of two data sets, Kruskal–Wallis followed by Dunn's post-tests for multiple comparisons). The confidence interval applied to all tests was 95%.

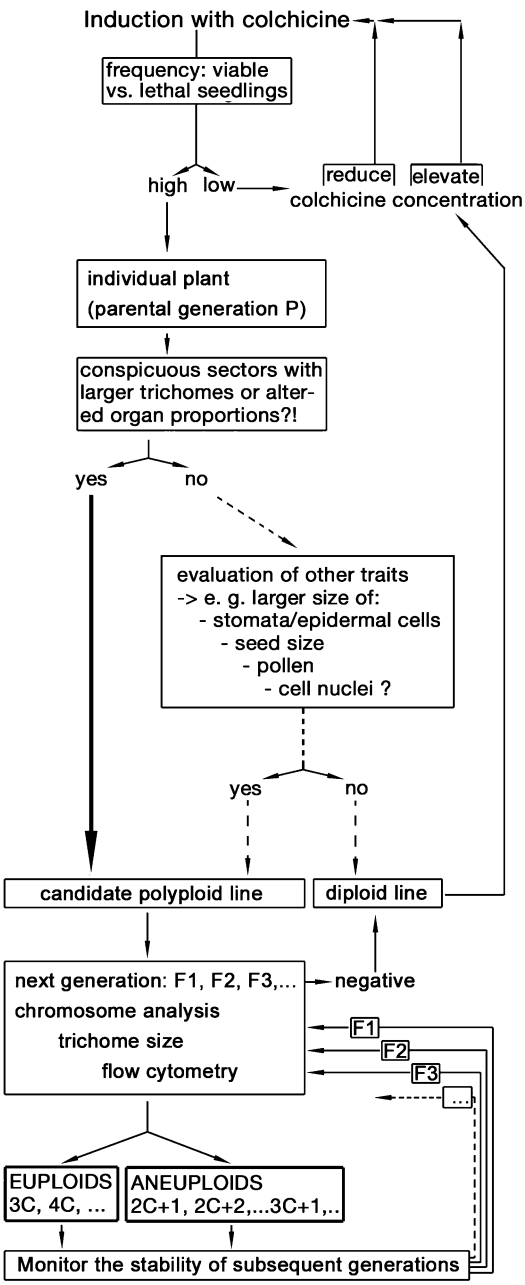

Fig. 1 Flow chart of the procedure for induction, identification and assessment of polyploids in *Arabidopsis thaliana* (for details see text). The big arrow directly leading from "conspicuous sectors…" to "candidate polyploid line" indicates the shortest and most convenient way to isolate the highest number of poly-/tetraploids with the least effort

Analysis of the polyploidy grade

We routinely analysed nuclei from rosette leaves from treated plants by flow cytometry. Where appropriate we analysed tissue from sectors of the colchicine treated plants beforehand in addition to the evaluation of trichome morphology. Flow cytometry was essentially performed as described (e.g. Henry et al. 2005) using the high-resolution kit from Partec. Briefly, leaves were chopped with a sharp razor blade in 0.3 ml of nuclei extraction buffer (solution A of the Partec kit) and filtered through 20 or 30 µm Cell Trics filters (Partec). The flow through was combined with 1.2 ml of DAPI solution (solution B of the Partec kit) and analysed in a PAS II flow cytometer (Partec) equipped with a HBO lamp for UV excitation. The PAS II FlowCytometer distributes the measured particles according to their fluorescence intensity into 1024 different channels. Routinely, several thousand particles were measured per leaf and measurements were often repeated. We compared the peak positions of the 2C, 4C, 8C, 16C and 32C nuclei between diploid and tetraploid plants whose ploidy was already known or had been assessed by chromosome counts of metaphases. Due to endopolyploidy, flow cytometry measurements from a diploid *Arabidopsis* plant not only exhibit a 2C peak but also further peaks up to 32C. Consequently, plants with a higher basic ploidy level e.g. tetraploids lack the 2C peak. However, flow cytometry is not sensitive enough to discriminate between certain euploids and aneuploids, e.g. 4C vs. 4C + 1. Bearing this in mind, the chromosome number of plants was assessed by counting metaphase chromosomes of root tips. These chromosome counts uncovered a number of Col-0 plants originating from the most intensive colchicine induction procedure, which displayed irregular mitotic chromosome figures (see "Results").

We followed standard protocols for metaphase chromosome preparation (e. g. Maluszynska and Heslop-Harrison 1991; Zhong et al. 1996) with slight modifications. Briefly, roots from seedlings grown on agar plates were dissected and incubated for 1 h at 4°C in 2 mM hydroxychinolin until the tissue was transferred to Carnoy's solution (ethanol:acetic acid; 3:1; v:v). Alternatively, roots were dissected and immediately submerged in 1 ml Carnoy's solution to fix the root tips for at least 12 h, washed three times for ca. 5 min with H_2O_{bidest} and incubated for at most 15 min at 37°C in enzyme solution (10% Macerozyme R-10 from Duchefa; 2% Cellulase "Onozuka R-10" from Duchefa in 100 mM Citric-buffer pH4.8). Roots were washed three times with H_2O_{bidest} and placed on small Petri dishes to separate the root tips (they often separate automatically upon digestion). About 10 root tips were transferred onto a slide (SuperFrost®Plus, Menzel), squashed with the tip of a needle and covered with a drop of 60% acetic acid for ca. 1 min. After adding 800 µl Carnoy's solution for 2 min the slide was submerged in 70% ethanol, air-dried and 60 µl DAPI solution (4′,6-diamidino-2-phenylindol-dihydrochloride, 1 µg/ml) was added. The slide was covered with a cover slip and stored for 10 min in the dark. Afterwards the cover slip and DAPI were washed off with H_2O_{bidest}, the slide was air-dried, covered with 10% glycerol and a new cover slip and analysed under the epifluorescence microscope. For each line and generation we evaluated between 5 and 20 metaphases.

