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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue

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Abstract Genotypes of *Nicotiana attenuata* collected from Utah and Arizona were transformed with 17 different vectors (14 unpublished vectors based on 3 new backbone vectors) using an *Agrobacterium*-mediated procedure to functionally analyze genes important for plant–insect interactions. None of the 51 T1–T3 transgenic Utah lines analyzed by the flow cytometry were tetraploid, as opposed to 18 of 33 transgenic Arizona lines (55%). Analysis of T0 regenerants transformed with the same vector carrying an inverted repeat (IR) *N. attenuata* pro-systemin construct confirmed the genotype dependency of tetraploidization: none of the 23 transgenic Utah lines were tetraploid but 31 (72%) of 43 transgenic Arizonas were tetraploid. We tested the hypothesis that the differences in polysomaty of the explant tissues accounted for genotype dependency of tetraploid formation by measuring polysomaty levels in different seedling tissues. Hypocotyls, cotyledons, and roots of Utah and Arizona genotypes contained similar percentages of 4C nuclei (61 and 60; 7 and 5; and 58 and 61%, respectively). Since we used hypocotyls as explant sources and the nonoccurrence of tetraploid Utah transformants does not correspond to the high percentage of 4C nuclei in Utah hypocotyls, we can rule out a direct relationship between tetraploid formation and polysomaty level. We hypothesize that the difference between the Utah and Arizona genotypes results from the failure of polyploid Utah callus to regenerate into fully competent plants. We propose that future work on post-transformation polyploidy concentrate on the processes that occur during callus formation and plant regeneration from callus.

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Introduction

Agrobacterium-mediated transformation has revolutionized the analysis of gene function in the plant sciences. Lines transformed with endogenous and heterologous genes in sense or antisense/inverted repeat (IR) orientations are frequently used to alter the expression of particular genes and thereby understand their function. Transformation success of plants regenerating from callus is usually determined by antibiotic screens, PCR, and Southern analysis and confirms the function of the resistance marker, the presence of the transgene, and the number of transgene copies. However, functional analyses can be confounded when genetic changes, such as the doubling of chromosomes or even whole genomes that are much larger than those directly related to the transgene, occur in the transformation process. Tetraploidization of plants after regeneration from calli with or without transformation was demonstrated more than 2 decades ago (Jacobson 1981; Imai et al. 1993; Lavia et al. 1994), but the analysis of ploidy levels has not yet been routinely integrated into post-transformation screening programs. More recently, flow cytometry, which allows plant transformants to be reliably and rapidly analyzed (Goldman et al. 2004; Ellul et al. 2003; Sigareva et al. 2004; Ducreux et al. 2005) has demonstrated that transformation can double the chromosome number of the transformed plants. In diploid tomato, 24.5–80% of transformants were tetraploid (depending on cultivar and method; Ellul et al. 2003) and up to 92% of originally triploid bermuda grass *Cynodon dactylon* × *transvaalensis* cv. TifEagle transformants were found to be hexaploid (Goldman et al. 2004).

The production of tetraploids by the transformation system for *Nicotiana attenuata* Torrey ex. Watson (Krügel et al. 2002), a native diploid tobacco used for the analysis of ecologically important traits (Baldwin 2001), is analyzed

