Phenotypic, genetic and genomic consequences of natural and synthetic polyploidization of *Nicotiana attenuata* and *Nicotiana obtusifolia*

S. Anssour¹, T. Krügel¹, T. F. Sharbel², H. P. Saluz³, G. Bonaventure¹ and I. T. Baldwin¹,²,*

¹Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Beutenberg Campus, Hans-Knöl-Str. 8, 07745 Jena, Germany, ²Apomixis Research Group, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany and ³Leibniz Institute for Natural Product Research and Infection Biology e.V., Hans-Knöll-Institute (HKI) Beutenbergstr. 11a, 07745 Jena, Germany

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**Background and Methods**

Polyploidy results in genetic turmoil, much of which is associated with new phenotypes that result in speciation. Five independent lines of synthetic allotetraploid *N. × obtusiflora* (*N × O*) were created from crosses between the diploid *N. attenuata* (*Na*) (*C*) and *N. obtusifolia* (*No*) (*O*) and the autotetraploids of *Na* (*NaT*) and *No* (*NoT*) were synthesized. Their genetic, genomic and phenotypic changes were then compared with those of the parental diploid species (*Na* and *No*) as well as to the natural allotetraploids, *N. quadrivalvis* (*Nq*) and *N. clevelandii* (*Nc*), which formed 1 million years ago from crosses between ancient *Na* and *No*.

**Key Results**

DNA fingerprinting profiles (by UP-PCR) revealed that the five *N × O* lines shared similar but not identical profiles. Both synthetic and natural polyploidy showed a dosage effect on genome size (as measured in seeds); however, only *Nq* was associated with a genome upsizing. Phenotypic analysis revealed that at the cellular level, *N × O* lines had phenotypes intermediate of the parental phenotypes. Both allo- and autotetraploidization had a dosage effect on seed and dry biomass (except for *NaT*), but not on stalk height at first flower. *Nc* showed maternal (*Na*) cellular phenotypes but inherited maternal (*No*) biomass and seed mass, whereas *Nq* showed maternal (*Na*) cellular phenotypes but inherited paternal (*Na*) biomass and seed mass patterns. Principal component analysis grouped *Nq* with *N × O* lines, due to similar seed mass, stalk height and genome size. These traits separated *Nc*, *No* and *Na* from *Nq* and *N × O* lines, whereas biomass distinguished *Na* from *N × O* and *Nq* lines, and *NaT* clustered closer to *Nq* and *N × O* lines than to *Na*.

**Conclusions**

Both allo- and autotetraploidy induce considerable morphological, genetic and genomic changes, many of which are retained by at least one of the natural polyploids. It is proposed that both natural and synthetic polyploids are well suited for studying the evolution of adaptive responses.

**Key words:** *Nicotiana*, polyploidy, genome size, DNA fingerprinting, phenotypic variation, Solanaceae.

**INTRODUCTION**

Polyploidy is an ongoing process which has played an important role in the creation of plant diversity; both autoploidy (multiplication of a single genome) and allopolyploidy (fusion of two distinct genomes) are important mechanisms for generating new species (Otto and Whitton, 2000). Some estimates suggest that 70% of angiosperms and 95% of ferns are polyploids (Masterson, 1994; Bennett and Leitch, 1997; Otto and Whitton, 2000), including important cultivated plant species such as alfalfa (*Medicago sativa*), banana (*Musa sapientum*), coffee (*Coffea arabica*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and wheat (*Triticum aestivum*) (Elliot, 1958; Wendel, 2000). Furthermore, recent studies demonstrated that some typical diploid plants such as *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000; Wolfe, 2001) and maize (*Zea mays*; Helentjaris et al., 1988; Gaut and Doebley, 1997) experienced a polyploidization event in their past and are now considered paleopolyploids. Although ubiquitous in nature, the phenomenon of polyploidy requires that during early generations, neopolyploids overcome several genetic obstacles including chromosomal breaks and homologous synapse formation (Pires et al. 2004), increased transposon activity (Madlung et al., 2005), differential gene expression (Comai et al. 2000; Ping et al. 2008) and epigenetic changes (Madlung et al., 2005; Chen, 2007).