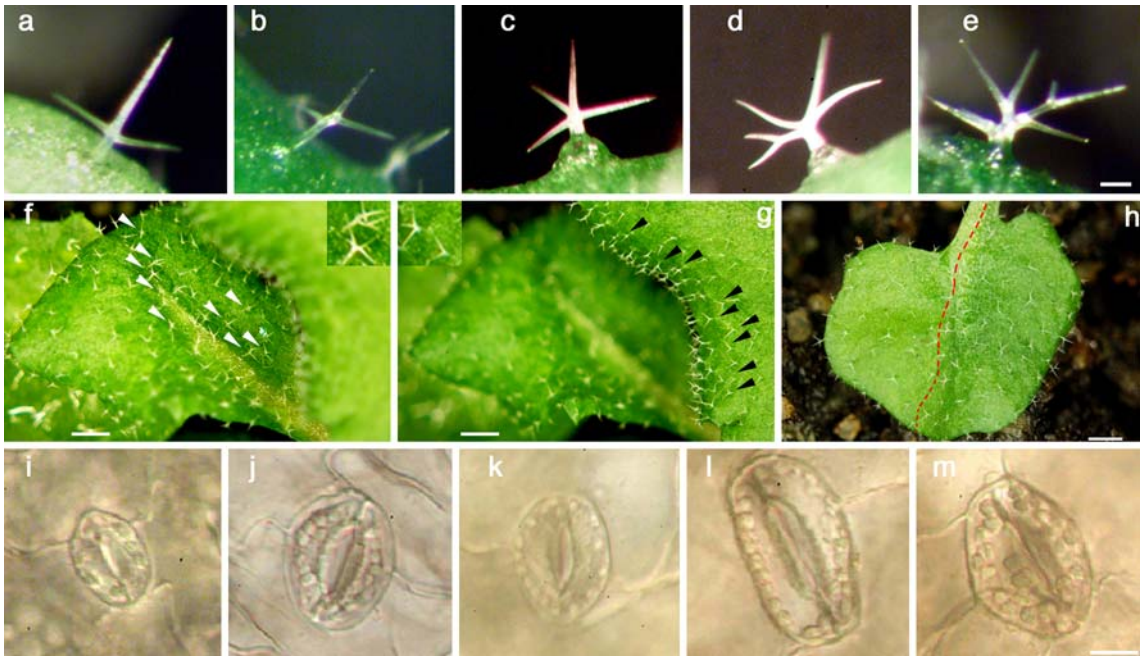

Fig. 2 Cell size and tissue size effects of polyploidy. Shown is the trichome morphology of diploid Col-0 (**a**), diploid L*er*-0 (**b**), tetraploid Col-0 (**c**), octoploid Col-0 (**d**) and aneuploid Col-0 (**e**) plants. Sectors of colchicine treated plants as visualized by trichome morphology and leaf morphology (**f**–**h**); (**f**) polyploid trichomes (white arrowheads) on a leaf of a sectored plant ecotype Yo-0 (inset shows a magnification of such trichomes), (**g**) a neighboured leaf with diploid trichomes (black arrowheads; inset shows a magnification); (**h**) sectored leaf of a treated CIBC-5 ecotype, diploid sector (left) separated by a stippled line from the polyploid sector (right). Sizes of stomata from diploid Col-0 (i), tetraploid Col-0 (j), octoploid Col-0 (k) and two different (4x + 1 and 6x + 2) aneuploid Col-0 plants, respectively (l + m). Note that there are elevated numbers of chloroplasts in the stomata of plants with higher ploidies (see also Fig. 7). Scale bars: 0.1 mm in **e** is same for **a**–**e**; 1 mm in **f**–**h**; 10 μm in **m** is same for **i**–**m**

Results

The generation of polyploids involves three major steps: induction with colchicine, identification of candidate lines (sectors) and assessment of polyploid lines by analysing the progeny of candidate lines (Fig. 1). The rationale of our procedure is based on the straightforward verification of the architecture of trichome cells as a morphological surface "marker", which discriminates between diploid and polyploid *Arabidopsis* sectors and lines, respectively (Fig. 2). The treated parental generation was examined with respect to the presence of polyploid sectors. Plants with polyploid sectors were always counted as potential parents for polyploid lines, because we observed that, if a polyploid sector developed reproductive organs, it regularly produced polyploid progeny. The identification of sectored plants therefore works as a short cut in our strategy (Fig. 1).

Induction via colchicine treatment

The initial tests started with high concentrations of colchicine and either extreme durations of exposition to this agent or high numbers of treatments. In these cases, most seedlings became necrotic and died. However, the data indicate that the tolerance to colchicine varies between ecotypes. For instance, 12% of Col-0 seedlings survived 4 h of submersion in 0.5% (w/v) colchicine giving one tetraploid line (named P9A) and six aneuploid lines, three of which are presented in this work solely for comparison (4x + 1, 6x + 1 and 7x[+ 3], respectively; see below). Less than 1% of L*er* survived (Table 1). We also tested this procedure with the ecotypes Kas-2 (N1264), Nd-0, Mt-0 (TD), and Oy-0 (TD), with comparable numbers (60–120) of seedlings (not shown). These showed survival frequencies between 0% (Mt-0 TD) and 7.5% (Nd-0). However, except with Col-0, we did not obtain any plants with polyploid sectors. The nine-fold treatment did not lead to polyploid sectors in L*er*-0 (Table 1) but also not in Mt-0 (TD) and Nd-0 (survival frequency of 45.8 and 20.7%, respectively; not shown). A three-fold or one-fold treatment (with different concentrations) always produced polyploids. Plants with Col-0 background are less sensitive and outrun L*er*-0 plants when one aims to obtain many polyploids. We have continued to treat diverse ecotypes with the one-drop method and concentrations of 0.5 and 0.1% colchicine, respectively, with variable results (Supplementary Table 1). For some ecotypes, it was not possible to obtain any plants with polyploid sectors so far. Interestingly, high survival frequencies do not guarantee a high frequency of polyploid

sectors (compare Col-0/DR5 and Col/CYCAT1 at 3x 0.5% with the same at 3x 0.1%). The result with RI-lines is conspicuous. They show low or moderate survival frequencies but very high frequencies of survival with polyploid sectors.

