# Functional consequences of natural and synthetic polyploidization of *Nicotiana attenuata* and *N. obtusifolia*- special focus on expressed anti-herbivore response

# Dissertation

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# 1. GENERAL INTRODUCTION

## 1.1. What is polyploidy?

Polyploidy is the condition in which diploid cell or organism acquires one or more additional homologous set of chromosomes (Ohno et al., 1967). There are several categories of polyploids depending on the total number of chromosome sets which is indicated by the prefix, tri- (3), tetra- (4), penta- (5), hexa- (6) and octa- (8) preceding the word 'ploid'. Polyploids arise when rare mitotic or meiotic errors cause the formation of non haploid gametes; for instance, diploid gametes, which arise infrequently, might fuse with ones and produce triploid zygotes. These later are unstable and can either be sterile or contribute to the formation of polyploid gametes, depending on the species (Comai, 2005).

Polyploids can be sorted into two types: autopolyploids or allopolyploids. The former are generated by duplication of single set of chromosomes, as a result of a mutation in chromosome number, whereas the latter by the union of distinct set of chromosomes, as a result of both concurrent hybridization and mutations in chromosome number (Comai, 2005) (Fig.1). In some cases the separation between autopolyploids and allopolyploids can be ambiguous, especially when the chromosome sets of allopolyploids differ proportionally to the divergence of the parental genomes; the closer the parents, the more similar the resulting allopolyploid is to an autopolyploid (Wolf, 2001).

## 1.2. Prevalence of polyploidy in nature

Polyploidy is common in plants with relatively high frequency (1 per 100,000), especially among ferns and flowering plants, including both wild and cultivated species. Estimates suggest that polyploidy is responsible for 2-4% of speciation events in angiosperms and 7% in ferns (Otto and Whitton, 2000; Blanc and Wolfe, 2004). Many of our cultivated plants are derived from autopolyploidy (duplication of single genome), e.g. alfalfa and potato, or from allopolyploidy (union of distinct genomes), e.g. wheat, oat, cotton, coffee and canola (Elliott, 1959; Wendel, 2000). In animals, the frequency of polyploids occurrence is much lower and restricted only to few frog and fish species such as goldfish (Ohno, 1967), salmon, and salamanders, whereas higher vertebrates do not tolerate polyploidy, but still 10% of spontaneous abortions in humans are due to polyploidy (Jaillon et al., 2004).
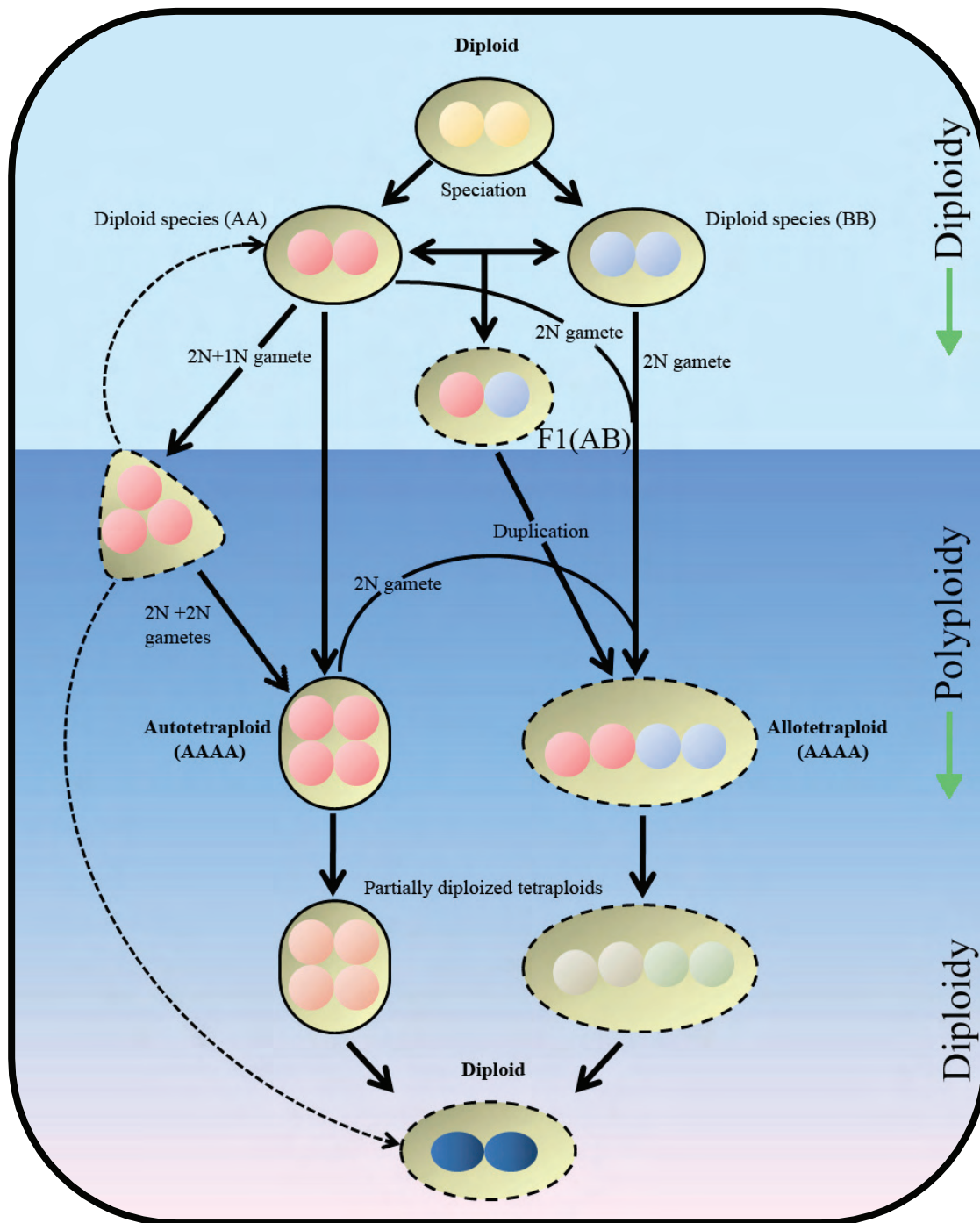
**Figure. 1.** Diagram illustrating the formation of auto and allo-polyploids, and their gradual diploidization.

This diagram was simplified to show only few possible paths that lead to the formation of auto and allopolyploids. New paths can be also drawn to show the contribution of triploid gametes to the formation of allopolyploids. Genomes that are illustrated by ovals reflect the increased gene number. Different colors of circles (or ovals) represent diverged genomes. Dashed nuclear contours represent unstable ploids. A and B represent distinct genome species and N is the gametic type. This figure was modified and adapted from Comai L. (2005).

**1.3. Implications of polyploidy in the evolution of eukaryotic genomes**

In nature, established polyploids undergo an evolutionary trajectory of diploidization to avoid extinction; during this gradual process the genomic redundancy is reduced (Wang et al., 2005). The molecular basis of diploidization is still not clear, but it presumably occurs through genetic alterations that differentiate duplicated genes. In this concern, duplicated genes can be lost, maintained as duplicates, or experience subfunctionalization and neofunctionalization (Fig.1). Bioinformatic and theoretical analyses indicate that these genetic alterations are often not random and strongly affect the outcome of the formed species (Lynch and Conery, 2000; Zhang and Kishino, 2004; Comai, 2005).

Studies of eukaryotic genomes, such as *Arabidopsis* (Arabidopsis Genome Initiative, 2000), revealed that many sequenced diploid genomes display signatures of duplications in their ancestry (Paterson, 2005; Comai, 2005). All Angiosperms probably have experienced at some point in their evolutionary history a polyploidy event, and thus, considered as paleopolyploids. It seems that this evolutionary phenomenon is not only bound to flowering plants; several unexpected such as baker's yeast (*Saccharomyces cerevisiae*), teleost fish and an early ancestor of the vertebrates, show also a polyploidy ancestries (Fig. 2).

The effects of polyploidy on the evolution of higher plant species have been conservative. Particularly, allopolyploidy results in the appearance of radically new gene combinations most of which are unadaptive in stable environments and thus, discarded by natural selection. However, when exposed to changing environment, in which many new ecological niches are being open up, neopolyploids are likely to adapt (Stebbins, 1966). Polyploidy serves also the purpose of stabilizing interspecific hybrids, by reducing the amount of genetic segregations and by reducing infertility. Polyploidy might also generate new phenotypes and contributes in the stabilization and the establishment of neopolyploids in their new habitats.
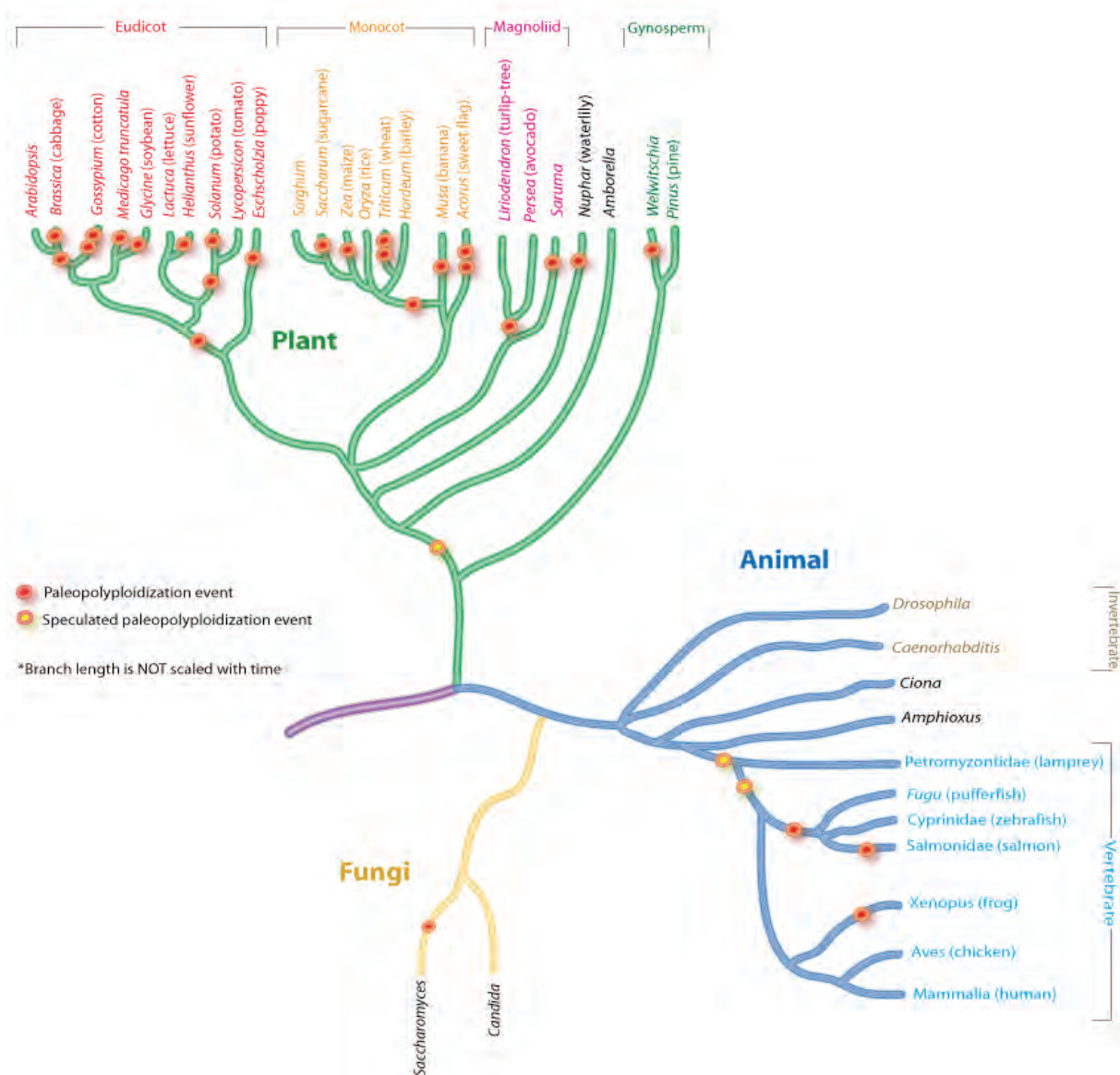
**Figure. 2: Phylogenetic tree of eucaryotes illustrating known paleopolyploidy events.**

Red dots indicate well-supported paleopolyploidy and yellow dots indicate putative cases. Branch lengths were not scaled to time. Figure created by Peter Zhang based on Wolfe (2001), Adams and Wendel (2005), Cui et al. (2006).