Table 1 Vectors used for transformation

Vector	Type	Gene function	Accession number	Reference for cloning	Resistance in vector
pNATLOX1	AS	*N. attenuata* lipoxygenase *NaLOX3*	AY254349	Halitschke and Baldwin (2003)	Nourseothricin
pNATPI1	AS	*N. attenuata* trypsin proteinase inhibitor partial sequence	AY184823	Zavala et al. (2004)	Nourseothricin
pRESC2PIA2	CE	*N. attenuata* trypsin proteinase inhibitor, full length	AF542547	Zavala et al. (2004)	Hygromycin
pNATNC	EV	–	–	–	Nourseothricin
pRESC2NC	EV	–	–	–	Hygromycin
pRESC2RCA	AS	*N. attenuata* RUBISCO activase	BU494545	Völckel and Baldwin (2003)	Hygromycin
pRESC2SYS1	AS	*N. attenuata* pro-systemin	AY456270	Unpublished	Hygromycin
pRESC2SYS2	CE				
pRESC2TFN	AS	*N. attenuata* transcription factor WRKY3	AY456271	Unpublished	Hygromycin
pRESC5LOX	IR	*N. attenuata* lipoxygenase *NaLOX3*	AY254349	Halitschke and Baldwin (2003)	Hygromycin
pRESC5PMT	IR	*N. attenuata* putrescine-N-methyl transferase	AF280402	Winz and Baldwin (2001)	Hygromycin
pRESC5SYS2	IR	*N. attenuata* pro-systemin	AY456270	Unpublished	Hygromycin
pRESC2ETR1	CE	*Arabidopsis thaliana* functional restricted. ethylene receptor 1, *etr1-1*	AC020665	Chang et al. 1993	Hygromycin
pSOL3LOX	IR	*N. attenuata* lipoxygenase *NaLOX2*	AY254348	Halitschke and Baldwin (2003)	Hygromycin
pSOL3PIA	IR	*N. attenuata* trypsin proteinase inhibitor partial sequence	AY184823	Zavala et al. (2004)	Hygromycin
pSOL3NC	EV	–	–	–	Hygromycin
pSOL4PIA	IR	*N. attenuata* trypsin proteinase inhibitor partial sequence	AY184823	Zavala et al. (2004)	Nourseothricin

Construction of the first three vectors published (see references in Methods section); construction of remaining vectors is described in Supplementary Material S1

AS antisense expression, IR Inverted repeat, CE Constitutive expression, EV empty vector

in this study. Two genotypes of *N. attenuata,* both from field collections—one from Utah (Baldwin et al. 1994) and one from Arizona (Glawe et al. 2003)—are routinely transformed in our laboratory to study the interaction between *N. attenuata* and its herbivores. We transformed the Utah and Arizona genotypes with the following *N. attenuata* genes in antisense or IR orientations: RUBISCO activase, pro-systemin, WRKY3 transcription factor, lipoxygenase 3, putrescine-*N*-methyl-transferase, and trypsin proteinase inhibitor. In addition, we constitutively over-expressed the *N. attenuata* trypsin proteinase inhibitor, the pro-systemin gene, and a mutated ethylene receptor 1 of *Arabidopsis thaliana* (Table 1). Transgenic lines had previously been subjected to the routine post-transformation analysis of antibiotic screens and Southern hybridizations. Here we add flow cytometry to the analysis to determine the tetraploidy of the Utah and Arizona genotypes and test a mechanism that could account for the transformation-induced tetraploidization.

Materials and methods

Plant material

The Utah genotype was collected in 1992 from plants growing at the DI ranch, Santa Clara, southwestern Utah, USA (Baldwin et al. 1994). These seeds were used to establish an inbreeding line from which seeds of plants in the 7th, 11th, 12th, 14th, or 15th generation have been used for transformation. For an inbreeding-free explant source, seeds were collected from one plant at the same site in 2004. The Arizona genotype was collected in 1996 from a 20-plant population near Flagstaff, Arizona, USA (Glawe et al. 2003). Plants grown from these seeds were selfed in 2001 and their bulk-collected seeds (1st generation) were used for transformation. From this 1st generation an inbred line to the 7th generation was established. Seed germination and rearing of plants were performed as described in Krügel et al. (2002).

Plasmid construction

A summary of the vectors used and the genes of interest carried by these vectors is provided in Table 1. The construction of pNATLOX1 (11.2 kb) was described in Krügel et al. (2002); details for the construction of pNATPI1 (9.0 kb) and pRESC2PIA2 (11.1 kb) can be found in the Supplementary Material of Zavala et al. (2004). The construction of all other vectors is described in Supplementary Material S1. Maps of the backbone vectors pRESC501, pSOL3RCA, and pSOL4RCA as the basis for all described vectors are given in Supplementary Material S2, S3, and S4.