The prevalence of polyploids in plants likely reflects the evolutionary and ecological advantage of having extra gene copies. By increasing gene copy number and therefore functional redundancy, polyploidy releases selective constraints on the redundant genes, allowing them to accumulate new mutations and potentially new functions which in turn could improve fitness (Jiang et al., 1998). Importantly, polyploidy affects gene regulatory and expression networks (Pires et al., 2004; Schranz and Osborn, 2004), leading to dosage variation among phenotypic traits (proportionally increasing phenotypic traits) and/or to the emergence of new phenotypes that may contribute to speciation and the exploitation of new ecological niches (Donald, 2004). Compared with their progenitors, polyploids can develop different morphological, ecological, cytological and physiological phenotypes (Levin, 1983; Ramsey and Schemske, 2002; Chen, 2007; Gaeta et al., 2007).
Given the usefulness of polyploids for agriculture, it is not surprising that polyploidy has regained the interest of researchers during the last few years (Chen et al., 2007) with new efforts on Arabidopsis thaliana (Comai et al., 2000; Pontes et al., 2004), Brassica spp. (Luken et al., 2006; Gaeta et al., 2007), cotton (Jiang et al., 1998; Liu et al., 2001), Nicotiana spp. (Lim et al., 2006; Pearse et al., 2006) and wheat (Han et al., 2003). Among these, Nicotiana is perhaps the most extensively studied genus at the genetic, genomic and phenotypic levels. This is mainly due to its robust phylogenetic framework (Chase et al., 2003; Clarkson et al., 2004): 35 of the 75 total species are recognized as alloploids (Clarkson et al., 2004; Leitch et al., 2008). Studies of Nicotiana species have provided critical information on the genetic and genome evolutionary influence of polyploidy on gene conversion, sequence elimination events, rDNA loci changes, transposon activation, tandem and dispersed sequence evolution (Kovarik et al., 1996, 2004, 2008; Clarkson et al., 2004; Melayah et al., 2004; Lim et al., 2006; Petit et al., 2007), chromosomal rearrangements, chromosomal breaks, homologous synapse formation and genome size evolution (Kenton et al., 1993; Kitamura et al., 1997; Chase et al., 2003; Lim et al., 2004; Leitch et al., 2008). More recently, species of this genus were used to study the evolution of polygenic defences (Lou and Baldwin, 2003; Qu et al., 2004; Pearse et al., 2006; Wu et al., 2006). An interesting genome evolution study was carried out by Leitch et al. (2008), which demonstrated that the allotetraploids N. clevelandii (Nc) and N. quadrivalvis (Nq) underwent a genome upsizing (increase in genome size) of 2.5 % and 7.5 %, respectively, during their evolutionary history. However, whether the uniparental DNA sequence elimination and genome upsizing occurred during polyploid speciation or were the result of early and drastic genomic changes (within the initial generations) during polyploidization remains unknown.

In this study, five independent lines of the allotetraploid N. × obtusiflora (N × o) were synthesized by crossing N. attenuata (Na) with N. obtusifolia (No) to simulate Nc and Nq alloploidy systems. Nc and Nq are allotetraploids derived from amphidiploidy involving two diploid species, an ancestor of N. attenuata as the paternal genome donor and an unknown maternal genome donor (Goodspeed, 1954). Recent advances in plastid DNA (Clarkson et al., 2004) and plastid (Zheng et al., 2004) phylogenetic and molecular clock analyses show that an ancestor of N. obtusifolia was the missing maternal genome donor. Two different polyploidization events involving Na and No ancestors led to the formation of Nc and Nq approx. 1 million years ago (Chase et al., 2003; Knapp et al., 2004). Na is an annual plant found in the Great Basin Desert and north along the Sierra Mountains into California and Oregon, whereas No is a perennial plant found in Mexico and south-western USA. Both Na and No have distinct cytological and morphological characteristics (Goodspeed, 1954). Nq and Na are annual plants found in sandy washes along the California coast, and in drier habitats in southern California, respectively (Goodspeed, 1954).

To infer the evolutionary dynamics that occurred during Nc and Nq polyploidization events, genetic, genomic and morphological changes generated after re-synthesizing Nc and Nq were examined. Because allopolyplody is usually accompanied by a genome automultiplication step, these changes were also compared with those of synthetic autotetraploids of Na and No.

MATERIALS AND METHODS

Plant growth

Seeds of all species were germinated and plants were grown as described by Krügel et al. (2002). Briefly, seeds were sterilized for 1 h with 0.1 m M gibberellic acid, and germinated on sterile agar with Gamborg B5 (Duchefa, St Louis, MO, USA) with 26 °C/16 h 100 % light and 24 °C/8 h dark. Na seeds were soaked in 1:50 (v/v) diluted liquid smoke; however, the other species studied did not require this treatment to synchronize their germination. After 10 d, plants were transferred into soil in Teku pots. Once established, plants were transferred to 1-L pots in soil and grown in a glasshouse at 26–28 °C under 16 h supplemental light from Philips Sun-T Agro 400 Na lights (Eindhoven, The Netherlands).

Confirmation of polyploid formation and breeding

Na seeds were collected from a native Utah population (Baldwin et al., 1994) and subsequently inbred for 17 generations. No seeds were collected in 2004 at the Lytle ranch preserve (Saint George, UT, USA) and inbred for one generation. Seeds of Nq and Nc were kindly supplied by Dr Verne A. Sisson (Oxford Tobacco Research Station, Oxford, NC, USA) and originally collected by Goodspeed (1954).

Synthetic allotetraploidization. Reciprocal crossings between Na and No were attempted; for this, unopened flowers of Na (or No) were emasculated 1 d before anthesis. The next day, pollen from a freshly dehisced anther of the selected No (or Na) plant was applied to the exposed stigma. Unfortunately, only crosses of Na (♂) to No (♀) produced viable embryo and endosperm. Attempts to reverse-cross [No (♀) to Na (♂)] in vivo and in vitro were not successful. This result is probably due to the size differences between Na and No styles. Indeed, Na’s style is longer than that of No, suggesting that No’s pollen tube might not reach Na’s ovary. A recent study demonstrated that the length of the style influences the success of interspecific crosses amongst different Nicotiana species in the section Alatae; pollen tubes from members of short pistil species could only grow to a distance proportional to, but not greater than, their own pistil lengths. Therefore, the fertilization success of males from short pistil species is dramatically reduced when they are crossed with females from long pistil species (Lee et al., 2008). Another explanation could be that the observed embryo abortion is caused by genetic barriers present in Na. Indeed, pre- or post-fertilization incompatibility occurs quite often when breeding two different species or genera and obstructs the hybridization of several species including important crops such as rice (Suputtittada et al., 2000).