**1.4. Polyploidy in the genus *Nicotiana***

Polyploidy is common in the genus *Nicotiana* with approx 35 of the 75 total species recognized as allopolyploids (Clarkson et al., 2004; Leitch et al., 2008), including, *N. tabacum* (section *Nicotianae*), *N. rustica* (section *Rusticae*), *N. arentsii* (section *Undulatae*), *N. clevelandii* and *N. quadrivalvis* (section *Polydicliae*), *N. nudicaulis*, *N. repanda*, *N. nesophila* and *N. stocktonii* (section *Repandae*). All species from the section *Suaveolentes* are polyploids (a total of 23 species). Unlike other *Nicotiana* polyploids which contain 48 chromosomes (the diploid chromosome number for the genus is 2n = 24), the evolution of the polyploids from this section has been accompanied by changes in chromosome number (2n ranges from 32 to 48), probably through chromosome deletions or fusions.

Recently, cytological and morphological evidence combined with plastid and nuclear gene sequence data (Chase et al., 2003; Clarkson et al., 2004), revealed all parental species for nearly all *Nicotiana* allotetraploids. These studies, showed also that most of parental species giving rise to *Nicotiana* polyploids are related to each other; in this concern, both parental species of *N. arentsii* belong to the same section (*Undulatae*) and those of *N. rustica* belong to closely related sections (*Paniculatae* and *Undulatae*) (Clarkson et al., 2004); whereas, the parental species of *N. tabacum* and polyploids from the sections: *Repandae*, *Polydicliae* and *Suaveolentes* are distantly related. More recently, molecular clock analysis conducted by Clarkson et al. (2005) and Kovarik et al. (2008), showed that *Nicotiana* polyploids vary considerably in age; from 200 000 years (*N. tabacum*, *N. rustica* and *N. arentsii*) to 10 Myr (allopolyploids from section *Suaveolentes*).

Up-to-date, studies of *Nicotiana* species have provided critical information on the genetic and the genomic evolutionary impact 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 (Kitamura et al., 1997; Chase et al., 2003; Lim et al., 2004; Leitch et al., 2008). However, there is still a lack of information on how polyploidy influence the expression of functioning physiological systems that allow the polyploids to respond to their natural environment.
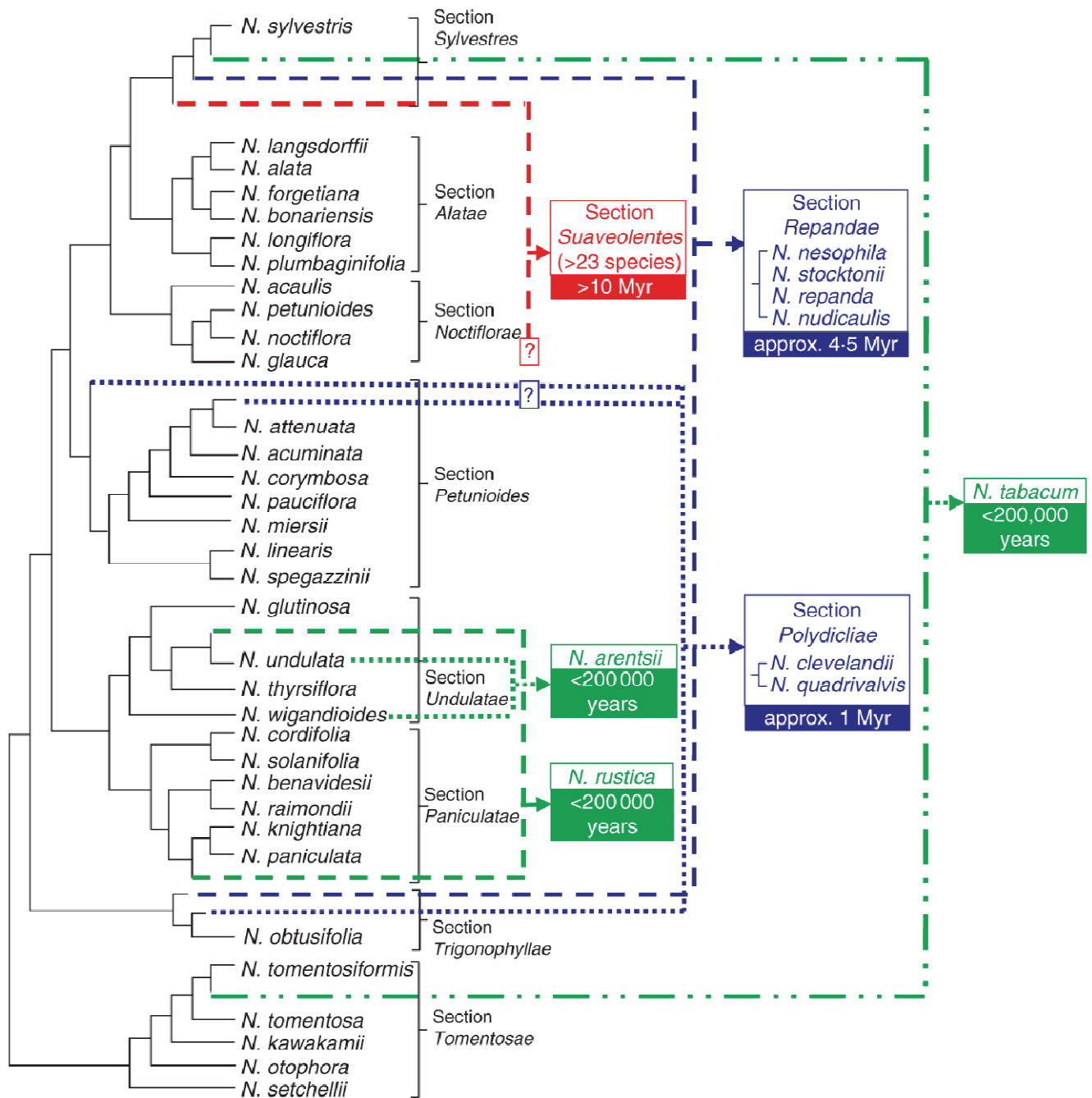
**Figure. 3: Phylogenetic relationships of *Nicotiana* species with proposed origins of polyploids**

Figure modified and adapted by Leitch et al. (2007) using recent phylogenetic information and data from Knapp et al. (2004). Ambiguities concerning one of the parental genome donors for sections *Polydicliae* and *Suaveolentes* are indicated by question marks.

**1.5. The natural *N. quadrivalvis* and *N. clevelandii* allopolyploid system**

*N. quadrivalvis* (*Nq*) and *N. clevelandii* (*Nc*) 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), glutamine synthetase (Qu 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 of years ago (Chase et al., 2003; Knapp et al., 2004). The allopolyploids *Nq* and *Nc* constitute an excellent system to study polyploidy, since their parental diploid species are available with sufficient genetic and molecular information.

The wild tobacco *N. attenuata* is an annual plant occurring in the Great Basin Desert and north along the Sierras into California and Oregon native to the Great Basin Desert, USA. Seeds of *N. attenuata* germinate in nitrogen-rich soil, after being exposed to smoke (Baldwin et al., 1994). *N. obtusifolia* is a perennial plant occurring in Mexico and southwestern USA. Both *Na* and *No* have distinct cytological and morphological characteristics. *Nq* and *Nc* are annual plants found in sandy washes along the California coast, and in drier habitats in southern California, respectively (Goodspeed, 1954).

In nature, both diploid and allopolyploids are attacked by herbivores from more than 20 taxa, including mammalian browsers which can consume entire plants, intracellular sucking and leaf-chewing insects, such as *Manduca sexta* which is the specialist herbivore of *N. attenuata* (Fig. 5).
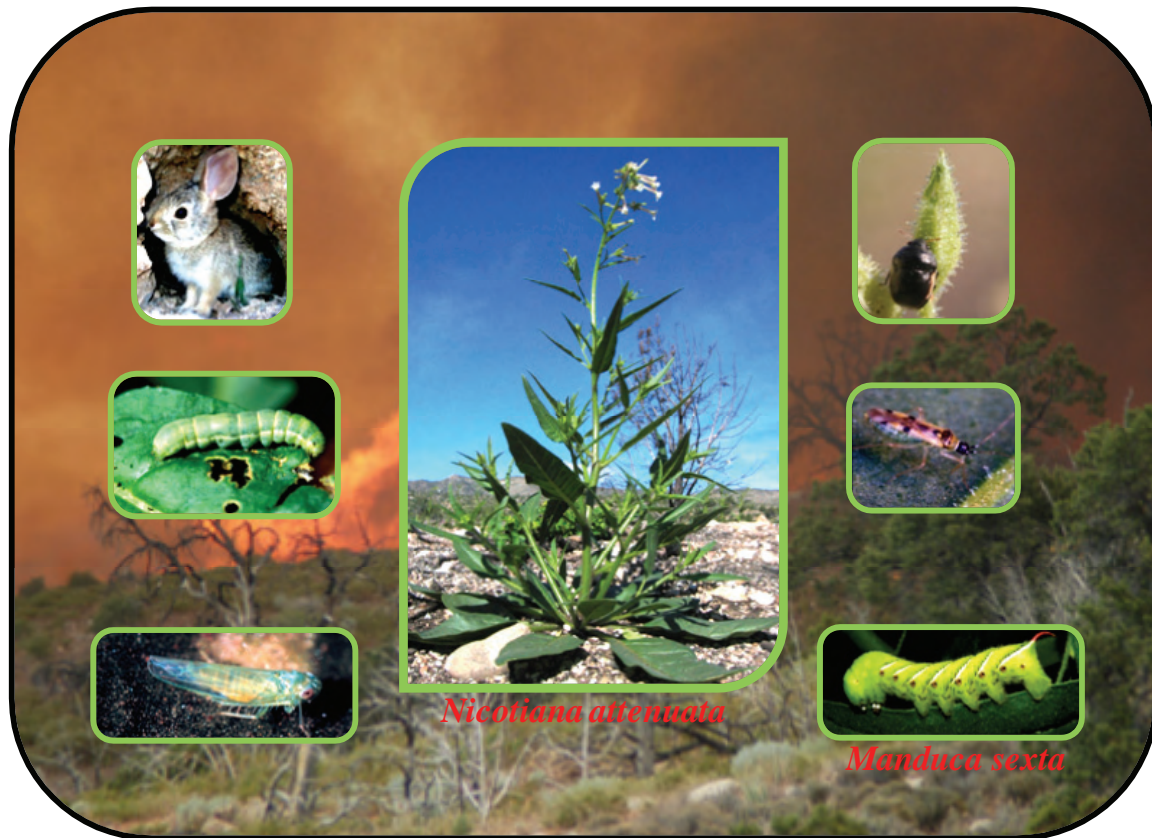
**Figure. 4:** The wild tobacco *N. attenuata* and its native herbivore species. Photo courtesy: D. Kessler.

## 1.6. The defensive response of the model plant *N. attenuata* against its specialist herbivore *M. sexta*

The anti-herbivore responses of *N. attenuata* againt *Manduca sexta*, Solanaceous-specialist lepidopteran herbivore, have been extensively studied in both greenhouse and native **N**orth **A**merican populations for more than a decade (Baldwin, 2001). Recent studies, suggest that *N. attenuata* anti-herbivore response against *Manduca sexta* is highly specific, and a large part of the plant's transcriptome is involved (Hui et al., 2003; Voeckel and Baldwin, 2004). This specificity is achieved during herbivory, when *M. sexta* oral secretions and regurgitants (OS) are introduced into wounds during feeding. In this concern, fatty acid-amino acid conjugates (FACs) from larval oral secretions (OS) bind to hypothetical receptors in the cell membrane at the attack site and activate unknown short-distance mobile signals. These signals activate mitogen-activated protein kinases (MAPKs) that include salicylic acid–induced protein kinases (SIPK) and a wound induced protein kinase (WIPK) which phosphorylate transcription factors (such as WRKYs), that in turn activate phytohormone signaling such as jasmonic acid (JA), salicylic acid (SA) and jasmonic acid–isoleucine (JA-Ile), and their

associated biosynthetic genes such as lipoxygenase 3 (LOX3), isochorismate synthase (ICS) and jasmonate-resistant4 (JAR4).