Generation of transgenic plants

The transformation procedure is described in detail in Krügel et al. (2002). Briefly, hypocotyls from 8-day-old seedlings germinated on Gamborg's B5 medium were cut with a scalpel in two to three, 3 mm long pieces after the tip of the scalpel was dipped into a suspension containing the vector-harboring *Agrobacterium tumefaciens* (strain LBA 4404, Life technologies-Gibco BRL). On different phytagel-based media, the explants and resulting calli/plants went through five stages: co-cultivation (3 days), callus growth (14–21 days), shoot regeneration (14–21 days), shoot maturation (14–21 days), and rooting (21 days). After rooting, the plants were transferred to soil in Magenta boxes (77 mm × 77 mm × 77 mm) and finally planted in 2-l pots in the greenhouse for breeding.

Characterization of transformants

For each T0 plant, 60 T1 seeds are germinated on plates with germination medium containing 35 mg/L hygromycin as described in Krügel et al. (2002) for seedling selection. If 75% of the seedlings survived after 7 days, the line was considered to be a putative transformant. For nourseothricin-resistant T0 plants, half a cotyledon of 60 T1 seedlings was transferred to plates with callus-inducing medium containing 250 mg/L nourseothricin as described in Krügel et al. (2002) for callus selection. If 75% of the cotyledon halves showed callus growth within 7 days, the line was considered a putative transformant.

Flow cytometry

Nuclei were extracted and stained using the Partec Cystain UV-precise P kit (containing the dye 4′,6′-diamidino-2-phenylindole, DAPI) and 30 µm mesh filters (Partec, Münster, Germany) according to the manufacturer's instructions. Samples were mascerated together with *Brassica oleracea* cv. Rosella, *Hordeum vulgare* cv. Sultan, or *Secale cereale* cv. Petkus Spring as internal standards. Measurements were taken on the flow cytometer CCA-II (Partec, Münster, Germany) with UV excitation by a mercury arc lamp. The gain was generally set to 435 but was occasionally fine-tuned between 433 and 440. To measure transformants, plants were grown to the elongation stage and leaves from the upper part of the elongated shoot were used. For those lines identified in Table 2, flow cytometry was performed on cotyledons of 8-day-old seedlings.

Chromosome counting

Five-day-old seedlings that had been germinated as described in Krügel et al. (2002) were incubated for 2 h in 2 mM 8-hydroxychinolin at room temperature. After being fixed in alcohol/acetic acid (3:1: v:v), seedlings were macerated in 1 N HCl at 60°C for 4 min. After separation from the seedling, root tips were stained with carmine acetic acid, squeezed between a microscope slide and cover slip, and immediately observed under a microscope with 100×10 magnification and oil immersion. Pictures were taken with an Axioskop2 with digital camera and Axiovision 3.0 software for image acquisition (Carl Zeiss, Oberkochen, Germany).

Results and discussion

Distribution of tetraploid transformants

Ploidy levels were determined by dividing the average fluorescence of 2C nuclei of the sample by the average fluorescence of 2C nuclei of the standard, yielding the ratio R. For measurements with the standard *Brassica oleracea* cv. Rosella, a sample with an R of 3.32±5% CV was considered diploid and a plant with R=6.64±5% CV tetraploid. For *Hordeum vulgare* cv. Sultan the ratios were 0.655±5% and 1.31±5% CV, and for *Secale cereale* cv. Petkus Spring, the ratios were 0.408±5% CV and 0.816±5% CV. A small number of samples was measured without a standard; as all samples were measured with a similar gain, the location of the 2C peak in the histogram of a diploid plant could be readily determined. When the 2C peak was absent and the 4C peak was very prominent, a plant was considered tetraploid. The flow cytometric ploidy determinations were confirmed by counting metaphase chromosomes for pRESC2RCA-transformed Arizona lines