Sector analysis in colchicine treated plants

If a plant survives the colchicine treatment, it might remain unaffected and diploid. Alternatively, if colchicine has suppressed mitosis in a cell of the shoot apex a mosaic plant will grow. In this plant the root, hypocotyl, cotyledons and first primary leaves will be diploid.

Polyploidy often leads to an increase in cell size. We reasoned that an increase in the size of epidermal cells would be readily detectable and that this could act as a reliable indicator for polyploid sectors in colchicine treated plants. In fact, the morphology of trichomes turned out to be perfectly suited for this purpose. We found that the way trichomes react to a change of polyploidy in their sector is not just by an overall increase in size but also an increase in the number of branches (Fig. 2). This roughly correlated with the degree of polyploidy in progeny originating from sectors with altered versus non-altered trichomes. Sectors that were sufficiently large were also analysed by flow cytometry analysis. We assume that trichome cells that are already endopolyploid in diploid plants might have an even more elevated degree of endopolyploidy in polyploid plants. They at least had larger nuclei in the cases examined (SFig. 1 in supplementary material). The identification of polyploid sectors by surface markers alone was not a sufficient parameter for our strategy. Most important was whether epidermal (L1) sectors reflected polyploid tissues in the corresponding sub-epidermal layers (L2, L3) because these are the origins for (polyploid) gametes and progeny, respectively. This was regularly the case (see below).

Stability of newly induced tetraploid *Arabidopsis* plants

We analysed the relative stability of the newly induced tetraploids over three consecutive generations in about two dozen lines from the Col-0 and L*er*-0 ecotypes as schematically outlined in Fig. 3. We took advantage of the fact that an accumulation of multiple branched trichomes is a reliable marker for a sector/plant, which gives polyploid progeny in all (even the parental) generations. Single colchicine treated seedlings were given a code number, which henceforth identifies the corresponding line in our lab (Table 2; first column: ecotype; second column: line/code-no.). Plants, that survived and displayed polyploid sectors (as judged by trichome and organ morphology) were grown to maturity and harvested. Seeds of each line (F1 generation) were germinated and the root tips of ten young seedlings were taken for chromosome analysis (Fig. 4). The F1 generation might consist of different ploidies dependent on the size of the two competing sectors, diploid vs. polyploid, in the parent plant (Fig. 3). We evaluated the metaphase figures and calculated the average chromosome number for each line (Table 2, third column: F1chr.). The chromosome analysis of the F1 progeny indicated more or less polyploid progeny for virtually all plants, as indicated by the chromosome index (F1chr.-column in Table 2). Note that, due to the variable quality of the metaphase figures taken, the chromosome count does not precisely sum up to even numbers. This is also true for the control diploid and tetraploid plants. This is because it was sometimes difficult to distinguish all the chromosomes in the mitotic figures. We considered a value of ca. 19 together with a corresponding 4C flow cytometry value to indicate a tetraploid. Five seedlings of each line were further grown and inspected for trichome morphology (Fig. 3). In all except three cases, 5/5 seedlings showed polyploid trichomes and/or abnormally structured/sized organs. One of the five mature plants was selected for flow cytometry analysis (Table 2; fourth column: F1flc.). This step already separated lines with respect to their ploidy (F1flc-column in Table 2): these included three diploids, twelve tetraploids, one pentaploid, two hexaploids and two octoploids. The penta- and hexaploids were not followed through all generations. The hexa- and octoploids, respectively, exhibited instability through all generations and produced progeny of mixed ploidy (the former more than the latter). Plants with a basic ploidy level higher than diploid lacked the 2C peak. Plants with a basic ploidy level higher than tetraploid in addition lacked the 4C peak (Fig. 5). All the plants that turned out to be tetraploid or octoploid were harvested and further analysed (F2 generation). We proceeded as in the first generation, i.e. ca. 10 seedlings were taken for chromosome analysis (Table 2; fifth column: F2chr.), five seedlings were grown, evaluated for their trichomes and one of the five adult plants was harvested for flow cytometry (Table 2; sixth column: F2flc.) to give the progeny of the next generation and so on. In all but one instance, the tetraploid lines remained tetraploid. The octoploids exhibited chromosome number instability as deduced from their average number of metaphase chromosomes. All tetraploids of the F2 generation remained tetraploid in the next generation (Table 2, seventh and eighth column: F3chr. and F3flc., respectively).