By inhibiting ICS, non-expressor of pathogenesis-related (NPR1) negatively regulates SA production and thereby SA/JA antagonism, allowing the expression of JA-mediated direct defenses such as nicotine, phenolics, trypsin protease inhibitors (TPIs) that diminish the performance of *M. sexta* larvae on elicited plants. Particularly, TPIs play a central role in this plant-herbivore interaction (Zavala et al., 2004), these inhibitors bind to proteases present in *M. sexta* larvae midgut slowing their growth and increasing their mortality (Glawe et al., 2003) (Fig. 6). Herbivore-elicited TPI activity is a polygenic trait under both transcriptional and post-transcriptional control. JA, SA, ethylene, and abscisic acid are all known for their role in the signaling network that regulates TPI transcription accumulation (Pena-Cortes et al., 1995; O'Donnell et al., 1996; Koiwa et al., 1997; Halitschke and Baldwin, 2003; Horn et al., 2005).
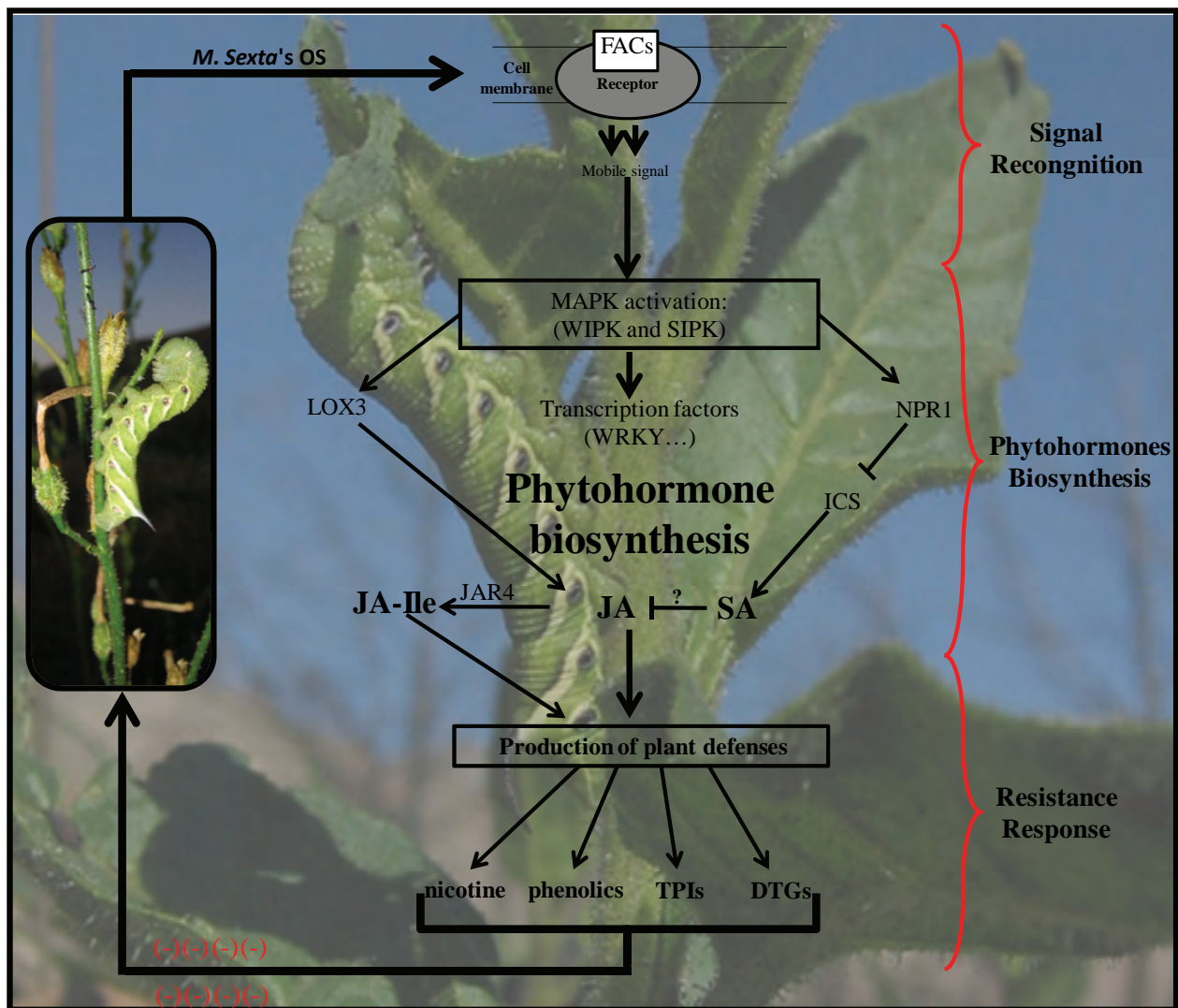


**Figure. 5:** An overview of the OS-elicited signaling cascade that activates direct defenses in *N. attenuata*'s leaves. Photo courtesy: D. Kessler

**3. Chapter I. Phenotypic, genetic and genomic consequences of natural and synthetic polyploidization of *Nicotiana attenuata* and *Nicotiana obtusifolia***

## 3.1. Introduction

The prevalence of polyploids in nature likely reflects the evolutionary and ecological advantage of having extra gene copies. Indeed, 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. (Lukens 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. 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). 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*) derived from amphidiploidy, involving two diploid ancestors, *N. attenuata* (as the paternal donor) and *N. obtusifolia* (as the maternal donor) approx. 2 Myrs ago (Chase et al., 2003), underwent a genome upsizing (increase in genome size) of 2.5 % and 7.5 %, respectively, during their evolutionary history. However, whether the 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, we simulated *Nc* and *Nq* allopolyploid systems, by synthesizing five independent lines of the allotetraploid *N. ×obtusiata* (*N ×o*) [*N. attenuata* (*Na*) (as the paternal donor) × *N. obtusifolia* (*No*) (as the maternal donor)], and autotetraploids of *Na* (*Na*T) and *No* (*No*T). To infer the evolutionary dynamics that occurred during *Nc* and *Nq* polyploidization events, we examined the genetic, genomic and morphological changes that occurred in the synthetic allopolyploids and compred them with those of *Nc* and *Nq*. Because allopolyploidy is usually accompanied by a genome automultiplication step, these changes were also compared with those of synthetic autotetraploids of *Na* and *No*.

## 3.2. Material and methods

### 3.2. 1. 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 mM 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).

### 3.2. 2. 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 (Suputtitada 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 × 77 × 77), maintained in growth chambers [26 °C/16 h, light (200-250 μm s$^{-1}$ m$^{-2}$), 24 °C/8 h dark] and transferred to the glasshouse. The resulting diploid hybrid seeds were germinated; seedlings at the cotyledon stage were treated with 0.6 % colchicine for 24 h to induce polyploidy and thereby restore fertility. Seeds from the colchicine-treated plants were collected and assigned to individual capsules to avoid sampling differences from the potentially chimeric $F_1$ polyploids. Viable allopolyploids were

subsequently inbred for five generations. It is important to mention that $N \times o$ plants from early generations ($F_2$ to $F_4$) produced seed capsules containing a mixture of aborted ovules, empty seed coats and unequally developed seeds, most of which did not germinate. However, the fertility of these plants increased from generation to generation through self-pollination.

### *Synthetic autotetraploidization*

Seeds of *Na* and *No* were germinated until the seedling stage and 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. *Na* and *No* tetraploids were inbred for four and five generations, respectively.

### 3.2. 3. 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 OTTO I 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 $Na_2HPO_4.12H_2O$, 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 $N \times o$ (lines 1-5, $F_5$) 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 (PeqLab 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, 1x Flexi buffer (Promega), $MgCl_2$ (4 mM, dNTPs (0.2 mM)] using seven universal primers (Table S1 in Supplementary data). The amplification cycle, run on Verti$^{TM}$ 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 5 s; (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 (18 × 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).

### 3.2. 4. 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 (Axioscope, 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 ×o* (lines 1-5, $F_5$), *Na*T ($F_5$), *No*T ($F_5$), *Nq* and *Nc*.

### 3.2. 5. 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.

## 3.3. Results

### 3.3.1. 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 (*Na*T) and *No* tetraploid (*No*T) 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.
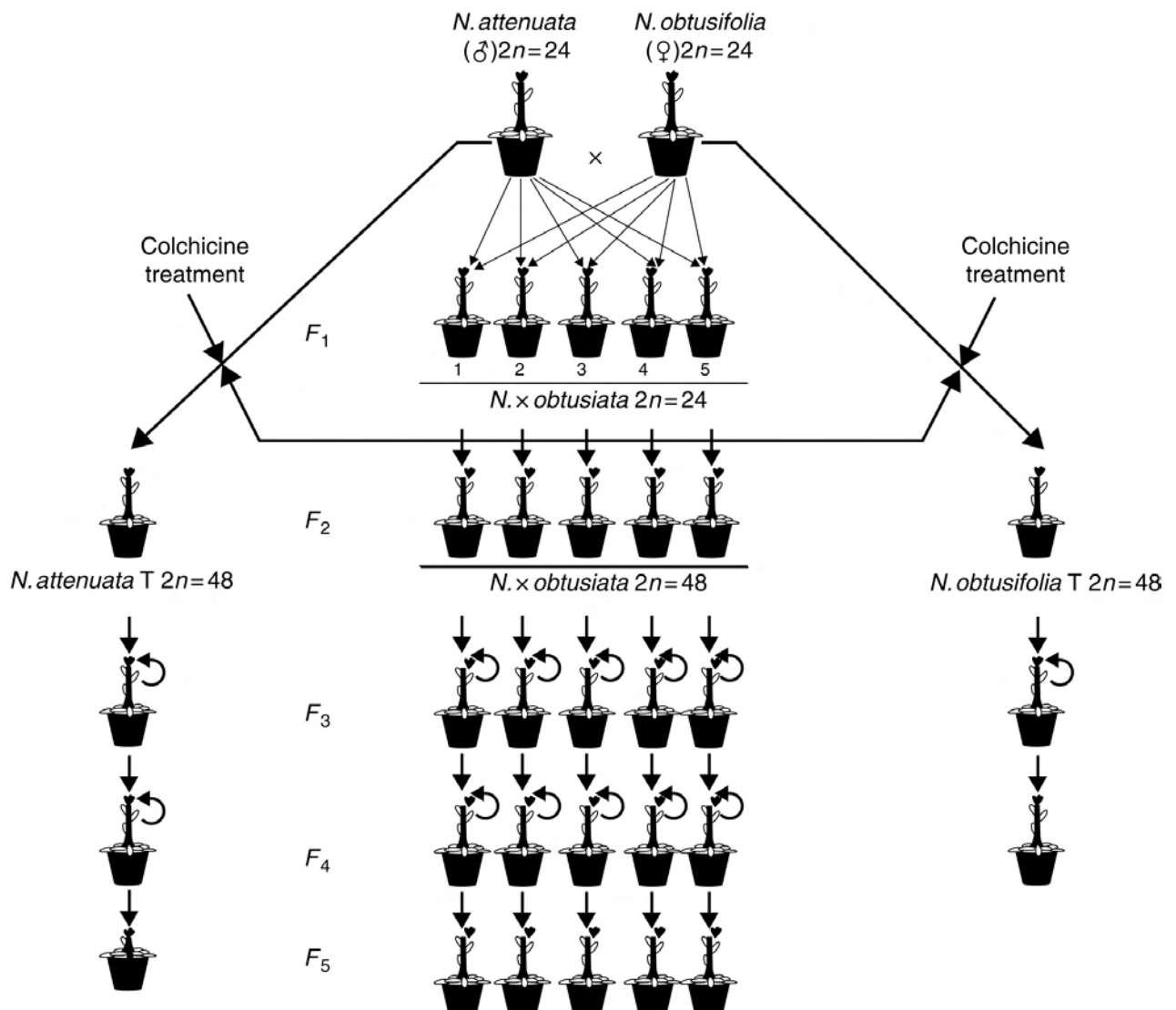


**Figure 1**. Breeding scheme of *N. ×obtusiata* lines 1-5 and autotetraploids of *N. attenuata* and *N. obtusifolia*

*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. *Na*T and *No*T 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 (Supplemental Fig. S1), 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* (Supplemental Fig. S 1). Both synthetic and natural polyploid flowers retained the opening rhythms of *Na* flowers.