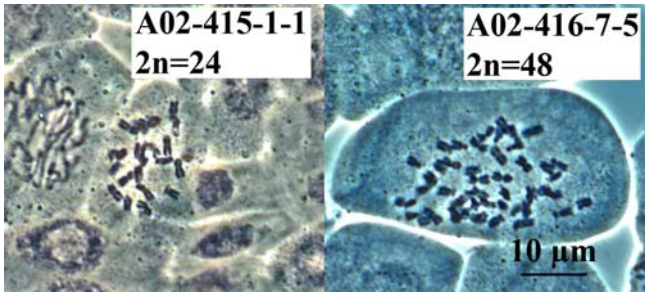

Fig. 1 Metaphase plates in root tips of transgenic *N. attenuata* of the Arizona genotype at 1000× magnification. T3 seeds of lines growing in T2 were used. The T2 lines analyzed are given in the figure and described in Table 2

Table 2 Ploidy levels of transgenic plants

Construct name	Transformant	Generation of transformants	Inbred generation of starting material	Standard	Ploidy
Utah genotype transformants					
pNATLOX	A300-1-1	T2	6×	NST	2×
pNATNC	A03-412-4-1[a]	T2	15×	HV	2×
pNATPI1	A315-1-5-1	T3	7×	BO	2×
	A339-1-1-1	T3	7×	BO	2×
pRESC2ETR1	A03-328-8-1	T2	14×	NST	2×
	A03-408-7-1	T2	14×	NST	2×
pRESC2NC	A03-09-3-1	T2	14×	BO	2×
pRESC2RCA	A991-2-1-1	T3	13×	BO	2×
	A02-19-4-7-1	T3	13×	BO	2×
	A02-31-9-3-1	T3	13×	BO	2×
	A02-177-4-2-1	T3	11×	BO	2×
pRESC2SYS1	A137-7-x-1	T3	11×	BO	2×
	A148-3-x-1	T3	11×	BO	2×
	A160-5-x-1	T3	11×	BO	2×
pRESC2SYS2	A430-14-x-1	T3	14×	BO	2×
pRESC2TFN	A02-489-x-x-1	T3	14×	NST	2×
	A02-491-x-x-1	T3	14×	NST	2×
	A784-x-x-1	T3	12×	NST	2×
	A793-x-x-1	T3	12×	NST	2×
pRESC5LOX	A03-499-1	T1	15×	NST	2×
	A03-499-3-1	T2	15×	HV	2×
	A03-507-6	T1	15×	NST	2×
	A03-507-6-1	T2	15×	SC	2×
	A03-514-2	T1	15×	NST	2×
	A03-515-3	T1	15×	NST	2×
	A03-533-1	T1	15×	NST	2×
	A03-534-5	T1	15×	NST	2×
	A03-534-5-1	T2	15×	SC	2×
	A03-542-4	T1	15×	NST	2×
	A03-542-5-1	T2	15×	SC	2×
	A03-562-2-1	T2	15×	HV	2×
pRESC5PMT	A03-108-3-1	T2	14×	BO	2×
	A03-108-3-1-1[a]	T3	14×	HV	2×
	A03-145-1-1-1[a]	T3	14×	HV	2×
pSOL3LOX	A04-52-2-1	T2	14×	HV	2×
	A04-57-1-1	T2	14×	SC	2×
	A04-59-2-1	T2	14×	HV	2×
	A04-65-10-1	T2	14×	SC	2×
	A04-67-4-1	T2	14×	HV	2×
pSOL3NC	A04-266-1	T1	14×	HV	2×
pSOL3PIA	A04-141-4	T1	14×	BO	2×
	A04-142-4	T1	14×	BO	2×
	A04-143-5	T1	14×	BO	2×
	A04-160-1	T1	14×	BO	2×
	A04-169-4	T1	14×	BO	2×
	A04-186-1	T1	14×	BO	2×
pSOL4PIA in A03-108-3	A04-103-2	T1	1×	BO	2×
	A04-105-2	T1	1×	BO	2×
	A04-106-2	T1	1×	BO	2×
	A04-107-2	T1	1×	BO	2×
	A04-111-2	T1	1×	BO	2×