Viable embryo and endosperm produced through crosses between Na (♂) and No (♀) were rescued using the ovule culture method of Chung et al. (1988) with some modifications. Briefly, the swollen capsules were removed from the plants at various intervals following pollination, and the surfaces of the
ovaries were sterilized for 5 min in 5 mL aqueous solution of 0.1 g dichloroisocyanuric acid (Sigma-Aldrich, Steinheim, Germany), supplemented with 50 μL of 0.5 % (v/v) Tween-20 (Merck, Darmstadt, Germany) and rinsed three times in sterile water. Individual ovules were then carefully removed from ovaries and distributed over the medium in Petri dishes. The medium was the same as that used by Chung et al. (1988), but with no mannitol and 4 % sucrose. The plates were then maintained in a growth chamber (Percival, Perry, IA, USA) at 26 °C (16 h light (155 μm s⁻¹ m⁻²), 8 h dark). Germination started with the emergence of cotyledons 11 d after the ovule rescue procedure. After the first roots emerged, plants were transplanted to a new medium containing 0.292 g L⁻¹ Peters Hydro-Sol (Scotts, Heerlen, The Netherlands), 103.1 mg L⁻¹ MS-vitamins and 6 g L⁻¹ plant agar (both Duchefa, Haarlem, The Netherlands). Resulting seedlings that had strong roots were planted into soil [Tonsubstrat (Klasmann–Deilmann, Geeste-Groß, Hesepe, Germany) and chunky sand 9 : 1] in magenta boxes (77 x 77 m). Resulting seedlings at the cotyledon stage were treated while in the cotyledon stage for 24 h with 0.3 % and 0.6 % colchicine, respectively. Viable tetraploid seedlings were then potted and grown in the glasshouse as described above. Viable allopolyploids were subsequently inbred for five generations. It is important to mention that No tetraploids were inbred for four and five generations, respectively.

Genome analysis

Genome size measurement. DNA content was examined in ten seeds from each line in a Partec Flow Cytometer PA (Partec, Münster, Germany) using the flow-cytometric seed screen method (Matzk et al., 2000) and Poa pratensis as internal standard. Briefly, seeds were loaded with two metal balls (3 mm diameter) into each well of a deep-well (2 mL) plate. To this was added 80 μL of OTTOI buffer [0.1 M citric acid monohydrate, 0.5 % (v/v) Tween-20 (pH 2–3)], and the plate was shaken to grind the seeds on a GenoGrinder 2000 for 1 min (50 rpm). Then, 2 mL of OTTO I was added to the grinded seed/buffer mixture and filtered through a 50-μm mesh. Finally, 2 mL of OTTO II buffer [0.4 M Na₂HPO₄, 12H₂O, 2 mL DAPI solution (pH 8.5)] was added to the filtered solution and stored at a cold temperature in the dark before measurement.

UP-PCR DNA fingerprinting. Leaf material (approx. 2 g) was collected from rosette-stage leaves of Na, No, Nc, Nq and N x o (lines 1–5, F₅) from two individuals per species. DNA was extracted using the Amersham Bioscience plant DNA extraction kit. DNA quality and quantity were determined using a Nanodrop spectrophotometer ND-1000 (PegLab Biotechnologie GMBH, Erlangen, Germany). Then, UP-PCR reactions as described by Bulat et al. (2000) were performed in 10 μL of reaction mix [primers (25 pM), 10 ng of genomic DNA, 1 X Flexi buffer (Promega), MgCl₂ (4 mM), dNTPs (0.2 mM)] using seven universal primers (Table S1 in Supplementary data available online). The amplification cycle, run on Verti™ 96-well thermal cycler (Applied Biosystems, Foster City, MO, USA) consisted of an initial denaturation step at 94 °C for 2 min, followed by two cycles at 94 °C for 30 s; (45–60 °C) for 10 s and 72 °C for 1 min, then 35 cycles of 94 °C for 5s; (45–60 °C) for 5 s and 72 °C for 35 s and a final 2 min extension step at 72 °C. The annealing temperatures were ranged according to the sequence of the primers. The PCR products were separated on 8 % (w/v) polyacrylamide gels (16 × 16 cm) using vertical electrophoresis (SE 600) (Hoefer, Holliston, MA, USA). The gels were stained with ethidium bromide and photographed under UV light (210–285 nm).

Phenotypic characterization

Photographs were taken with a Canon D30 digital camera, and seed micrographs were taken using SPOT software (Visitron System, Puchheim, Germany) on a dissecting microscope (Axioskop, Zeiss, Jena, Germany).

Cellular phenotype measurements. Guard cell length, epidermal cell area and stomata density were measured for all species on fully expanded rosette-stage leaves. For each species, strips of lower epidermal peels from the middle portion of the leaves were removed with fine forceps. Peels were mounted in drops of distilled water on a glass slide and visualized under an inverted microscope for transmitted light (Axiovert 200M, Zeiss, Jena, Germany). Flat portions of peels were identified and photographed. Guard cell length (n = 30), stomata density (n = 10) and epidermal cell area (n = 20) were measured on leaf epidermis using the Axio Vision LE software.