*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. *Na*T and *No*T 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 (Supplemental Fig. S1). *Nc* and *Nq* seeds are brown and rotund, as well as reniform shaped with fluted-reticulate and fluted-plicate ridged surfaces, respectively (Fig. 2). Allotetraploid 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* (Supplemental Fig. S1).
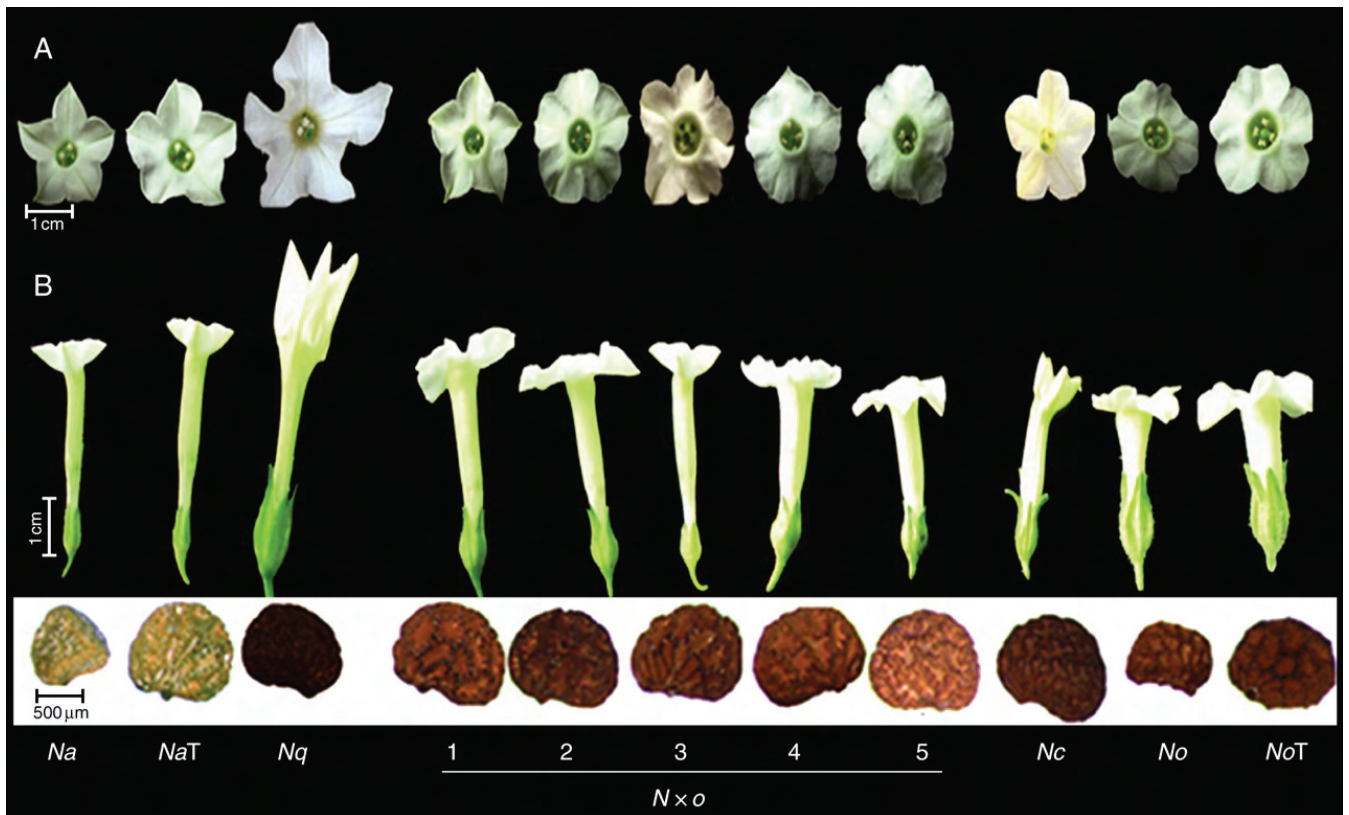
**Figure. 2.** Corolla limbs, flowers and seed morphologies of *N. attenuata* (*Na*), *N. attenuata* autotetraploid (*Na*T) (F5), *N. obtusifolia* (*No*), *N. obtusifolia* autotetraploid (*No*T) (F4), *N. ×obtusiata* (*N ×o*) (lines 1-5, F5), *N. clevelandii* (*Nc*) and *N. quadrivalvis* (*Nq*).

(A) Corolla limbs: *Na*T and *No*T corolla limbs are about 1.5 times larger than those of *Na* and *No*. *N ×o* corolla limbs are on average 1.7 times larger than those of *Na*. *Nq* corolla limbs are 3 times larger than those of *Na*, whereas *Nc* corolla limbs are 1.1 times larger than those of *No*.

(B) Flower tubes: *Na*T and *No*T flower tubes are similar to those of *Na* and *No*, respectively. *N ×o* (lines 1-4) flower tubes are similar in length to those of *Na*, whereas those of *N ×o* line 5 are similar to *No*'s. *Nq* flower tubes are 1.4-fold longer than those of *Na*, whereas *Nc* flower tubes are similar in length to those of *No*.

(C) Seeds: *Na*T and *No*T seeds have the colour, shape and surface appearance of *Na* and *No* seeds, respectively. *N ×o* (lines 1-5) seeds have intermediate parental color and *Na*'s seed shape. *N ×o* (lines 1-4) seeds have the surface appearance of *Na*, whereas seeds from

$N{\times}o$ line 5 have that of *No*. *Nc* and *Nq* seeds have the brownish pigmentation typical of *No* seeds.

***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*. $N{\times}o$ 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). $Nq, N{\times}o$ lines 1-5 leaves are as long as those of *Na*, whereas *Nc* leaves are as long as those of *No* (Supplemental Fig. S1).
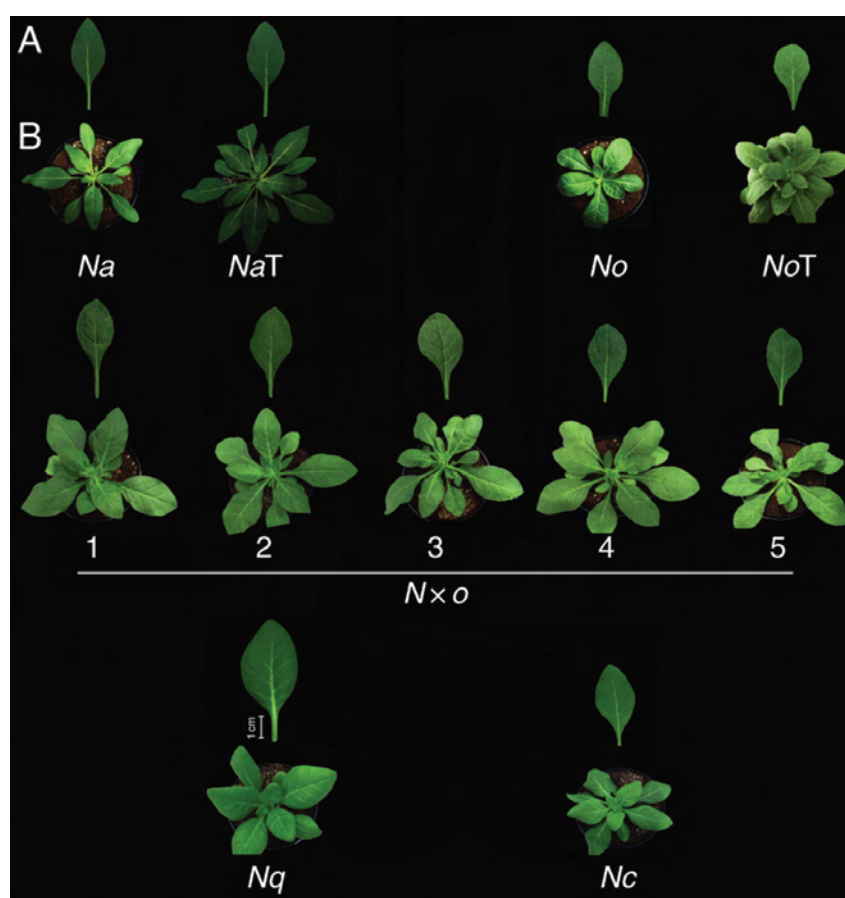


**Figure. 3.** (A) leaves and (B) rosette-stage plants of *N. attenuata* (*Na*), *N. attenuata* autotetraploid (*Na*T) ($F_5$), *N. obtusifolia* (*No*), *N. obtusifolia* autotetraploid (*No*T) ($F_4$), *N. ×obtusiata* ($N{\times}o$) (lines 1-5, $F_5$), *N. clevelandii* (*Nc*) and *N. quadrivalvis* (*Nq*).

 $N{\times}o$ (lines 1-5) leaves have long petioles and an intermediate parental shape. *Nq* and *Nc* produce ovate-elliptical leaves with long and short petioles, respectively. Synthetic polyploids rosette-stage plants develop approx. 3 d before either parent (photographs were taken at the same stage).

### 3.3.2. 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 *Na*T (5.98 pg) and *No*T (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 (Supplemental Fig. S2). 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 upsizing of 1.5 % compared with the theoretical size (Fig. 4).
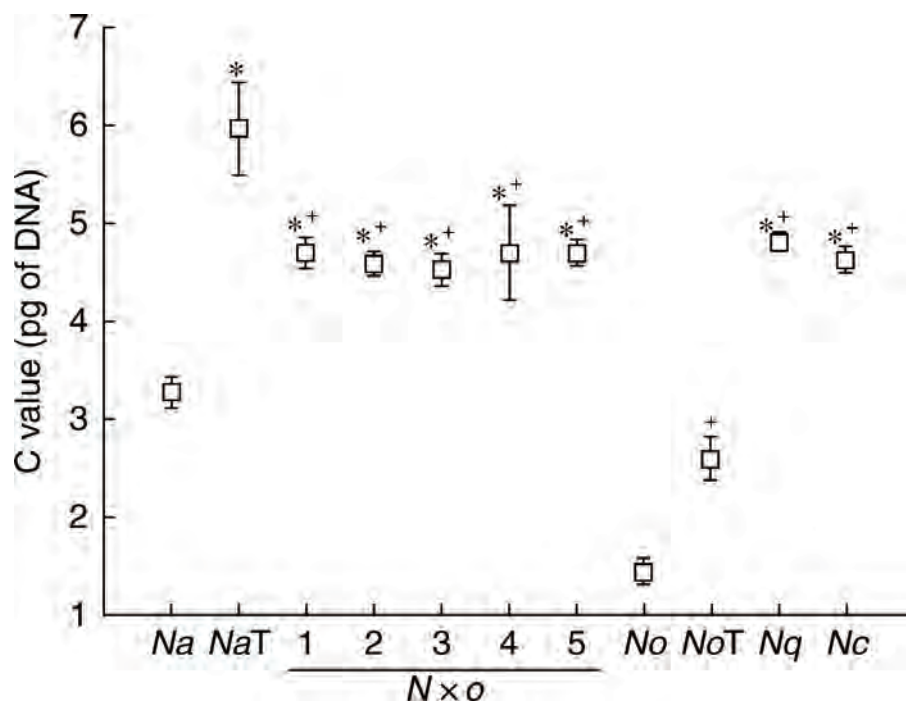


**Figure. 4.** Genome sizes (mean C value in pg DNA $\pm$ SE) of *N. attenuata* (*Na*), *N. attenuata* autotetraploid (*Na*T) ($F_5$), *N. obtusifolia* (*No*) ($F_5$), *N. obtusifolia* autotetraploid (*No*T) ($F_4$), *N. ×obtusiata* (*N ×o*) (lines 1-5, $F_5$), *N. clevelandii* (*Nc*) and *N. quadrivalvis* (*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* ($P < 0.05$).