Table 2 Continued

Construct name	Transformant	Generation of transformants	Inbred generation of starting material	Standard	Ploidy
	A04-215-1	T1	1×	HV	2×
	A04-216-1	T1	1×	HV	2×
	A04-226-1	T1	1×	HV	2×
	A04-227-1	T1	1×	HV	2×
Arizona genotype transformants					
pRESC2NC	A03 367-2	T1	1×	HV	4×
	A03 389-7	T1	1×	HV	4×
	A04-364-1[a]	T1	1×	HV	2×
pRESC2PIA2	A966-x-x-1	T3	1×	BO	2×
	A981-x-x-1[b]	T3	1×	BO	2×
	A989-x-x-1	T3	1×	BO	4×
	A995-x-x-1	T3	1×	BO	4×
pRESC2RCA	A02-306-12-1-1	T3	1×	BO	4×
	A02-348-8-1-1	T3	1×	BO	4×
	A02-363-1-2-1	T3	1×	BO	4×
	A02-414-2-3-1	T3	1×	BO	4×
	A02-415-1-1-1	T3	1×	BO	2×
	A02-416-7-5-1	T3	1×	BO	4×
	A02-418-5-1-1	T3	1×	BO	2×
	A02-424-14-5-1	T3	1×	BO	4×
	A02-434-16-1-1	T3	1×	BO	2×
	A02-443-4-1-1[c]	T3	1×	BO	2×
	A02-458-8-1-1	T3	1×	BO	4×
	A02-484-3-2-1	T3	1×	BO	4×
	A02-485-2-2-1	T3	1×	BO	2×
	A02-504-4-1-1	T3	1×	BO	2×
pRESC5PMT	A04-108-6	T1	1×	BO	4×
	A04-108-6-1[a]	T2	1×	HV	4×
	A04-139-1	T1	1×	BO	4×
	A04-140-3	T1	1×	BO	4×
	A04-144-3	T1	1×	BO	2×
	A04-145-1	T1	1×	HV	4×
	A04-157-3	T1	1×	BO	2×
	A04-161-3	T1	1×	BO	2×
	A04-189-1	T1	1×	HV	2×
	A04-190-1	T1	1×	HV	2×
	A04-196	T0	1×	BO	4×
	A04-220	T0	1×	BO	4×
	A04-163-4	T1	1×	HV	2×

All transformants were confirmed by their resistance to hygromycin or nourseothricin. The digits beyond the line number are the numbers for a specific individual in a certain generation after T0. Example: A03-328-8-1 is a T2 offspring of transformation event A03-328 (T0). An × means that no number was assigned to the individual that gave rise to the next generation

NST no internal standard, BO *Brassica oleracea* cv. Rosella, *Hordeum vulgare* cv. Sultan, SC *Secale cereale* cv. Petkus Spring

[a]Measured with one cotyledon of an 8-day-old plant

[b]This diploid line has been used for the experiments described in Zavala et al. (2004). All conclusions drawn in this work are due to the experimental conditions described there and not to tetraploidization

[c]Not transformed according to hygromycin-resistance screen

A02-415 ($2n$=24) and A02-416 ($2n$=48, Fig. 1), the former being diploid by flow cytometry and the latter tetraploid (Table 2). A majority of the ploidy determinations of the transgenic lines presented in Table 2 were conducted after hygromycin resistance screens and Southern blot analysis had established that the lines had been transformed.

That none of the 51 transgenic Utah lines were found to be tetraploid was not influenced by the vectors used for the transformation or by the generation of inbred lines used for transformation. Even when the Utah genotype was subjected to two consecutive transformations (pSOL4PIA transformed into A03-108-3, which had been transformed with pSOL3LOX), none of the regenerated lines were tetraploid.