By analysing metaphase chromosome figures, we also detected six aneuploid lines amongst the seven Col lines, which resulted from the most intensive colchicine induction procedure (see "Materials and methods"). The variability of chromosome numbers observed for these lines by far

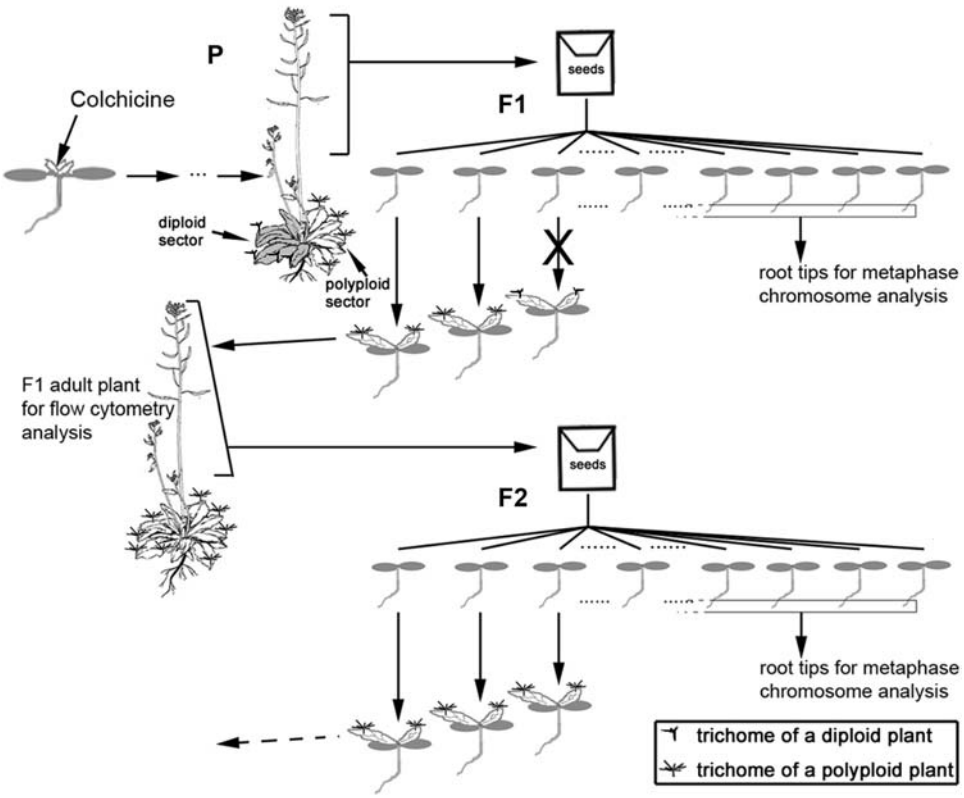

Fig. 3 Selection and assessment scheme for polyploid lines in consecutive generations (see text)

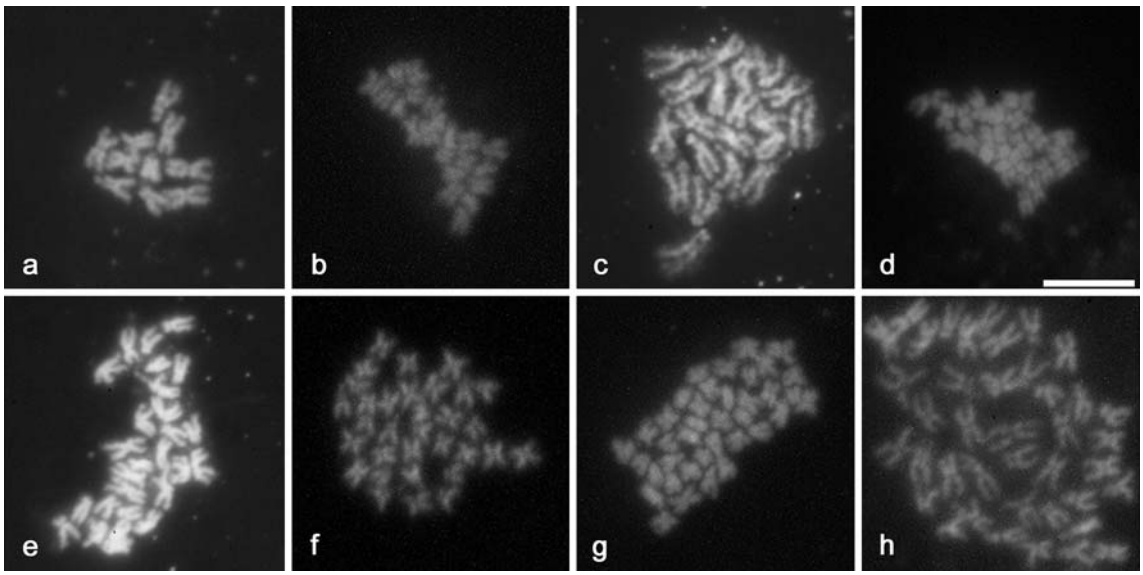

Fig. 4 Metaphase chromosomes of dividing root tip cells. Shown are metaphase chromosomes of different ploidies from the Col-0 ecotype (except the hexaploid, which is of mixed ColxLer background): **a** 2x, **b** and **c** 4x, **d** 4x + 1, **e** 6x, **f** 6x + 2, **g** 7x[+ 3] and **h** 8x. The chromosomes in **a**, **c**, **e** and **h** have not reached full condensation. Scale bar in **d** is same for **a**–**h**: 10 μm

exceeded that which results from differences in the quality of particular metaphases as mentioned above. We estimated the most likely chromosome numbers of these plants by evaluating metaphase figures of siblings of generation F2. Since this study focuses on tetraploids, we did not follow further generations of these plants. For a comparison of cell size features (see below), we selected siblings of the same generation from three of these six lines with an estimated chromosome number of 4x + 1, 6x + 1 and 7x[+ 3]. We suspect that the aneuploid constitution of these lines resulted from the strength of the colchicine induction procedure.