### 3.3.3. 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 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 (Supplemental Fig. S3), 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 revealed that all synthetic hybrids grouped closer to *Na* than to *No*, whereas *Nq* and *Nc* grouped closer to *No* than to *Na* (Fig. 5B).
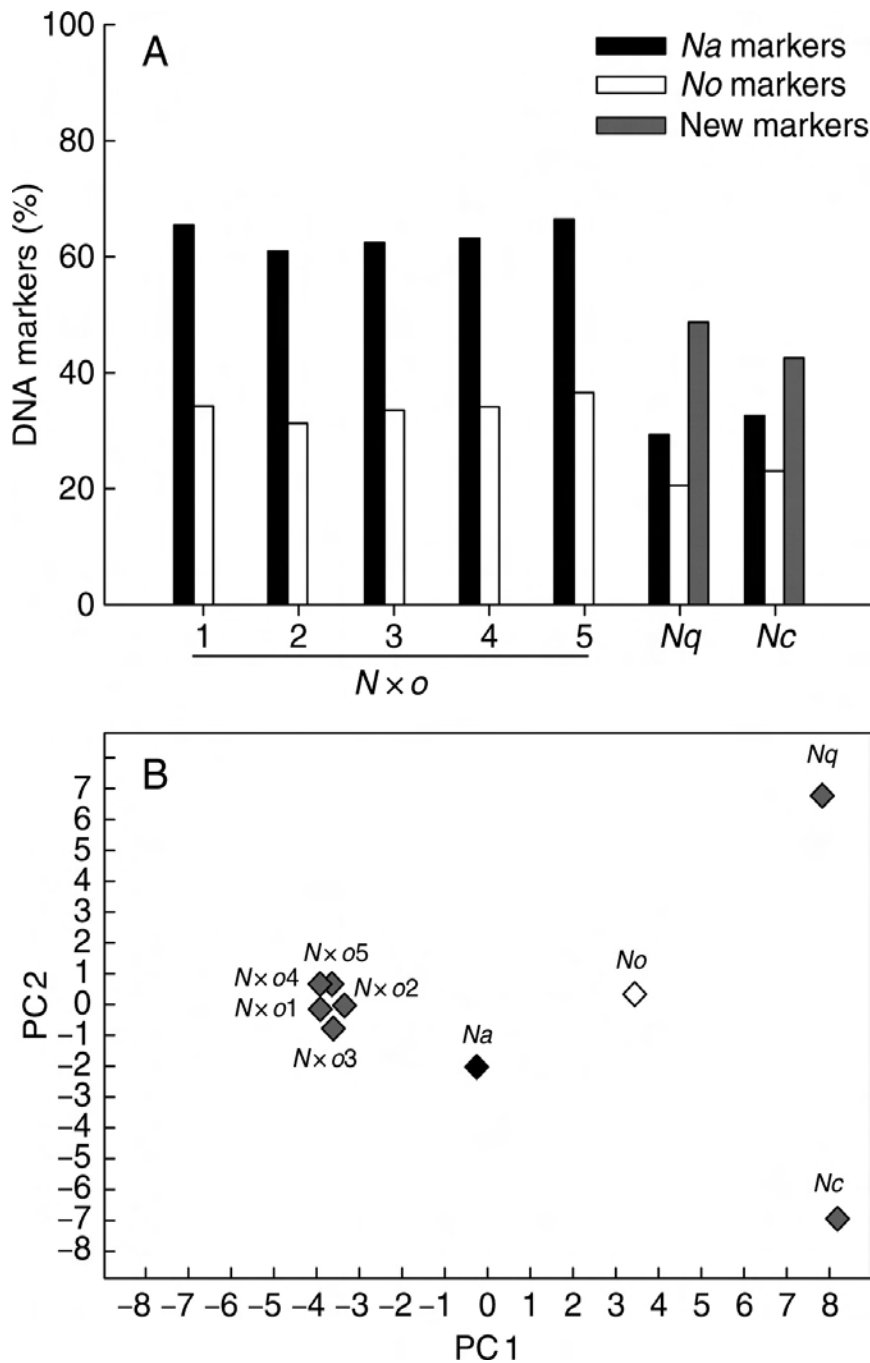
**Figure 5.** Analysis of UP-PCR DNA fingerprinting profiles of *N. attenuata*, *N. obtusifolia*, *N. × obtusiata* (lines 1–5), *N. clevelandii* (*Nc*) and *N. quadrivalvis* (*Nq*). (A) Percentages of *Na*- and *No*-specific DNA markers and new markers present in each species. DNA markers unique to *Na* or *No* were first identified and then recorded in the allotetraploid. DNA markers present only in the allotetraploid 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.

### 3.3.4. Variation among phenotypic traits after polyploidization

Phenotypic variation often arises with the formation of polyploids and might contribute to their success in nature (Osborn 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 *Na*T were about 1.5 and 1.3 times larger than those in *Na* and *No*, respectively. In $N \times o$ (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 *Na*T and *No*T were about 1.5-fold heavier than in *Na* and *No*, respectively. Dry biomass in *Na*T did not significantly differ from that in *Na*, whereas biomass in *No*T was about 1.3-fold that in *No.* Seed and dry biomass in $N \times o$ (lines 1-5) were on average about 1.5 times what they were in *Na*, but height at first flower in $N \times o$ (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 $N \times o$ 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

$N \times o$ lines, whereas biomass distinguished $Na$ from $N \times o$ and $Nq$ lines. $Na$T grouped closer to $Nq$ and $N \times o$ lines than to $Na$ (Fig. 7).



**Figure 6.** Quantitative phenotypical traits (mean±SE) were measured in *N. attenuata* (*Na*), *N. attenuata* autotetraploid (*Na*T) (F5), *N. obtusifolia* (*No*), *N. obtusifolia* autotetraploid (*No*T) (F4), *N ×obtusiata* (*N ×o*) (lines 1-5, F5), *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).

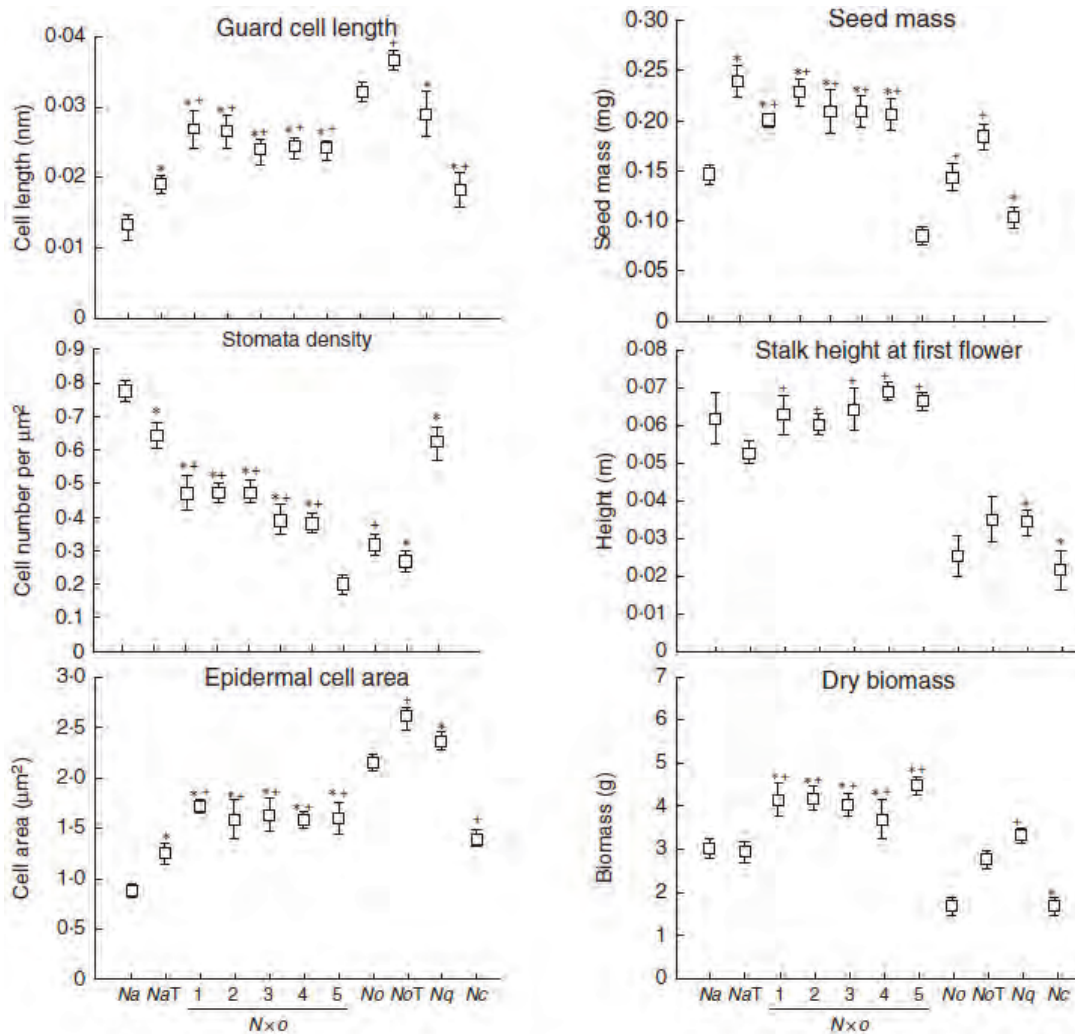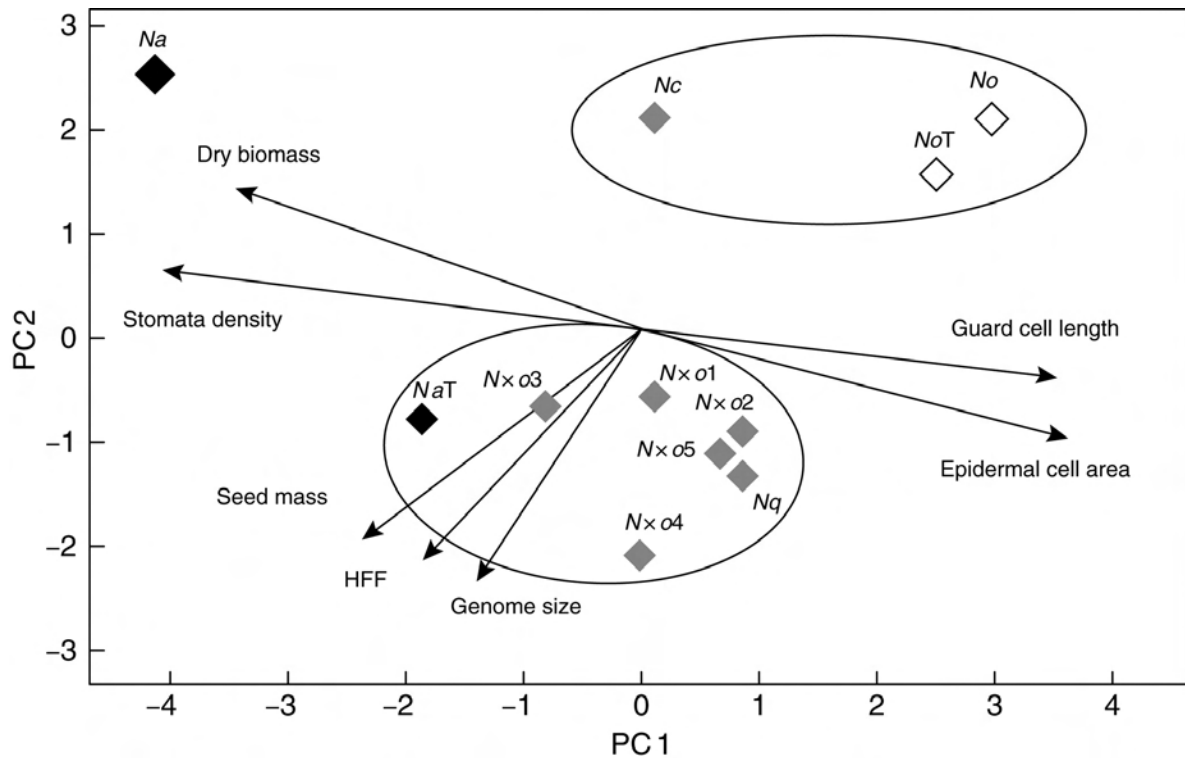**Figure 7.** Principal component analysis (PCA) of quantitative phenotypic traits measured in *N. attenuata* (*Na*), *N. attenuata* autotetraploid (*Na*T) ($F_5$), *N. obtusifolia* (*No*), *N. obtusifolia* autotetraploid (*No*T) ($F_4$), *N. ×obtusiata* (*N ×o*) (lines 1-5, $F_5$), *N. clevelandii* (*Nc*) and *N. quadrivalvis* (*Nq*).

The quantitative phenotypic values used in this analysis are those shown in Fig. 5. Percentages of total variance explained by PC 1 and 2 are 45.5 % and 36.2%, respectively.

### 3. 4. Discussion

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* 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-proteinase inhibitor genes were retained in both *Nc* and *Nq*, whereas paternally inherited (*Na*) trypsin-proteinase 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*, *Na*T, *No*T, *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 *Na*T (5.98 pg) and *No*T (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 $F_1$ 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: *Na*T theoretical (6.62 pg), *No*T theoretical (2.92 pg)], the observed genome sizes of *Na*T and *No*T 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 *Nq* and *Nc* was estimated as +7.5% and +4.5%, respectively. The discrepancy in the results is due to a difference in the estimation of *Na* genome size: 2.5 pg in Leitch et al. (2008) and 3.5 pg shown here, which leads to different *Nc* and *Nq* genome expansion estimations. The difference in the estimation of *Na* genome size might be due to a difference in *Na* accessions used by different groups. In addition, an effect of intensive inbreeding of *Na* 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- (*Na* and *No*) specific DNA markers and new markers. *N ×o* (lines 1-5) displayed an average of DNA fingerprinting profiles consisting of 70 % parent-specific markers from *Na* and 28.5 % from *No*, and 1.5 % of new markers (Fig. 5A). These results demonstrate that after five generations, synthetic allopolyploids undergo a certain amount of recombination which results in the loss and gain of new DNA markers. *N ×o* lines show almost entirely additive DNA fingerprinting profiles of the two parental genomes. The predominance of *Na*-specific markers (*Na* markers are twice as frequent as those of *No*) in *N ×o* DNA fingerprinting profiles reflects the DNA content from *Na* in the *N ×o* genome (*Na*'s genome size is almost twice as large as *No*'s).