The situation was quite different for transgenic Arizona lines (Table 2): there more than half of the lines were tetraploid. It was not possible to distinguish tetraploid from diploid plants by visually examining external morphological features. The occurrence of tetraploid transformants did not appear to influence the transgenes inserted into the vectors. For example, for more than 10 independently transformed lines that were analyzed after being transformed with vectors pRESC2RCA and pRESC5PMT, the tetraploidization rates were 57 and 50% (Table 3). To rule out the prosaic explanation for transformed tetraploids, namely, that they resulted from naturally occurring Arizona tetraploids among the seeds used for the transformation, we examined the ploidy levels of 22 individual seedlings from

Table 3 Summary of tetraploidy occurrence from Table 2

Genotype	Vector	*N*	Tetraploid number	Tetraploid frequency (%)
Utah	All	51	0	0
Arizona	All	33	18	55
	pRESC2NC	3	2	67
	pRESC2PIA2	4	2	50
	pRESC2RCA	14	8	57
	pRESC5PMT	12	6	50

Table 4 Occurrence of tetraploidy in T0 transformants of Utah and Arizona genotypes of *N. attenuata* containing the vector pRESC5SYS2 (*N. attenuata* pro-systemin gene in an inverted repeat orientiation) and for untransformed seedlings of the Arizona genotype

	N	Tetraploid number	Tetraploid (%)
T0 Utah	23	0	0
T0 Arizona	43	31	72
Wild-type Arizona	22	0	0

Hordeum vulgare cv. Sultan served as internal standard

the seed source used for the transformation; all were found to be diploid (Table 4).

Since all of the Utah lines listed in Table 2 passed the antibiotic screen and were therefore fertile, the lack of tetraploids among the T1–T3 generation Utah may have resulted from the discarding of infertile tetraploid T0 plants during the post-transformation breeding process. To test this hypothesis, we analyzed 23 T0 Utah and 43 T0 Arizona lines that had been transformed with the vector pRESC5SYS2 before the hygromycin resistance test was carried out (Table 4). As we found no T0 Utah lines that were tetraploid, we conclude that our transformation system does not produce tetraploid transformants of the Utah genotype. This experiment also highlighted the efficiency of using flow cytometric analysis before the antibiotic resistance screen for Arizona plants: only 12 of 43 transformants were diploid.

Compared to Arizona explant source, sources of the Utah genotype are from a higher inbreeding generation (6× to 15×) (all 1×, Table 2). To exclude the possibility that the nonoccurrence of tetraploidy for Utah is due to inbreeding, we transformed Utah and Arizona explant sources with vector pRESC2RCA. For both genotypes low and high inbreeding generations were used (Table 5). Unfortunately only one transformant resulted from the transformation of wildtype Utah material, probably due to the nontransformability of the offspring of this specific plant DI04/18. Nevertheless, Table 5 shows that the general statement, "no tetraploid Utah transformants occur," remains true; in contrast, tetraploid plants occurred for both Arizona inbreeding stages. The low percentage of tetraploid of Arizona 1× is probably due to the low *n* and highlights the high variability of percentages which forbids any statistical analysis beyond a statement about whether tetraploidy occurs. Despite this limitation, we can conclude that inbreeding up to the 7th generation played no role in the absence of tetraploidy for the Arizona genotype.