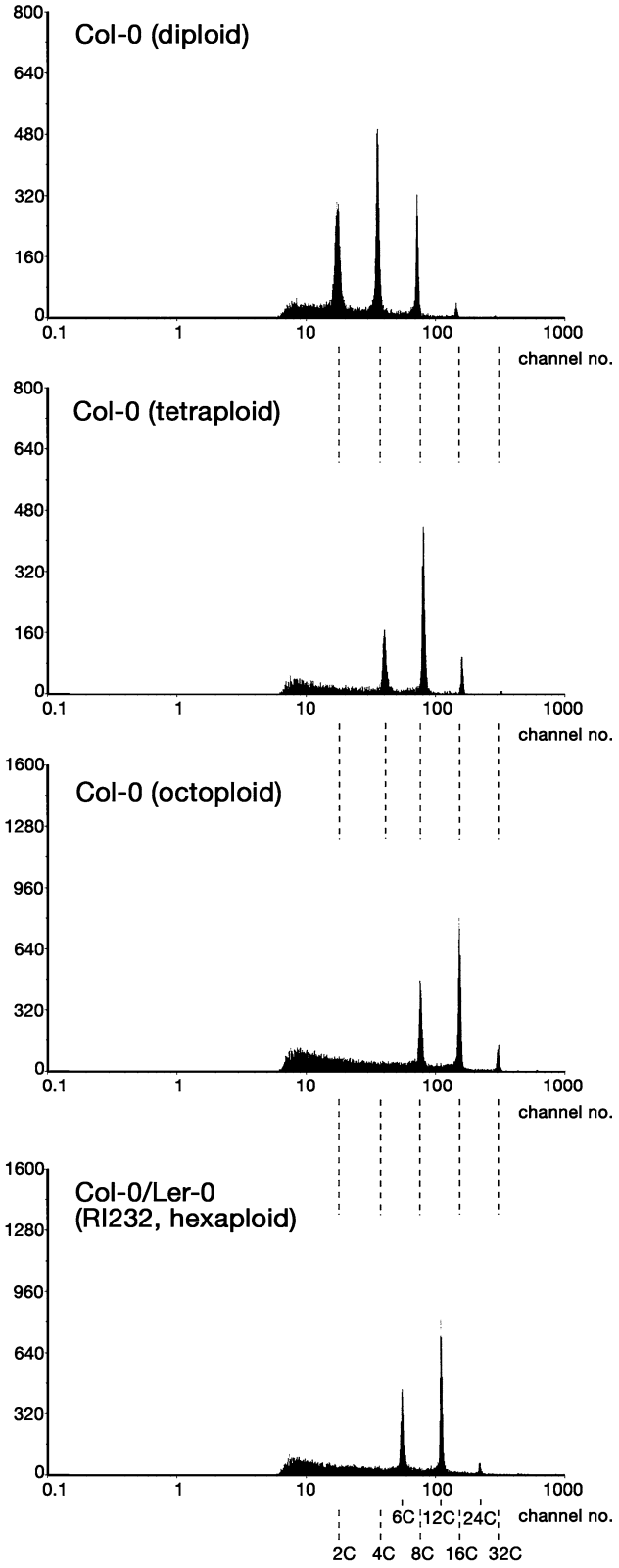

Fig. 5 Flow cytometry of *Arabidopsis* polyploids. The number of measured particle counts (nuclei) versus the channel of the Partec II analyser is given. Note the absence of the 2C peak in the tetraploid and the absence of the 2C + 4C peaks in the octoploid plant respectively. Note also the position of the peaks of the hexaploid plant

Cell size features of polyploid lines

The numerous lines assessed, spanning a large range of ploidies, gave us the opportunity to test a number of characters, in particular cell size, in more detail. This analysis revealed some known but also new interesting features. The effect of polyploidy in the epidermis is summarised by the average number of branches per trichome. The trichome index gives the proportion of a particular trichome branch class in the corresponding population (Fig. 6). The average branch number of a trichome starts at 3.00, 3.00 and 2.34 in diploid L*er*-0, Col-0 and Zü, respectively, continues with 4.04, 3.33 and 2.94 in tetraploid L*er*-0, Col-0 and Zü, respectively, and reaches 4.27 in hexaploid Col-0xL*er*-0 (RI232), 5.19 in octoploid Col-0 and 3.83, 4.51 and 4.52, respectively, in the aneuploids (4x + 1, 6x + 1, 7x[+ 3]). Plants with numerous six- and seven-branched trichomes are more likely hexa- or octoploid than tetra- or diploid, respectively, (Fig. 6). Interestingly, the higher the degree of ploidy, the more trichomes disperse along a higher number of trichome branch classes. Diploids have two- to four-branched trichomes. Tetraploids have predominantly two- to five-branched trichomes. Trichomes of hexa- and octoploids scatter along three to eight branched variants. Aneuploids resemble plants with higher ploidies in this respect, irrespective of their actual chromosome number (Fig. 6). Thus, an elevated number of trichome branches in comparison to the corresponding progenitor ecotype generally tends to indicate higher ploidy. The new lines we obtained allowed us to compare the impact of ploidy (2x vs. 4x) and the impact of ecotype (Col-0, Ler-0, Zü) simultaneously. We obtained significant values for the general impact of both factors on trichome branch number ($P < 0.001$, two-way ANOVA). Therefore, we separated the ecotypes subsequently and performed the analyses of ploidy effect within a given ecotype. This revealed significant differences between Ler 2x vs. Ler 4x and Zü 2x vs. Zü 4x, respectively, (*t* tests, $P < 0.0001$ for both). Within Col-0 multiple comparisons (one-way ANOVA) were performed. Euploids and aneuploids were also separately compared because analyses indicated differences in variance between these groups (e.g. Col-0 2x vs. Col-0 7x[+ 3] with $P < 0.0001$, *F* test). Comparison of Col-0 euploids displayed significant differences ($P < 0.001$) except that one comparison, Col-0 2x vs. Col-0 4x, was critical and gave non-significant ($P > 0.05$) and significant ($P < 0.01$) values depending on the transformation of data to obtain a Gaussian distribution (sin*x* vs. exp*x*). One-way ANOVA of aneuploids did not reveal significant differences. The hexaploid line was excluded from these comparisons since it is of mixed Ler-0/Col-0 origin.