Seed biomass, plant dry biomass and stalk height measurement. Seed mass (from samples of ten seeds weighed to the nearest 10 μg), dry biomass (plants at bolting stage) and height at first flower were measured on 25–30 individuals of Na, No, N x o (lines 1–5, F₅), NqT (F₅), NoT (F₅), Nq and Nc.

Statistical analysis

Quantitative phenotypic traits (length of guard cells, area of epidermal cells, density of stomata, stalk height at first flower, seed and dry plant biomass) were analysed with unpaired t-test using Statview (SAS Institute, Cary, NC, USA). Principal component analysis (PCA) was conducted using the TIGR Multiple Array Viewer software package (TMev version 4.0).

For the analysis of UP-PCR DNA fingerprinting profiles, a numerical matrix was generated by recording all DNA markers. A binary code was then used to express the presence (1) or absence (0) of these markers. For analysis, only DNA
markers present in both replicates from each line were considered.

RESULTS

Botanical description

Nicotiana attenuata (Na) is an annual diploid plant found in the Great Basin Desert and north along the Sierra Mountains into California and Oregon, USA. *Nicotiana obtusifolia* (No) (syn. *N. trigonophylla* Dunal) is a perennial diploid plant found in the desert of the south-western United States and Mexico. *Na* (♂) and *No* (♀) were used to produce five independent lines of synthetic allotetraploids *N. ×* obtusiata (*N × o*). *Na* tetraploid (*NaT*) and *No* tetraploid (*NoT*) are synthetic autotetraploids obtained by treating *Na* and *No* with 0.3 % and 0.6 % colchicine, respectively (Fig. 1). *Nicotiana quadrivalvis* (*Nq*; syn. *N. bigelovii* pursh) and *N. clevelandii* are natural allotetraploid descendants of the ancestors of *Na* and *No*, formed approx. 1 million years ago (Leitch et al., 2008) and found in the eastern part of California.

Flowers. *Na* flowers open at twilight but close by midday. *Na* corolla limbs are white and cleft into acute lobes. In contrast, *No* flowers remain open all day long; their corolla limbs are dull white and have a circular, crenate shape. *NaT* and *NoT* flowers retained the corolla shape and colour, tube length and opening rhythm of *Na* and *No* flowers, respectively. *N × o* lines 2–5 retained the shape and colour of intermediate parental corolla limbs, but flowers of *N × o* line 1 retained the shape and colour of *Na* corolla limbs (Fig. 2). Flowers of *N × o* lines 1–4 inherited the length of *Na*, whereas flowers of *N × o* 5 inherited that of *No*. The corolla limbs of synthetic autotetraploids were about 1.5-fold larger than those of their diploid counterparts (Fig. S1 in Supplementary data, available online), while those of the allotetraploids were 1.7-fold larger than those of *Na*. *Nq* corolla limbs are white and cleft into broadly triangular-ovate sub-acute lobes, whereas *Nc* corolla limbs are cream-coloured and cleft into broadly ovate unequal lobes (Fig. 2). Compared with *Na* and *No* flowers, *Nq* flower tubes were 1.4-fold longer than those of *Na* and corolla limbs were 3 times as large as those of *Na* (Fig. S1). Both synthetic and natural polyploid flowers retained the opening rhythms of *Na* flowers.

![Breeding scheme of N. × obtusiata lines 1–5 and autotetraploids of N. attenuata and N. obtusifolia. Allotetraploids: five emasculated N. obtusifolia (No) flowers were pollinated with excised N. attenuata (Na) anthers. Seedlings were then produced using the ovule culture method as described by Chung et al. (1988). Seedlings of the F₁ hybrid were treated with 0.6 % colchicine to produce fertile plants. All the lineages were inbred for five generations. Autotetraploids: NaT and NoT were generated by treating their diploids with 0.3 % and 0.6 % colchicine, respectively. Na and No tetraploids were inbred for four and five generations, respectively.](https://academic.oup.com/aob/article-abstract/103/8/1207/178637)
Seeds. Na seeds are grey-brown and reniform shaped, with a fluted-reticulate surface, whereas those of No are brown rhomboidal with finely wavy ridges on their surfaces. NaT and NoT seeds retained the colour, shape and surface appearance of Na and No seeds, respectively. N × o lines 1–5 seeds retained the intermediate parental colour but kept the seed shape of Na seeds. N × o lines 1–4 seeds retained the surface appearance of Na seeds, whereas N × o line 5 retained that of No (Fig. 2). Seeds of autotetraploids were twice as large as those of their diploid counterparts (Fig. S1). Nc and Nq seeds are brown and rotund, as well as reniform shaped with fluted-recticate and fluted-plicate ridged surfaces, respectively (Fig. 2). Allo-tetraploid seeds are about as large as those of the parents added together; seeds in Nq, Nc and N × o lines 1–5 are about 1.5-fold larger than those in Na and No (Fig. S1).

Leaves. Rosette-stage leaves of Na are ovate-elliptic, oblong with long petioles, whereas those of No are elliptical with short-winged petioles. Autotetraploidization did not produce any changes in the shape and size of the leaves of Na and No. N × o lines 1–5 produced leaves with long petioles and intermediate parental phenotypes. Nq and Nc produced ovate-elliptical leaves with long and short petioles, respectively (Fig. 3). Nq, N × o lines 1–5 leaves are as long as those of Na, whereas Nc leaves are as long as those of No (Fig. S1).