Although formed from independent *Na* and *No* crossings, the five lines of *N ×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 *Nq* and *Nc* have similar percentages of *Na-* and *No-*specific markers. *Nq* inherited 29.5 % of *Na*'s markers and 21 % of *No'*s, whereas *Nc* inherited 33 % of *Na's* markers and 23.5 % of *No's*. *Nq* and *Nc* showed a high level of recombination; new markers accounted for 49.5 % and 43.5 % of *Nq*'s and *Nc*'s DNA fingerprinting profiles, respectively (Fig. 5A).

Compared with the *N ×o* profiles, those of *Nq* and *Nc* show a significant reduction in *Na*-specific markers (*Na* markers ratios are twice as infrequent in *Nq* and *Nc* as in *N ×o* lines). A possible explanation emerges from the study carried out by Lim et al. (2007), who concluded that during the evolutionary histories of *Nq* and *Nc*, a massive DNA invasion occurred, involving *Na* subtelomeric repeat sequences onto *No* chromosomes, followed by their replacement. Such genomic changes may have led to the decline of *Na*-specific markers and the emergence of new ones. Paternal DNA sequence elimination has also been revealed by genetic studies in *Nicotiana* allopolyploids (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 *Na* and *No* that gave rise to *Nq* and *Nc* have undergone large genomic changes, eventually reducing the number of *Na*-specific DNA makers. Principal component analysis based on DNA markers present in each species revealed that all *N ×o* lines grouped closer to *Na* than to *No*, whereas *Nb* and *Nc* grouped closer to *No* than to *Na* (Fig. 5B). The method used in this study is an efficient and reliable way to generate DNA fingerprint 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 *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 ×o* lines retained intermediate parental phenotypes (Fig. 6). Both synthetic allo- and autopolyploidy resulted in dosage effects on seed and dry biomass (except for *Na*T), 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 *Na* and *No*, 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 ×o* lines might be a consequence of non-additive parental gene regulation, whereas the dosage effect in *Na*T and *No*T 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 allopolyploids (Chen, 2007), leading to *de novo* phenotypical variations.

Concerning the natural polyploids, *Nc* had paternal (*Na*) cellular phenotypes, but inherited maternal (*No*) seed and dry biomass, whereas *Nq* had paternal maternal (*No*) cellular phenotypes but inherited paternal (*Na*) 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 *Nq* and *N ×o* (lines 1-5) grouped together due to similar 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, and *Na*T clustered closer to *Nq* and *N ×o* lines than to *Na*. Because similar phenotypic traits may result from similar metabolic regulation, it may seem that only *Nq* (which groups together with *N ×o* lines) was re-synthesized but not *Nc*.

The present study demonstrates that both auto- and allotetraploidization induce significant and complex genomic and phenotypic changes. After five generations,

autotetraploids showed a dosage effect on genome size and most of the parental phenotypes, whereas allotetraploids showed a dosage effect on genome size, seed and dry biomass, but retained a combination of *Na* and *No* DNA fingerprinting profiles and cellular phenotypes. After polyploidy speciation, *Nc* and *Nq* 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 sympatry with the ecological interactions of the parents, the polyploid probably benefits from maintaining aspects of the parental gene expression program 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.

**4. Chapter II. Variation in anti-herbivore defense responses in synthetic *Nicotiana* allopolyploids correlates with changes in uni-parental patterns of gene expression**

**4.1. Introduction**

Genome doubling (polyploidy) has been and continues to be a pervasive force in plant evolution. Modern plant genomes harbor evidence of multiple rounds of past polyploidization events, often followed by massive silencing and elimination of duplicated genes. Recent studies have refined our inferences of the number and timing of polyploidy events and the impact of these events on genome structure. Many polyploids experience extensive and rapid genomic alterations, some arising with the onset of polyploidy. Survivorship of duplicated genes are differential across gene classes, with some duplicate genes more prone to retention than others.

Recently, the dynamic changes affecting parental DNA sequences have received attention. Using *Zingeria* and *Tragopogon* species, both Kotseruba et al. (2003) and Lim et al. (2008) demonstrated that DNA sequence elimination may target only one or the other progenitor. In addition, using *Gossypium* allopolyploids, Adams et al. (2004) showed that the epigenetic silencing of parental homologous genes might be developmentally regulated, with one homolog silenced in some organs and the other silenced in other organs. These studies have provided mechanisms for the genetic changes that occur during polyploidy and specifically how the expression of one or the other parental genes may be modified; however, how these changes in parental gene expression influence the expression of functioning physiological systems that allow the hybrids to respond to their natural environment is still not clear. It is commonly assumed that the responses of most neoallopolyploids will be dysfunctional, and these dysfunctional hybrids are rapidly removed by natural selection. Hence, the adaptive radiation of polyploidy lineages is thought to be the result of the rapid winnowing of the bursts of genetic and functional diversity that results early in the neopolyploidization process. This assumption has not been thoroughly tested in any system.

The natural allopolyploids, *N. quadrivalvis* (*Nq*) and *N. clevelandii* (*Nc*), allopolyploids derived from amphidiploidy involving two diploid ancestors, *N. attenuata* (as the paternal donor) and *N. obtusifolia* (as the maternal donor) approx. 2 Myrs ago (Chase et al., 2003), have been particularly useful for understanding how complex polygenic traits evolve. In 2003, Lou and Baldwin reported that *Nq* and *Nc* retained different components of *Na's* jasmonic acid (JA)-mediated defense response to attack from *Manduca sexta* larvae, most of which are fully mimicked by applying *M. sexta* oral secretions (OS) to wounds (Fig.

1; Wu and Baldwin, 2009). Most aspects of *Na*'s recognition response were retained with modifications in *Nq*, but many have been lost in *Nc*. Wu et al. (2006) demonstrated that maternally inherited (*No*) trypsin-proteinase inhibitor (TPI) genes, which encode for protease inhibitors that reduce *M. sexta* performance, were retained in both *Nc* and *Nq*, whereas paternally inherited (*Na*) trypsin-proteinase inhibitor genes were deleted. However, when these changes occurred, either directly after polyploidization or during the intervening 2 Myrs in either parents or allopolyploids, remains a mystery. Answering this question requires an understanding of the changes that occur rapidly after neopolyploidization and can be addressed by comparing responses in both parental lines and newly resynthesized allopolyploids.

The first attempts, carried out by Pearse et al. (2006), to synthesize *Na* and *No* allopoylploids were unsuccessful, thus the authors used *N. miersii* as a maternal surrogate for *No* (the species most phylogenetically related to *No*) to create the synthetic neoallopolyploid, *N. ×mierata*. By eliciting plants with OS and methyl jasmonate (MeJA) and comparing the changes in TPI activity, secondary metabolites and released volatile organic compounds (VOCs) in the parents with those of the neoallopolyploid lines, the authors concluded that parental signaling cascades eliciting these defense responses had been reshuffled in the neopolyploids in a "plug-and-play" fashion to allow different secondary metabolite responses to be elicited by the diversity of OS- and JA-elicited signaling systems found in the parents. The analysis of metabolic responses provided by this study would benefit from information about how the expression of parental genes is altered in the synthetic polyploids, specifically, which parent genetically dominates and how this influences the defensive response of the synthetic allopolyploids.

Here we examine the changes in *Na* and *No* anti-herbivore gene expression, phytohormone accumulation and TPI activity in 5 independent allopolyploids lines of *N.×obtusiata* (*N×o*) and synthetic autopolyploids of *Na* and *No* (*Na*T and *No*T) characterized in Anssour et al. (2009). We first dissect the specific transcript accumulation (levels and timing) of some of the early anti-herbivore responsive genes in the OS-elicited signaling cascade (Fig. 1), namely wound-induced protein kinase (WIPK), lipoxygenase 3 (LOX3), the non-expressor of PR1 (NPR1) and jasmonate-resistant 4 (JAR4) of *Na* and *No*. Then, we measure the phytohormone levels of jasmonic acid (JA), JA-Ile and salicylic acid (SA); finally, we analyze the kinetics of parental TPI transcript accumulation and TPI activity in the synthetic polyploids. Variations in the expression of anti-herbivore signaling and resistance

genes and in the levels of phytohormones and TPI activity among the synthetic polyploid lines are compared to their parental lines.

## 4.2. Material and methods

### 4.2.1. Plant material

*Plant breeding*

*Na*'s seeds originated from a native population in Utah (Baldwin et al., 1994) and inbred for 17 generations. *No*'s seeds were collected in 2004 at the Lytle ranch preserve (Santa Clara UT, USA) and inbred for 1 generation. The polyploids' seeds, inbred for 5 generations, were produced from synthetic polyploids described in Anssour et al. (2009).

*Plant growth*

Seeds from all studied species and lines were germinated and grown as described by Krügel et al. (2002). Briefly, seeds were treated with smoke before being sterilized for 1 h with 0.1 mM gibberellic acid and germinated on sterile agar with Gamborg B5 media (Duchefa). After 10 days of growth, seedlings were transferred to soil-based growth medium in Teku pots (Waalwijk, The Netherlands) and, after an additional 10 days, transplanted to soil in 1 L individual pots and grown in a glasshouse at 26–28 °C under 16 h of light supplied by Philips Sun-T Agro 400 Na lights (Eindhoven, The Netherlands). Plants in the rosette stage of growth were used in all experiments.

*Plant treatment and sample harvest*

Plant treatments were conducted as described by Lou and Baldwin (2003) with some modifications. For W+OS treatments, +1 leaves from each species and lineage were damaged by rolling a fabric pattern wheel to create a standardized mechanical wound, then 20 μl of OS (one-one diluted with deionized water) from fourth- to fifth-instar *M. sexta* larvae was rubbed into the wounds. For MeJA treatment, MeJA was dissolved in heat-liquefied lanolin at a concentration of 7.5 mg/ml; 20 μl of the resulting lanolin paste was applied to +1 leaves to elicit the plants with 150 μg of MeJA. Untreated control plants were used in every experiment. After specific times, leaves were excised, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

### 4.2.2. Protein extraction and trypsin protease inhibitor (TPI) activity assay

Leaf tissue from each species and lineage were induced with *M. sexta* OS or with MeJA (one of six or seven plants/species or lineage/time point), and prepared for a TPI quantification. Briefly, plant tissues (approximately 150 mg) were crushed in liquid nitrogen, and 500 µl extraction buffer (0.1M Tris-HCl [PH=7.6], 2 mM polyvinylpyrrolidone,13 mM phenylthiourea, 30 mM diethyldithiocarbamate, 60 mM ethylene diamine tetraacetic acid) was added for every 100 mg of tissue. Leaf tissue was then completely suspended by vortexing. After being centrifuged at 4°C for 20 min, supernatant was transferred to a fresh tube. Total protein content in each sample was determined using a Bio-Rad protein assay kit (Bradford assay) against serial dilutions of BSA as a standard. Trypsin proteinase inhibitor (TPI) activity was determined by radial diffusion activity as described in van Dam et al. (2001).