Leaves having supernumerary trichome branches also had larger stomata (Fig. 7). This difference is significant

Table 2 Assessment of polyploidy grade of selected lines during three consecutive generations

Ecotype	Line code-no.	F1chr.	F1flc.	F2chr.	F2flc.	F3chr.	F3flc.
Col-0	N1092(diploid)[a]	9.7	2C	–	–	–	–
Col-0	N3432(tetrapl.)[b]	19.3	4C	–	–	–	–
Col-0	1326-12	18.2	4C	19.8	4C	19.8	4C
Col-0	1326-15	15.7	4C	20.1	4C	19.8	4C
Col-0	1326-18	14.9	2C	–	–	–	–
Col-0	1326-19	19.1	4C	19.6	4C	19.7	4C
Col-0	1326-26	19.7	4C	19.8	4C	19.6	4C
Col-0	1326-28	19.2	4C	19.3	4C	19.5	4C
Col-0	P9A	19	–	18.6	4C	–	4C
Col-0	3115-1	27.9	8C	19.2	4C/6C	23.8	4C
Col-0	3115-2	11.7	4C	9.2	2C	–	–
Col-0	3115-3	40.0	8C	33.9	8C	24.0	–
L*er*-0	1026-5	15.8	4C	20.1	4C	–	–
L*er*-0	1026-10	18.4	4C	19.1	4C	19.8	4C
L*er*-0	1026-19	11.8	2C	–	–	–	–
L*er*-0	1026-40	17.6	4C	19.5	4C	19.6	4C
L*er*-0	1026-41	16.8	4C	19.3	4C	20.0	4C
L*er*-0	1026-27	12.6	2C	–	–	–	–
L*er*-0	3116-1	17.8	6C	–	–	–	–
L*er*-0	3116-2	15.3	5C	–	–	–	–
L*er*-0	3116-6	20.0	4C	20.0	4C	19.6	4C
RI232-2		–	–	–	6C	28.6	6C

[a] Reference diploid line Col-0 N1092 used for comparison in all flow cytometry analyses

[b] Established and repeatedly assessed tetraploid Col-0 control line N3432 from Dr. O. Mittelsten-Scheid. For details see text

when di- and tetraploids are compared ($P < 0.0001$). The three analysed aneuploid lines did not reveal significant differences in multiple comparisons, which also indicated that a higher chromosome number does not always necessarily lead to a bigger cell size. Similarly, diploid Col-0 displayed a significant difference in the number of chloroplasts per stomata in comparison to tetraploid Col-0 ($P < 0.0001$). Aneuploids revealed a difference only with respect to Col-0 4x + 1 vs. Col-0 6x + 1 ($P < 0.05$). In random samples, we monitored other features such as size of cell nuclei, roots and seeds (SFig. 1). In all cases, these structures had larger sizes in polyploids in comparison to diploids. With respect to roots, the increased size of these organs is due to increase of cell size instead of cell number (not shown).

Discussion

Colchicine: effects and induction of polyploidy

The most important aspect of our colchicine treatments pertains to colchicine effects on the epidermal (L1) and sub-epidermal (L2, L3) layers. Following treatment, the apex can develop in three different ways. First, it might produce detectable diploid and polyploid sectors (Fig. 2). Second, diploid cells overgrow polyploid cells and most of the plant turns diploid again and produces diploid progeny. Conversely, polyploid cells overgrow diploid cells; i.e. polyploid progenies are mainly produced. In any case, it is a crucial prerequisite of our strategy that the altered polyploidy of the epidermis (L1 layer) on the treated plant reflects an altered polyploidy of inner cell layers (L2 and L3 layer, respectively) because these are the cells that give rise to generative cells. This is not self-evident because L1 and the inner layers (L2, L3) are developmentally separated early on (Duckett et al. 1994; Takada and Jürgens 2007). In fact, we found that once a polyploid sector had been identified on a plant, the same plant regularly delivered polyploid progeny (Table 2). This can be explained by two possible effects, which have been observed previously (Dawe and Freeling 1991; Tilney-Bassett 1986 and references therein). The first is that colchicine intruded into epidermal and subepidermal layers in most of the treatments. The second is that cells from one tissue "invaded" the adjacent lineage.

Colchicine is a highly poisonous secondary metabolite from *Colchicum autumnale* (and other Colchiceae), which inhibits microtubule polymerization by binding to tubulin. This study reveals ecotype and line differences in sensitivity to colchicine (some RI and transgenic lines; Table 1, Supplementary Tables 1 and 2). Whether the observed differences in sensitivity have a genetic basis, controlling