Variation among genome sizes after polyploidization

Genome size (C-value) was measured by flow cytometry in dry seeds using Poa pratensis (4.24 pg) as internal standard. Na and No genome sizes were 3.31 pg and 1.46 pg, respectively. Autotetraploidization induced a genome size dosage effect: the genome sizes of NaT (5.98 pg) and NoT (2.64 pg) were 1.8- and 1.6-fold the genome sizes of their diploid counterparts (Fig. 4). The analysis performed on four successive generations of N × o line 2 revealed that genome size remained stable (Fig. S2 in Supplementary data, available online). Both synthetic and natural allotetraploidization resulted in an increase of genome size. N × o (lines 1–5; average genome size of 4.77 pg), and the genome sizes of Nc (4.74 pg) were similar to the theoretical size of 4.77 pg (calculated sum of Na and No genome sizes); however, only Nq (4.84 pg) was associated with a genome upsizeing of 1.5 % compared with the theoretical size (Fig. 4).

Variation among DNA fingerprinting profiles after polyploidization

Polyploidy is frequently associated with a genetic rearrangement: parental DNA fragments may be gained or lost, or new fragments may appear (Song et al., 1995; Feldman et al., 1997). To determine the extent of genetic modification...
data, available online), which are mostly additive of the analysis revealed that all the synthetic hybrid lines share occurring after synthetic and natural polyploidization, a UP-PCR analysis was performed using seven universal primers and two biological replicates for each species. This analysis revealed that all the synthetic hybrid lines share similar but not identical profiles (Fig. S3 in Supplementary data, available online), which are mostly additive of the parental profiles; they retained on average 70% of the specific markers from Na and 28.5% of those from No. Nq and Nc retained a similar percentage of both parental markers. Nq contained 29.5% of the markers from Na and 21% of those from No, whereas Nc contained 33% of Na’s markers and 23.5% of No’s. New DNA markers accounted on average for 1.5% in N × o lines, whereas in the natural polyploids, they accounted for 49.5% and 43.5% of Nq’s and Nc’s markers, respectively (Fig. 5A). Principal component analysis (PCA) based on DNA markers present in each species. A numerical matrix for PCA was generated by recording all DNA markers. A binary code was used to express the presence (1) or absence (0) of these markers. UP-PCR DNA fingerprinting profiles were generated for each species using two biological replicates and seven universal primers. For analysis, only the DNA markers present in both biological replicates were considered. Percentages of total variance explained by PC 1 and 2 are 55% and 40.5%, respectively.

**Variation among phenotypic traits after polyploidization**

Phenotypic variation often arises with the formation of polyploids and might contribute to their success in nature (Osborn

**Fig. 3. (A) leaves and (B) rosette-stage plants of N. attenuata (Na), N. attenuata autotetraploid (NaT) (F3), N. obtusifolia (No), N. obtusifolia autotetraploid (NoT) (F3), N. × obtusiata (N × o) (lines 1–5), F3, N. clevelandii (Nc) and N. quadrivalvis (Nq).** N × o (lines 1–5) leaves have long and short petioles, respectively. Synthetic polyploids rosette-stage plants develop approx. 3 d before either parent (photographs were taken at the same stage).

**Fig. 4.** Genome sizes (mean C value in pg DNA ± s.e.) of N. attenuata (Na), N. attenuata autotetraploid (NaT) (F3), N. obtusifolia (No) (F3), N. obtusifolia autotetraploid (NoT) (F3), N. × obtusiata (N × o) (lines 1–5, F3), N. clevelandii (Nc) and N. quadrivalvis (Nq). Genome size was determined in ten seeds of each species using flow cytometric analysis of a single seeds. Significant differences were calculated using unpaired t-test: *, significantly different from Na (P < 0.05); +, significantly different from No (P < 0.05).

**Fig. 5.** Analysis of UP-PCR DNA fingerprinting profiles of N. attenuata, N. obtusifolia, N. × obtusiata (lines 1–5), N. clevelandii (Nc) and N. quadrivalvis (Nq). (A) Percentages of Na- and No-specific DNA markers and new markers present in each species. DNA markers unique to Na or No were first identified and then recorded in the autotetraploid. DNA markers present only in the autotetraploid but not in Na and No were considered new. (B) Principal component analysis (PCA) based on DNA markers present in each species. A numerical matrix for PCA was generated by recording all DNA markers. A binary code was used to express the presence (1) or absence (0) of these markers. UP-PCR DNA fingerprinting profiles were generated for each species using two biological replicates and seven universal primers. For analysis, only the DNA markers present in both biological replicates were considered. Percentages of total variance explained by PC 1 and 2 are 55% and 40.5%, respectively.
Polyploidy generates individuals that frequently out-compete sympatric progenitor species. The success of polyploids is partly attributed to genomic changes which confer phenotypic plasticity (Leitch and Leitch, 2008). Leitch et al. (2008) demonstrated that genomes of several established Nicotiana

et al., 2003). In this study, the aim was to determine the phenotypic and evolutionary consequences of synthetic and natural polyploidization of plants, from cellular to higher plant phenotypic scales.