### 4.2.3. Phytohormone analysis using liquid chromatography tandem mass spectrometry

For the phytohormone analysis, approximately 300 mg of crushed frozen leaf tissue sample from each species and lineages were transferred to a FastPrep tube containing 0.9 g of FastPrep matrix, 200 ng/ml of isotope labeled JA (1,2-$^{13}$C-JA) and pCA (as an internal standards) as well as 1 mL of extraction buffer (acetone: 50 mM citric acid [7:3 v/v]). Samples were then homogenized for 45 s at a speed 6.5 in a FastPrep homogenizer (Thermo Electron, http://www.thermo.com) and afterward centrifuged at maximum speed (16000 g) for 10 min at 4°C. Supernatants were transferred to fresh tubes to be subsequently evaporated in a vacuum concentrator (Eppendorf, http://www.eppendorf.com) to remove the remaining traces of acetone, and then extracted twice with 2 mL of ether. The ether phases of each sample were evaporated to dryness in the vacuum concentrator; the pellets were suspended with 70% methanol and the phytohormone measurements were conducted on a liquid chromatography tandem mass spectrometry system (Varian 1200; Varian, Palo Alto, CA, USA http://www.varianinc.com). 15 µL of each sample were injected onto a ProntoSIL column (C18; 5 mm, 50 × 2 mm; Bischoff, www.bischoff-chrom.com) attached to a precolumn (C18, 4 × 2mm; Phenomenex, www.phenomenex.com). The mobile phase, consist of 0.05% formic acid (solvent A) and 0.05% formic acid in acetonitrile (solvent B) used in a gradient mode with the following conditions: time/concentration (min/%) for B:0:00/15; 1:30/15; 4:30/98; 12:30/98; 13:30/15; 15:00/15 with a flow of (time/flow [min/mL]): 0:00/0.4; 1:00/0.4; 1:30/0.2; 10:00/0.2; 10:30/0.4; 12:30/0.4; 15:00/0.4. Compounds were detected in the electrospray ionization negative mode. Molecular ions [M-H](2) at m/z 137 and 209 and 141

and 213 generated from endogenous phytohomones and their internal standards, respectively, were fragmented under 15-V collision energy. The ratios of ion intensities of their respective daughter ions, m/z 93 and 97 and m/z 59 and 63, were used to quantify endogenous phytohomones.

### 4.2.4. Molecular cloning

cDNA molecular cloning and sequencing was performed as described by Wu et al. (2006). Briefly, total RNA was extracted from seven to ten replicated biological samples using TRIZOL reagent (Invitrogen, http://www.invitrogen.com) following the manufacturer's instructions. The cDNA synthesis was carried out using a first-strand cDNA synthesis kit (Invitrogen, http://www.invitrogen.com); 1 µg of total RNA from *No* samples was subjected to reverse transcription using oligo (dT) and Superscript II reverse transcriptase (Invitrogen, http://www.invitrogen.com). The obtained cDNA was used as a template to generate WIPK, LOX3, NPR1and JAR4 DNA fragments using PCR primers designed on *N. attenuata*'s published sequences. The PCR Amplification was done in a final volume of 50 µl containing 10 ng of cDNA, 13 µl PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each primer, 200 mM of each dNTP, and 1.25 units of Taq DNA polymerase. The PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 57 °C for 1 min (50 °C during the first two cycles), 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR fragments were gel-purified and cloned into pGEM-T Easy vectors (Promega, http://www.promega.com) and then sequenced. Sequencing was performed using an ABI PRISM 377 automated DNA sequencer (Global Medical Instrumentation, http://www.gmi-inc.com). Each clone was sequenced in both sense and antisense directions and at least four clones were sequenced for each fragment. All the sequences are presented in Supplemental Table S1.

### 4.2.5. SYBR green real-time PCR assay (qPCR)

q-PCR analysis was conducted using four to seven replicated biological samples for each time point in the kinetic analysis. The first step of reverse transcription was optimized to minimize errors which can be generated during cDNA synthesis; therefore, all total RNA samples were diluted to 0.5 mg/mL in 96-well PCR plates and the same enzyme master mix reaction was used for all samples. 2 µL of each diluted RNA sample was reverse-transcribed as described in the previous section; the obtained cDNA samples were further diluted with water to 40 µL. q-PCR was performed on an ABI PRISM 7700 sequence detection system

(Applied Biosystems, http://www.appliedbiosystems.com) using qPCR Core kits (Eurogentec, http://www.eurogentec.com). For each analysis, a linear standard curve, threshold cycle number versus log (designated transcript level) was constructed using serial dilutions of a specific cDNA standard; the levels of the transcript in all unknown samples were determined according to the standard curve. Actin, a housekeeping gene from *Na* and *No* that has been shown to have constant transcript levels by both RNA gel blotting and q-PCR after W+OS treatments (J. Wu, unpublished data) was used as an internal standard for normalizing cDNA concentration variations. The primers for the SYBR Green–based q-PCR were specifically designed to amplify in *N ×o* transcripts from only *Na* or *No*, but not both. A PCR test was performed for each pair of primers and the product was visualized on agarose gel (Supplemental Fig. S5). In contrast, actin primers were designed to amplify both *Na* and *No* transcripts. Primer sequences were designed following the PCR conditions recommended by the manufacturer.

### 4.2.6. Statistical analysis

The statistical analyses of data from each group of comparison at a specific time point after wounding and OS elicitations, and MeJA treatment were performed with StatView (StatView software, SAS Institute) using unpaired t-test.

### 4.3. Results

### 4.3.1. OS elicitation induces high levels of *Na*-WIPK and *No*-LOX3 transcripts and attenuated levels of *Na*- and *No*-NPR1 transcripts in *N×o* lines.

*N. attenuata*'s anti-herbivore response is rapidly initiated after the introduction of *M. sexta* OS into wounds. This defensive response is highly specific and its activation depends on the plant's ability to recognize fatty acid-amino acid conjugates (FACs) present in *M. sexta* OS. Recently, Wu et al. (2007) demonstrated that FACs rapidly activate two MAP kinases, WIPK and SIPK, which in turn stimulate the expression of NPR1 and JA biosynthetic genes, such as LOX3. To understand how the expression of these early anti-herbivore responsive genes are altered after synthetic auto- and allo-polyploidy, we measured the transcript accumulation of WIPK, LOX3 and NPR1 in the diploid and the synthetic polyploid lines subjected to wounding and OS elicitation performed on the +1 leaves, leaves which had just completed the source-sink transition.

In *Na* and *No*, WIPK transcripts rapidly accumulated after wounding and OS elicitation, reaching a maximum 1h after elicitation. The maximum level of WIPK transcripts

in *Na* was twice that in *No*. The accumulation of WIPK transcripts in both autotetraploids, *Na*T and *No*T, followed patterns similar to those in the diploids, *Na* and *No*; however, levels of WIPK transcripts were significantly higher (about 1.5-fold) in the autopolyploids compared to their respective diploids. After OS elicitation, the allopolyploid *N×o* lines expressed both parental WIPK transcripts, but the maximum transcript level of *Na*-WIPK (1h after elicitation) was five times that of *No*-WIPK (1h after elicitation).

In *Na* and *No*, the accumulation of LOX3 transcripts reached a maximum level 1.5h after OS elicitation and returned to basal levels after 3h. The maximum level of LOX3 transcripts in *Na* was twice that in *No*. The accumulation of LOX3 transcripts in both *Na*T and *No*T followed a similar pattern and did not differ significantly compared to their respective diploids. In *N×o* lines, the OS-elicited response was distinctly uniparental; OS elicitation induced the accumulation of *No*-LOX3 transcripts but did not induce any *Na*-LOX3 transcripts. The maximum level of *No*-LOX3 (1.5h after elicitation) was comparable with that of *No* (1.5h after elicitation).

The accumulation of NPR1 transcripts followed a similar pattern in *Na* and *Na*T, attaining maximum levels 2.5h after OS elicitation. The maximum level of NPR1 transcripts in *Na* was five times that in *No*. In *No* and *No*T, OS elicitation resulted in the accumulation of much lower levels of NPR1 transcripts. *N×o* lines accumulated both *Na* and *No*-NPR1 transcripts but the maximum levels were comparable to the low levels found in *No* (Fig. 1, Supplemental Fig. S1).
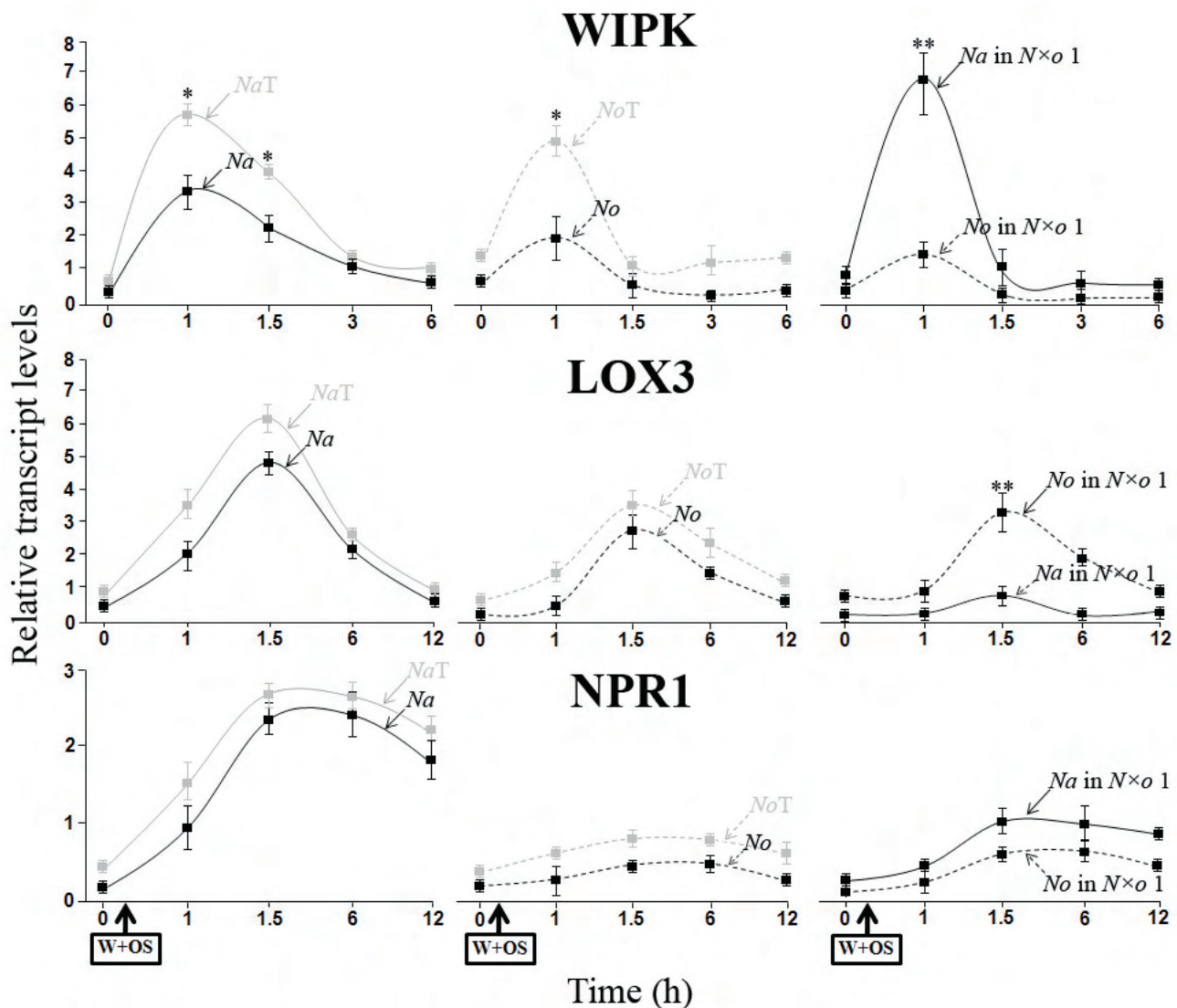
**Figure 1**. OS elicitation induces uni-parental transcript accumulation of WIPK and LOX3, but not NPR1 in the synthetic allopolyploids.

After elicitation with *M. sexta* oral secretions (OS), *N. attenuata* (*Na*) dramatically increases the levels of wound induced protein kinase (WIPK), lipoxygenase 3 (LOX3) and non-expressor of pathogenesis-related (NPR1) transcripts, whereas *N. obtusifolia* (*No*) accumulates comparatively lower levels of WIPK, LOX3 and NPR1 transcripts. Autopolyploid *Na* and *No* show dosage-dependent increases in the accumulation of WIPK transcripts, but not in those of LOX3 and NPR1. The synthetic allopolyploid, *N. ×obtusiata* line 1 (*N×o*1) exhibits uniparental patterns of transcript accumulation (of *Na*-WIPK and *No*-LOX3), and attenuated levels of *Na*-LOX3, and *Na*- and *No*-NPR1 transcripts. The transcript accumulation was analyzed by the SYBR green PCR. For this, single leaves from 7 to 8

replicate plants were wounded with a fabric pattern wheel and the wounds were immediately treated with 20 µL of deionized water mixed with 20 µL of *M. sexta* OS. Treated leaves were then harvested at the indicated times. All values were normalized to actin as an internal standard. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (*P<0.05; **P<0.01).