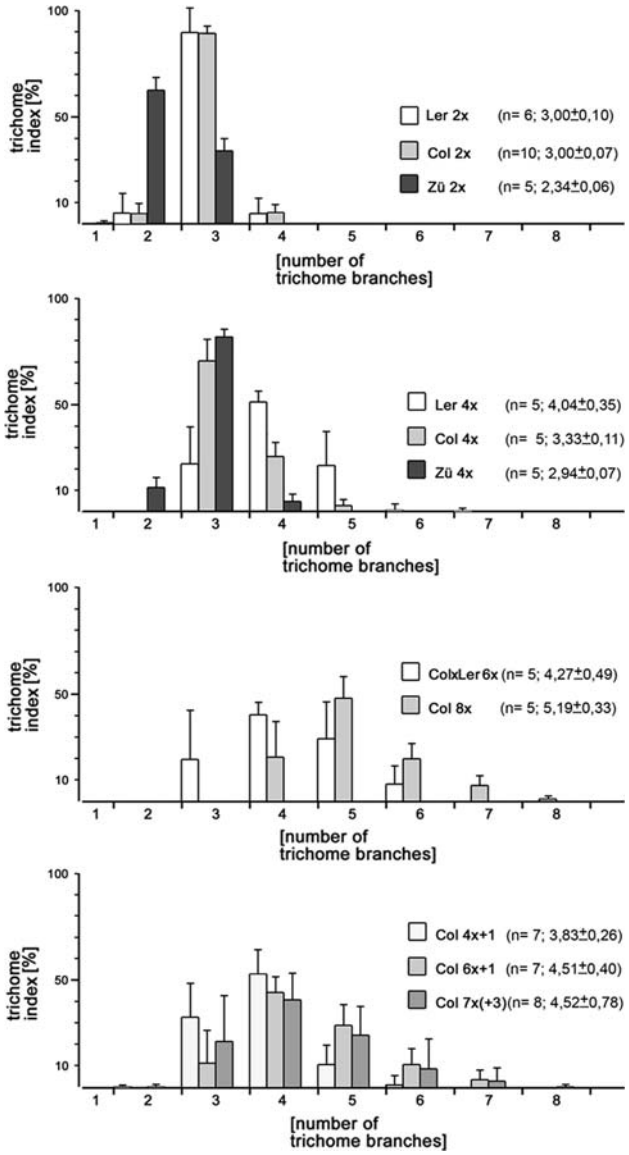

Fig. 6 The relation of basic polyploidy and branch numbers in *Arabidopsis* trichomes. The plots separate the data for different ploidy classes, i.e. diploid, teraploid, hexa plus octoploid and aneuploid respectively. Ecotypes and (an-)euploidies are indicated with different grey scales. The number of analysed plants, the mean of branches per trichome of all plants and their standard deviations are given on the right

processes such as uptake and detoxification of colchicine, remains to be determined.

Whether colchicine has mutagenic effects in addition to its toxic effects in *Arabidopsis* or other plants is not clear. In our experiments, we did not detect any of the mutants that most frequently occur in mutagenic screens (i.e. the albino, crème and fusca phenotypes; Jürgens et al. 1991; Mayer et al. 1991). Similarly, an independent study of over 40 colchicine induced *Brassica* allopolyploids did not provide any indication of a mutagenic effect of colchicine (Lukens et al. 2006). There is also no evidence for long term genomic instabilities caused by colchicine. In fact, these could be caused by other factors, such as scaling incompatibilities of cell volume versus intracellular structures (e.g. spindle geometry; Storchova et al. 2006).

Size effects in generated polyploids

When we examined the plants after colchicine induction we visually separated wild-type (diploid) plants from plants with putative polyploid sectors. These were defined as sectors carrying a conspicuously high number of trichomes with more than three branches (more than two in case of ecotype Zü). Actually, the subsequent analyses of the pedigree revealed that the trichome branch number tends to increase with polyploidy until it reaches a certain limit (Fig. 6). From an analysis of triploids, it is known that trichomes of such plants have values between diploids and tetraploids (Perazza et al. 1999). The statistical analyses of the di- and tetraploids demonstrate a general impact of both the genetic background (i.e. ecotype) and the grade of polyploidy. This does not exclude the possibility that some comparisons do not exhibit a difference (e. g. Col-0 vs. L*er*-0 diploid plants). A tendency to increase the number of trichome branches with increasing polyploidy grade is also given (despite particular exceptions) in the larger Col-0 polyploid series. Another interesting observation concerns the scattering of trichomes in different trichome branch classes. Thus, octo-, hexa- and aneuploid trichomes have four to seven trichome branch classes, as compared to three to four classes in diploids and tetraploids. This probably indicates that a plant with a high ploidy, e.g. an octoploid plant, can realize a broader range of ploidies in its cells. It has been shown in *Arabidopsis* and other plants that many cells have elevated ploidy levels due to endoreduplication (Galbraith et al. 1991; Schmuths et al. 2004). In addition, in aneuploids, cells might adopt variable ploidies due to enhanced genome instability. Very similar effects might influence the size of stomata (and in turn the number of chloroplasts they harbour). In diploids and tetraploids the difference is significant. Aneuploids display some variability in this respect but often the differences are not significant although they differ in chromosome number. Taken together, we could verify all known size effects (Melaragno et al. 1993; Perazza et al. 1999 and references therein) in polyploids, i.e. size increase of (epidermal) cells, organs and seeds as well as cell nuclei (Figs. 2, 6, 7 and SFig. 1).

Relative stability of neo-tetraploid lines

In this study, we focussed on the analysis of absolute chromosome numbers in tetraploid lines during three consecutive

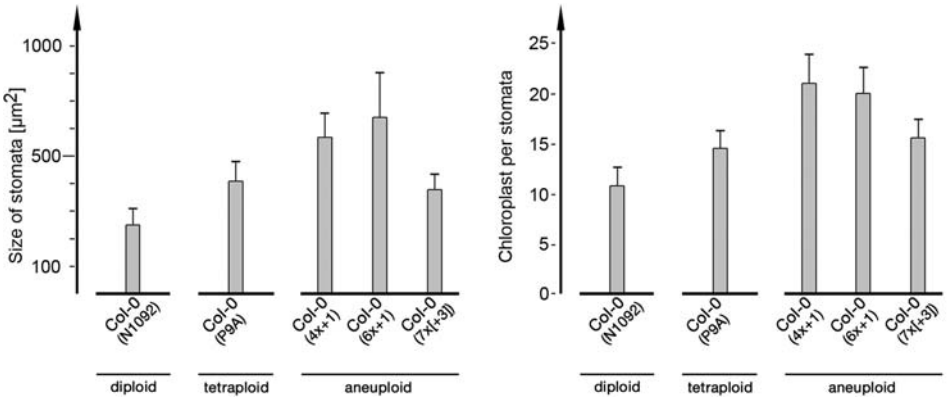

Fig. 7 Stomata in polyploids. The sizes of stomata (both cells) for corresponding lines are given in μm^2 (left). The number of chloroplasts per stomata from the same lines as in the left part is given in the right part. The corresponding lines and standard deviations are indicated

generations. We have addressed neither aspects concerning the correct number of particular chromosomes nor epigenetic variation.