**Cellular-scale phenotypes.** On the cellular level, guard cell length and epidermal cell area in No were 2.5-fold larger than in Na; however, the density of stomata cells in No leaves was 4 times lower than that in Na leaves. The epidermal and guard cells of NaT were about 1.5 and 1.3 times larger than those in Na and No, respectively. In N × o (lines 1–5), values for guard cell length, epidermal cell area and stomata density were between those of Na and No. Nc was characterized by Na-like phenotypes (the length of guard cells, the area of epidermal cells and the density of stomata in Nc were not significantly different in Na), whereas Nq was characterized by the phenotypes found in No (these phenotypes, for the length of guard cells, the area of epidermal cells and the density of stomata, were not significantly different in No; Fig. 6).

**Plant-scale phenotypes.** Measurements of seed mass, dry biomass and stalk height at first flower revealed that seed and dry biomass in Na were 2-fold more than in No, and height at first flower in Na was about 2-fold higher than in No. Seed and dry biomass in NaT and NoT were about 1.5-fold heavier than in Na and No, respectively. Dry biomass in NaT did not significantly differ from that in Na, whereas biomass in NoT was about 1.3-fold that in No. Seed and dry biomass in N × o (lines 1–5) were on average about 1.5 times what they were in Na, but height at first flower in N × o (lines 1–5) did not significantly differ from that in Na. Nq had the seed and dry biomass of Na, whereas Nc had the seed and dry biomass of No. Both Nq and Nc had No’s height at first flower (Fig. 6). Principal component analysis, using the quantitative phenotypes measured on each species, grouped Nq with N × o lines due to the similarity of seed mass, stalk height at first flower and genome size. These traits separated Nc, No and Na from Nq and N × o lines, whereas biomass distinguished Na from N × o and Nq lines. NaT grouped closer to Nq and N × o lines than to Na (Fig. 7).

**DISCUSSION**

Fig. 6. Quantitative phenotypical traits (mean ± s.e.) were measured in N. attenuata (Na), N. attenuata autotetraploid (NaT) (F2), N. obtusifolia (No), N. obtusifolia autotetraploid (NoT) (F2), N. obtusifolia (N × o) (lines 1–5, F2), N. clevelandii (Nc) and N. quadrivalvis (Nq). For each line, guard cell length (n = 30), stomata density (n = 10) and epidermal cell area (n = 20) were measured on leaf epidermis of each species using the Axio Vision LE software. Dry biomass measurements (n = 25–30) were made at the bolting stage. Seed mass (n = 300) and stalk height at first flower (n = 25–30) were also measured for each line. Significant differences were calculated using unpaired t-test: *, significantly different from Na (P < 0.05); †, significantly different from No (P < 0.05).
polyploids experienced genome expansion (e.g. via DNA retro-element insertion) or shrinking (e.g. via DNA sequence elimination) during their evolutionary histories. *Nicotiana clevelandii* (*Nc*) and *N. quadrivalvis* (*Nq*) in particular experienced inter-genomic mixing of parental genomes and genomic DNA expansion (Lim et al., 2006; Leitch et al., 2008). Eliminating DNA sequences also altered the defence system in *Nc* and *Nq*; Lou and Baldwin (2003) demonstrated that *Nq* and *Nc* retained different components of the parental JA-mediated defence system; most aspects of the recognition response were retained by *Nq* but lost in *Nc*. Furthermore, using cDNA, intron and promoter sequence analyses, Wu et al. (2006) showed that maternally (*No*) inherited trypsin-protease inhibitor genes were retained in both *Nc* and *Nq*, whereas paternally inherited (*Na*) trypsin-protease inhibitor genes were deleted. However, whether these changes resulted from *Nc* and *Nq* formation, directly after polyploidization or through long-term evolutionary modifications associated with speciation, cannot be uncovered by studying established lines. Only by comparing re-synthesized allopolyploids with their parental species can one evaluate this question. Here, an attempt was made to re-synthesize *Nc* and *Nq* by crossing *Na* and *No* and to synthesize the autotetraploids of the parental lines. A comparative analysis of the genetic, genomic and phenotypic changes that occurred in the synthetic and natural polyploids of this hybridization event has given us three insights into the evolutionary dynamics associated with polyploidy.

First, genome size (C-value) measurements performed on dry seeds of *Na*, *No*, *NaT*, *NoT*, *N × o* (1–5), *Nc* and *Nq* revealed that both synthetic and natural polyploidization have a dosage effect on genome size. The genome sizes of *NaT* (5.98 pg) and *NoT* (2.64 pg) were about 1.6 and 1.8 times the genome sizes of *Na* (3.31 pg) and *No* (1.46 pg), whereas the genome sizes of *N × o* (lines 1–5) (average size, 4.77 pg) were the sizes of the parents’ genomes combined. In contrast to other lines, *N × o* line 4 showed relatively large intra-population variation in genome size, suggesting that synthetic allopolyploidy induced differential genome stability among *N × o* lines; alternatively, the F1 generation of *N × o* line 4 may have had aberrant segregation of its chromosomes, which in turn may have resulted in an unstable karyotype among the progeny of this line. The genome sizes of *Nc* and *Nq* were 4.78 pg and 4.98 pg, respectively (Fig. 4). Compared with theoretical genome sizes [multiples of parental genome sizes: *NaT* theoretical (6.62 pg), *NoT* theoretical (2.92 pg)], the observed genome sizes of *NaT* and *NoT* are 9.7 % and 9.6%, respectively, smaller than expected, suggesting that possible chromosomal aberration (such as aneuploidy) might have occurred during synthetic autopolyploidization of both species; such genomic abnormalities are caused by meiotic irregularities which occur frequently during polyploidy. A recent example of aneuploidy was revealed in Lim et al. (2008). The authors showed that imbalances in parental chromosome contributions and inter-genomic translocation were frequent in several newly synthesized *Tragopogon* allopolyploids; in one case, a complete parental chromosome was lost.