Table 5 Comparison of tetraploidy occurrence after transformation with vector pRESC2RCA for different stages of inbreeding of the explant source

Genotype	Inbreeding generation of explant source	*N*	Tetraploids (%)
Utah	0×	1	0
	17×	19	0
Arizona	1×	11	27
	7×	24	62

Utah 0× are seeds from plant DI04/18 collected in 2004 from the same location where the founder seeds for the Utah inbred line have been collected. *Brassica oleracea* cv. Rosella was used as internal standard

Polysomaty in seedlings and tetraploidization

Polysomaty occurs when nuclei with a higher ploidy level than that found in particular tissues are found and results from endoreduplication of the chromosomal DNA without mitosis and cell division (Joubès and Chevalier 2000). Polysomaty was described half a century ago (Bradley 1954; Swift 1950) but only recently has it been shown to be widespread among herbaceous plants (Barow and Meister 2003) or correlated with the frequency of polyploidy regenerants. When *Lycopersicon esculentum* cv. Moneymaker callus was regenerated (without transformation), 58% of the regenerants were polyploid when hypocotyls were used as explants, 12% were polyploids when cotyledons were used as explants, and only 1.5% were polyploids when leaf explants were used. This correlated well with the percentages of nuclei which were diploid in hypocotyls, cotyledons, and leaves (22, 60, and 93%, respectively) (Bulk et al. 1990). A similar correlation was observed by Sigareva et al. (2004) who both transformed and regenerated three different genotypes of *Lycopersicon esculentum*. Regenerants from hypocotyl explants were 25, 36, and 27% diploid, while regenerants from leaves were 85, 82, 100% diploid (the latter with $n=4$). Hence, the tetraploidization observed in the Arizona lines may result from transformed tetraploid cells in the explant tissues (Bulk et al. 1990; Ellul et al. 2003), and the differences in the degree of polysomaty in the explants of Utah and Arizona genotypes may account for the differences in tetraploid formation.

To test this hypothesis, we measured the percentage of 4C nuclei in hypocotyls, cotyledons, and roots of 8-day-old Utah and Arizona seedlings (Fig. 2; Table 6). The cotyledons of *N. attenuata* harbored 5 and 7% 4C nuclei in Utah and Arizona, respectively, which contrasts with the observations in solanaceaous plants that cotyledons are highly polysomatic (Barow and Meister 2003; Ellul et al. 2003; Sigareva et al. 2004). On the other hand, the high percentage of 4C cells in the roots (58 and 61%; Table 6) is similar to that reported from the roots of *Lycopersicon pimpinellifolium* (Barow and Meister 2003). More importantly, the explant tissues used for the transformation, namely the