The detected tetraploid lines displayed a high degree of stability in chromosome number because, once they had passed the selection in F1 they remained stable. There was only one exception, Col-0 line 3115-2. The selected F1 plant only produced diploid progeny (note the near-to-diploid F1 chromosome index; Table 2). Such instabilities are also known for established lines. We observed this in the tetraploid lines: Wilna ecotype, N3247 and Wa-1 (N1587), which is a natural tetraploid (Henry et al. 2005; Schmuths et al. 2004). Interestingly, L*er*-0 seems to be more colchicine-sensitive than Col-0. However, once established L*er*-0 tetraploids seem to be as stable as Col-0 tetraploids. The hexa- and octoploids produced progeny of mixed ploidy (the former more than the latter). They could only be maintained through successive generations by selection of true hexaploids and octoploids in the pedigree by means of flow cytometry analysis. Though we have not analysed these in this detail, we found the aneuploids isolated from the experiment with the strongest colchicine induction treatment (see "Materials and methods") to be very variable with respect to genome composition. The chromosome analysis revealed an approximate estimation of their chromosome number. It is likely that this composition will vary in the next generations and lead to divergent lines in this respect.

The relative stability of tetraploids has been previously observed, indicating that they can be easily maintained through selfing (Bouharmont 1965; Heslop-Harrison and Maluszynska 1994). The ability to produce progeny of the same ploidy requires some degree of diploidisation or chromosomal stabilisation in meiosis. In a tetraploid plant, this is acquired by a high frequency of bivalents versus a low frequency of uni-, tri- and tetravalents. Two studies report different but significant degrees of multivalent formation in *Arabidopsis* tetraploids. Both used different ecotypes and lines established for quite a different number of generations (Weiss and Maluszynska 2000; Santos et al. 2003). In addition, the Wilna line had undergone some chromosomal rearrangements in the rRNA gene clusters, probably promoting bivalent formation i.e. chromosomal stability (Weiss and Maluszynska 2000). Santos et al. (2003) demonstrated the importance of the history of a line because established lines had acquired a higher degree of diploidisation than newly induced lines (note that one of the lines analysed in this work N3427, identical to CS3427, was found to be unstable in our analyses, see above). The observed stability of our tetraploid lines seems to be in contrast with the aberrant meiotic products reported in previous work (Weiss and Maluszynska 2000; Santos et al. 2003). According to those studies, our new tetraploid lines should have produced mixed progeny with chromosome indexes significantly deviating from 20. However, the chromosome indexes of all tetraploids evaluated in F3 lie between 19.5 and 20.0, which indicates a homogeneous pool of tetraploid seedlings. There are at least two possible explanations for these discrepancies. The first is that unbalanced chromosomes had a greater impact on the viability of gametes in our lines. This "filtering" effect excluded aberrant gametes and was not detected since we did not analyse meiotic stages. A second possibility is that similar (or more extensive) rearrangements such as those observed by Weiss and Maluszynska (2000) promoted bivalent formation and chromosome stability. These could also not be detected by our analysis. Interestingly, rapid and early rearrangements seem to be common in re-synthesized *Brassica* allopolyploids (Song et al. 1995). Besides *Arabidopsis* and *Brassica* auto- and allopolyploids, respectively, other (allo- or amphidiploid) species such as *Triticum* and *Aegilops* are known to undergo rapid rearrangements including gene loss (Kashkush et al. 2002; Adams and Wendel 2005b and references therein). However, this is not a general rule as shown by cotton (Gossypium) species (Liu et al. 2001). Considering the current data in *Arabidopsis*, additional aspects are important in this context. For instance, our procedure selected for tetraploidy in every new generation.

Furthermore, Santos et al. (2003), and Weis and Maluszynska (2000) analysed meiotic stages but did not analyse the progeny during consecutive generations of a larger number of independently induced lines.

In conclusion, our data show that the strategy we have developed to augment the resources of tetra-/polyploid *Arabidopsis* lines works efficiently. The data presented indicate that the generated tetraploids exhibit a considerable stability in successive generations.

Acknowledgements We thank Dr. Daniel and his group (Bayerische Landesanstalt für Landwirtschaft) for generous provision of the Partec II Flow Cytometer and help, Dr. Stephan Haug (Lehrstuhl für Mathematische Statistik, TU München) for helpful advice in statistics and an anonymous reviewer for useful hints. We owe very special thanks to Farhah Assaad for critically reading the manuscript and useful discussions. We thank the Nottingham *Arabidopsis* Stock Centre (NASC) for lines. We are particularly indebted to the following colleagues for kindly providing lines: Ortrun Mittelsten-Scheid (Wien), Jolanta Maluszynska (Katowice), Marie-Theres Hauser (Wien), Thomas Debener (Hannover) and Jiri Friml (Ghent). The financial support by the Deutsche Forschungsgemeinschaft (Grant GI 140/12-1 to A.G. and R.A.T.R.) is gratefully acknowledged.

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