Among the allopolyploids, only the size of *Nq*’s genome was associated with an increase (+1.5%), when compared with theoretical genome size [calculated sum of *Na* and *No* genome sizes (4.77 pg)]. A possible explanation for this DNA expansion may stem from the GISH results performed by Lim et al. (2007), which showed that the genome evolution in this polyploid has been accompanied by increases in the number of existing repeats. A difference in the factors controlling these mechanisms may explain why *Nc*’s genome size, unlike *Nq*’s, has remained unchanged during evolution. Alternatively, a difference in their respective ecological niches and in the availability of soil nutrients such as nitrates and phosphates, which are essential for DNA synthesis, may explain the expansion (Leitch and Bennett, 2004; Leitch and
Leitch, 2008). However, the results shown here differ from those published by Leitch et al. (2008) in which the increase of the genome expansion of \(N_q\) and \(N_c\) was estimated as +7.5\% and +4.5\%, respectively. The discrepancy in the results is due to a difference in the estimation of \(N_a\) genome size: 2.5 pg in Leitch et al. (2008) and 3.5 pg shown here, which leads to different \(N_c\) and \(N_q\) genome expansion estimations. The difference in the estimation of \(N_a\) genome size might be due to a difference in \(N_a\) accessions used by different groups. In addition, an effect of intensive inbreeding of \(N_a\) on genome size cannot be excluded. This difference in genome size estimations highlights the danger of inferring the genome size of polyploids from their diploid parental progenitors.

Secondly, the examination of DNA fingerprinting profiles using UP-PCR showed that synthetic and natural allotetraploids displayed different ratios of parent- (\(N_a\) and \(N_o\)) specific DNA markers and new markers. \(N \times o\) (lines 1–5) displayed an average of DNA fingerprinting profiles consisting of 70\% parent-specific markers from \(N_a\) and 28.5\% from \(N_o\), and 1.5\% of new markers (Fig. 5A). These results demonstrate that after five generations, synthetic allotetraploids undergo a certain amount of recombination which results in the loss and gain of new DNA markers. \(N \times o\) lines show almost entirely additive DNA fingerprinting profiles of the two parental genomes. The predominance of \(N_a\)-specific markers (\(N_a\) markers are twice as frequent as those of \(N_o\)) in \(N \times o\) DNA fingerprinting profiles reflects the DNA content from \(N_a\) in the \(N \times o\) genome (\(N_a\)'s genome size is almost twice as large as \(N_o\)’s).

Although formed from independent \(N_a\) and \(N_o\) crossings, the five lines of \(N \times o\) show similar, but not identical DNA fingerprinting profiles, reflecting the efficiency of UP-PCR (PCR performed with long primers), which, in contrast to the standard RAPD analysis (PCR performed with short primers), produces reproducible DNA fingerprinting profiles. Analysis of the DNA profiles of natural polyploids revealed that both \(N_q\) and \(N_c\) have similar percentages of \(N_a\)- and \(N_o\)-specific markers. \(N_q\) inherited 29.5\% of \(N_a\)’s markers and 21\% of \(N_o\)’s, whereas \(N_c\) inherited 33\% of \(N_a\)’s markers and 23.5\% of \(N_o\)’s. \(N_q\) and \(N_c\) showed a high level of recombination; new markers accounted for 49.5\% and 43.5\% of \(N_q\)’s and \(N_c\)’s DNA fingerprinting profiles, respectively (Fig. 5A).

Compared with the \(N \times o\) profiles, those of \(N_q\) and \(N_c\) show a significant reduction in \(N_a\)-specific markers (\(N_a\) markers ratios are twice as infrequent in \(N_q\) and \(N_c\) as in \(N \times o\) lines). A possible explanation emerges from the study carried out by Lim et al. (2007), who concluded that during the evolutionary histories of \(N_q\) and \(N_c\), a massive DNA invasion occurred, involving \(N_a\) subtelomeric repeat sequences onto \(N_o\) chromosomes, followed by their replacement. Such genomic changes may have led to the decline of \(N_a\)-specific markers and the emergence of new ones. Paternal DNA sequence elimination has also been revealed by genetic studies in \textit{Nicotiana} allotetraploids (Skalicka et al., 2005; Wu et al., 2006) and in the grass family (Kotseruba et al., 2003). Another explanation could be that the genomes of the ancestors of \(N_a\) and \(N_o\) that gave rise to \(N_q\) and \(N_c\) have undergone large genomic changes, eventually reducing the number of \(N_a\)-specific DNA makers. Principal component analysis based on DNA markers present in each species revealed that all \(N \times o\) lines grouped closer to \(N_a\) than to \(N_o\), whereas \(N_b\) and \(N_c\) grouped closer to \(N_o\) than to \(N_a\) (Fig. 5B). The method used in this study is an efficient and reliable way to generate DNA fingerprinting profiles to study the genetic changes in the polyploid species; however, the analysis does not take into account modifications intrinsically associated with polyploidy. Indeed, Hegarty et al. (2006), using cDNA microarray, demonstrated that hybridization and polyploidization induce distinct effects on large-scale patterns of floral gene expression of resynthesized \textit{Senecio} polyploids; hybridization induces a ‘transcriptome shock’ which is ameliorated after genome doubling in subsequent generations. Epigenetic changes are also widespread after polyploidization; changes affecting DNA methylation can induce a rapid and stochastic process of differential gene expression during polyploid formation and evolution (Wang et al., 2004).