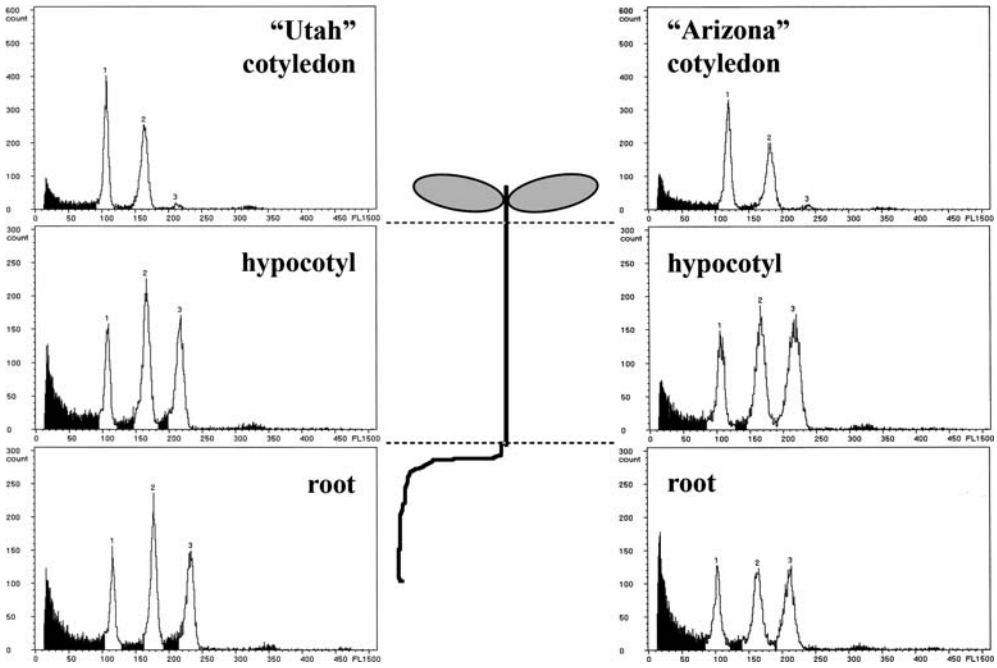

Fig. 2 Histograms of *N. attenuata* seedling tissue: the highlighted area under each peak is the integral of the number of nuclei counted. In order to obtain sufficient nuclei for the analysis, parts of several seedlings were pooled for each measurement (10 hypocotyls, 10 roots, 3 cotyledons). Peaks are numbered: 1, 2C peak of *N. attenuata*; 2, 2C peak of standard *Hordeum vulgare* cv. Sultan; 3, 4C peak of *N. attenuata*

Table 6 Percentage (± SD of three replicates) of 4C nuclei in different parts of 8-day-old *Nicotiana attenuata* seedlings (numbers refer to seedling parts pooled for the analysis)

Genotype	Hypocotyls (10)	Cotyledons (3)	Root (10)
Utah	61% ± 2[a]	7% ± 2	58% ± 4
Arizona	60% ± 4	5% ± 2	61% ± 5

No nuclei with a higher C level were observed

[a]Measurements seen in Fig. 2 were repeated 3 times

hypocotyls, had similar frequencies of 4C cells (60 and 61%, Table 6) in the Arizona and Utah genotypes. A direct relationship between tetraploidization rate in transformants and polysomaty of explant tissue should have yielded a flow cytometric histogram in the Utah hypocotyls similar to the upper left one measured from the Utah cotyledons (Fig. 2). As there was no polysomaty difference between the two genotypes, we can reject a direct relationship between the occurrence of tetraploidization in transformants of a genotype and the polysomaty level in its tissues.

However, our experiments and the results from Ellul et al. (2003) demonstrate that explant genotypes can influence the occurrence of tetraploid transformants. Ellul et al. (2003) reported that 80% of tetraploids resulted from one genotype and 30, 36, 28, and 43% from four others and that these frequencies were only weakly related to the polysomaty of explant tissue. However, Ellul et al. (2003) also showed that small changes in the transformation procedure led to changes in the percentage of tetraploids. Our own experiences (27% for Arizona 1×, Table 5) indicate that tetraploidy frequency varies hugely and to an extent that impairs statistical correlation when genotypes with low and high post-transformation tetraploidy are compared. The advantage of the experimental system introduced here is clear: One genotype with no post-transformation tetraploidy and another with a tetraploidy occurrence that can be detected even among a low number of transformants demonstrate that there is genotypic component in the occurrence of tetraploidy.

Conclusion

The occurrence of tetraploidy during transformation highlights the need to include ploidy tests in post-transformation-screening programs. Our observations suggest that tetraploid plants cannot always be distinguished by morphology, but that flow cytometry provides a rapid and robust determination of ploidy. When genotypes that produce a high percentage of tetraploids are to be transformed, a ploidy screen of T0 plants by flow cytometry can substantially reduce the post-transformation workload.

While in many transformation systems with callus-mediated regeneration, tetraploidy has been observed with different frequencies for different genotypes, the *N. attenuata* transformation system is unusual in having one genotype with no tetraploid transformants. As the clear-cut distinction between occurrence and nonoccurrence is easy to observe without statistical analysis, we were able to rule out three trivial causes: namely, that Arizona explant sources are tetraploid, that inbreeding is the single reason for no Utah tetraploids, and that the transgene triggers tetraploidy. As a fourth possible cause we were able to refute the hypothesis that tetraploidy occurrence resulted from differences in the polysomaty of explant tissue. Since the excluded causes are not directly related to callus-mediated transformation, future research on post-transformation tetraploidy should concentrate on the processes that take place during callus formation and subsequent plant regeneration.

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