Finally, phenotypic analysis revealed that, at the cellular level (leaf epidermal cell area, guard cell length, stomata density), \(N \times o\) lines retained intermediate parental phenotypes (Fig. 6). Both synthetic allo- and autopolyploidy resulted in dosage effects on seed and dry biomass (except for \(N_aT\)), but not on stalk height at first flower. This confirms previous studies demonstrating that polyploidy induces an increase in both seed and biomass (Knight and Beaulieu, 2008). Indeed, genome expansion is frequently associated with an increase in a plant’s transcriptome levels, which is subsequently reflected at both the proteome and phenotypic levels.

Cellular phenotypes such as guard cell length and epidermal cell size are usually considered indicative of ploidy level and genome size (Beck et al., 2003; Knight and Beaulieu, 2008). This relationship was clear in the synthetic autotetraploids of \(N_a\) and \(N_o\), but not in the allotetraploids, which showed intermediate parental phenotypes. Compelling evidence suggests that the phenotypic variations occurring after polyploidization are highly dependent on the gene expression regulatory networks for each species (He et al., 2003). The intermediate parental phenotype observed in \(N \times o\) lines might be a consequence of non-additive parental gene regulation, whereas the dosage effect in \(N_aT\) and \(N_oT\) phenotypes could be the outcome of additive parental gene regulation. Recently, Wang et al. (2006) demonstrated that, in contrast to the small effects of genome doubling on gene regulation in autotetraploids, the fusion of two distinct genomes in allotetraploids generated genome-wide non-additive gene regulation. Epigenetic changes may also reprogramme gene expression and developmental patterns of newly synthesized allotetraploids (Chen, 2007), leading to \textit{de novo} phenotypical variations.

Concerning the natural polyploids, \(N_c\) had paternal (\(N_a\)) cellular phenotypes, but inherited maternal (\(N_o\)) seed and dry biomass, whereas \(N_q\) had paternal maternal (\(N_o\)) cellular phenotypes but inherited paternal (\(N_a\)) seed and dry biomass, suggesting that, during polyploidy speciation, one or the other parental phenotypes is retained depending on the ecological requirements of each species. Principal component analysis performed on the synthetic and natural polyploids using the above-mentioned phenotypic traits (Fig. 7) revealed that \(N_q\) and \(N \times o\) (lines 1–5) grouped together due to similar seed mass, stalk height at first flower and genome size. These
traits separated \(N_c\), \(N_o\) and \(N_a\) from \(N_q\) and \(N \times o\) lines, whereas biomass distinguished \(N_a\) from \(N \times o\) and \(N_q\) lines, and \(N_oT\) clustered closer to \(N_q\) and \(N \times o\) lines than to \(N_a\). Because similar phenotypic traits may result from similar metabolic regulation, it may seem that only \(N_q\) (which groups together with \(N \times o\) lines) was re-synthesized but not \(N_c\).

The present study demonstrates that both auto- and allotetraploidization induce significant and complex genomic and phenotypic changes. After five generations, allotetraploids showed a dosage effect on genome size and most of the parental phenotypes, whereas all tetraploids showed a dosage effect on genome size, seed and dry biomass, but retained a combination of \(N_a\) and \(N_o\) DNA fingerprinting profiles and cellular phenotypes. After polyploidy speciation, \(N_c\) and \(N_q\) kept their large genome sizes, but show one or the other parental phenotypes, probably as a result of genetic drift or selection. Given that the newly formed allotetraploid species exists in sympathy with the ecological interactions of the parents, the polyploid probably benefits from maintaining aspects of the parental gene expression programme to meet the ecological challenges of parental environments. More detailed molecular and physiological studies using synthetic polyploids will help clarify functional predictions of this model and to underscore the molecular events that have shaped the evolution of polygenic adaptive traits during polyploidy speciation.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org/ and consist of the following. Fig. S1. Corolla limb size, seed size, flower tube and leaf length in \(N.\) attenuata \((Na)\), \(N.\) attenuata autotetraploid \((NaT)\) \((F_2)\), \(N.\) obtusifolia \((No)\), \(N.\) obtusifolia autotetraploid \((NoT)\) \((F_2)\), \(N.\) \times obtusiata \((N \times o)\) (lines 1–5, \(F_3)\), \(N.\) clevelandii \((Nc)\) and \(N.\) quadrivalvis \((Nq)\). Fig. S2. Genome sizes measured in seeds of \(N.\) obtusiata \((N \times o)\) lines 2 \((F_2\) to \(F_3)\) using flow cytometry for single seeds. Fig. S3. UP-PCR DNA fingerprinting profiles of \(N.\) attenuata, \(N.\) \times obtusiata lines 1–5, \(N.\) quadrivalvis, \(N.\) clevelandii, \(N.\) attenuata and \(N.\) obtusifolia. Table S1. Sequences of universal primers used in UP-PCR DNA fingerprinting profiles of \(N.\) attenuata, \(N.\) \times obtusiata lines 1–5, \(N.\) quadrivalvis, \(N.\) clevelandii, \(N.\) attenuata and \(N.\) obtusifolia.

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