**4.3.2. Both *cis* and *trans* regulatory elements act on the expression of WIPK, LOX3 and NPR1 gene elicited by *M. sexta* OS**

To get an insight into the genetic interactions acting on the expression of LOX3, WIPK and NPR1genes, we conducted a graphical analysis described by Zhang and Borevitz (2009). In this model, the authors suggest that the expression of a given gene is under the control of only *cis* genetic elements, if the specific parental expression difference is equal to the allele expression difference in the hybrid. Otherwise, both *cis* and *trans* elements are involved as the parental expression difference can be explained by *cis* effect (the allele expression difference in the hybrid), plus composite *trans* effect. A similar analysis comparing parental transcript accumulations among the auto and allopolyploids, elicited by *M. sexta* OS, revealed that both *cis* and *trans* regulatory elements act on the expression of WIPK, LOX3 and NPR1 genes (Fig. 2).
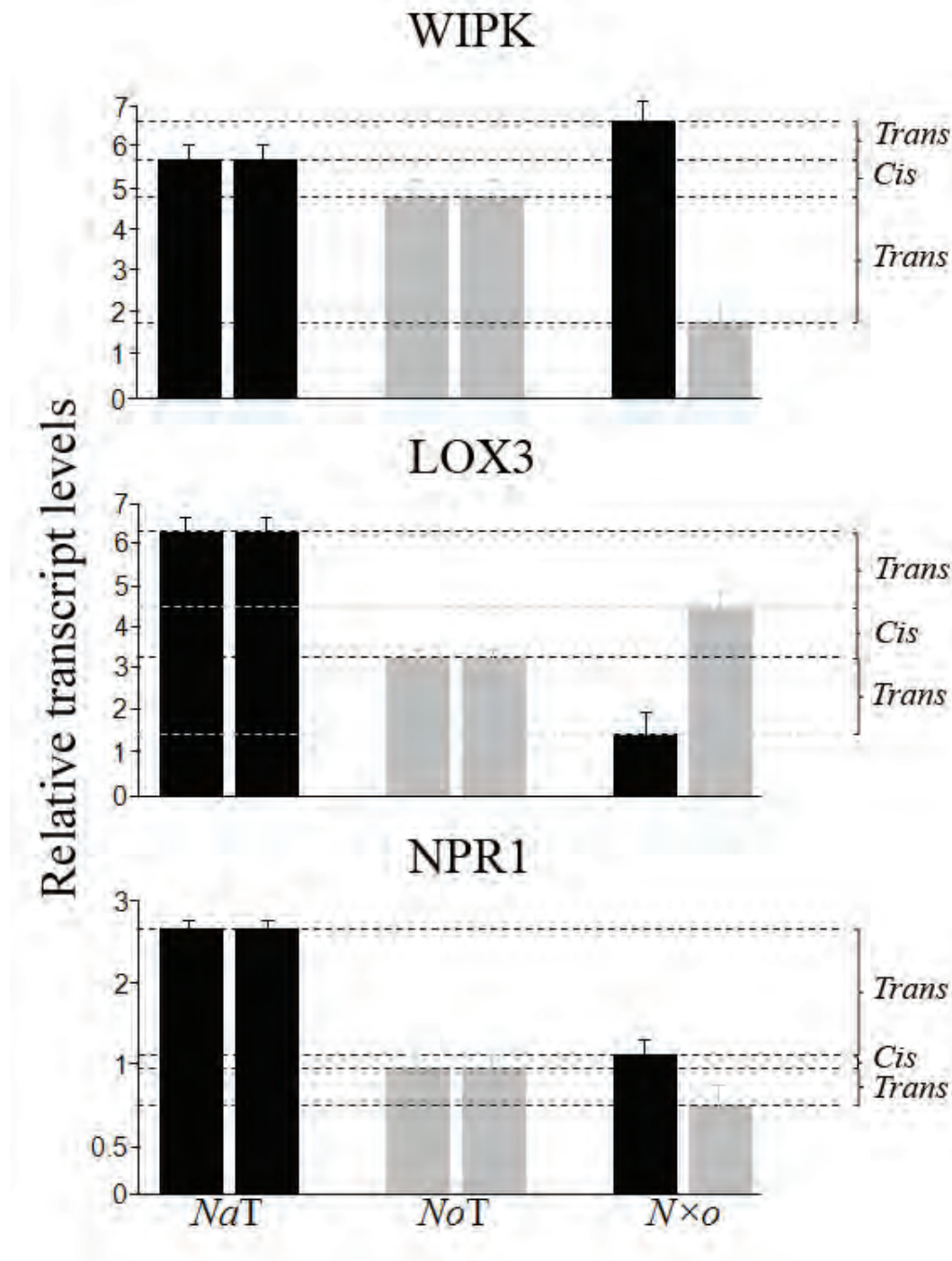
**Figure 2.** Both *cis* and *trans* regulatory elements act on the expression of WIPK, LOX3 and NPR1 gene elicited by *M. sexta* OS.

Wound induced protein kinase (WIPK), lipoxygenase 3 (LOX3) and pathogenesis-related (NPR1) transcripts accumulation was compared in *Na*T, *No*T and *N×o* lines using the approach described by Zhang and Borevitz (2009). Briefly, the authors consider that a given gene is under the control of only *cis* genetic elements if the specific parental expression difference is equal to the allele expression difference in the hybrid; otherwise, both *cis* and

*trans* elements are involved. Our analysis revealed that the parental WIPK, LOX3 and NPR1 transcript accumulation difference between *N. attenuata* (*Na*T) and *N. obtusifolia* (*No*T) is not equal to the difference of *Na* and *No* transcripts in the *N. ×obtusiata* (*N. ×o*) lines, suggesting that the regulation of the expression of these genes involves not only *cis*, but also *trans* elements. In this analysis, we considered only the maximal relative values of transcript accumulation elicited by *M. sexta* oral secretions (OS).

**4.3.3. The patterns of OS-elicited SA and JA accumulation suggest JA/ SA antagonism in the allopolyploid lines.**

OS-elicitation results in phytohormone bursts that spread throughout the attacked leaf to trigger defense responses, which are tailored by interactions amongst different phytohormones. In *Na, M. sexta* OS elicits a JA burst, and a much more modest response in SA levels, which is known to be down-regulated by an OS-elicited ethylene burst (Diezel et al., 2009) and the activity of NPR1 (Rayapuram and Baldwin, 2007). Hence, without the ethylene burst and the activity of NPR1, OS-elicitation in *Na* would result in a large SA burst, which antagonizes the JA burst and attenuates the JA-elicited defense responses (Diezel et al., 2009). In contrast to *Na*, *No* responds to OS elicitation with a large SA burst and an attenuated JA burst. To understand how synthetic polyploidy alters the accumulation of phytohormones during herbivory, we measured the levels of JA and SA after OS elicitation in the synthetic polyploids, and compared them to that of the parental diploids.

In *Na* and *Na*T, JA dramatically increased after OS elicitation, attaining maximum levels at 1.5 and 3h, respectively. The maximum level of JA in *Na*T was three times that in *Na*. In *No*, *No*T and *N×o* lines, OS elicitation induced only low levels of JA; the highest level was about half as much as that in *Na*. Compared to the diploid lines, both auto- and allopolyploids were delayed in attaining maximum JA levels, as was observed in previous studies (Lou et al., 2003; Pearse et al., 2006; Wu et al., 2006).

In *Na* and *Na*T, OS elicitation induced attenuated levels of SA, whereas in *No*, *No*T and *N×o* lines, it induced high levels of SA, which reached a maximum 1h after elicitation. The highest level of SA in *No*, *No*T and *N×o* lines was about four times that in *Na*. Statistical comparisons of JA and SA levels in *Na*, *No* and *N×o* lines showed that JA is negatively correlated with SA (r= -0.683, P<0.001) (Fig. 3, Supplemental Fig. S2).
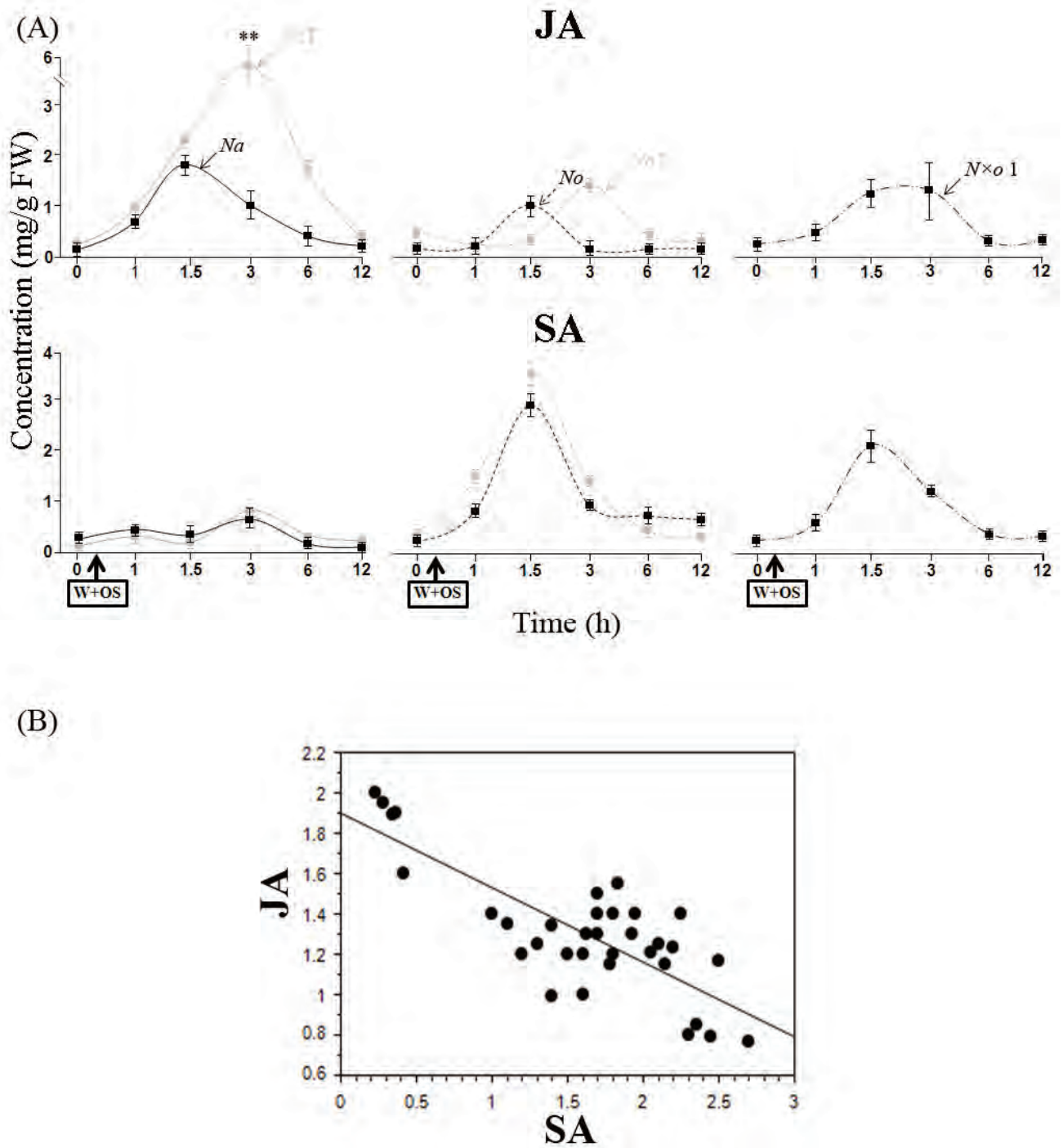
**Figure 3.** Patterns of OS-elicited SA and JA accumulation reveal SA/ JA antagonism in the allopolyploid lines.

(A) OS elicitation in *N. attenuata* (*Na*) and *N. attenuata* tetraploid (*Na*T: left panels) elicited strong jasmonic acid (JA) bursts and attenuated *salicylic acid* (SA) bursts. In contrast, OS-elicitation of *N. obtusifolia* (*No*), *N. obtusifolia* tetraploid (*No*T)(center panels) and *N.*

×*obtusiata* line 1 (*N*×*o*1)(right panels) produced small JA bursts, but large SA bursts. The JA burst was delayed in all tetraploid lines in comparison to those of the diploids.

(B) Correlations among levels of JA and SA in *Na*, *No* and *N*×*o* lines revealed that JA levels are negatively correlated with SA levels (r = -0.68, P< 0.01). The measurements of JA and SA levels were performed on leaves elicited with 20 µL of deionized water mixed with 20 µL of *M. sexta* OS and harvested at the indicated times. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (**P<0.01).

### 4.3.4. *N*×*o* lines enhance the accumulation of only one parental transcript of JAR4 and TPIs, and accumulate variable levels of active TPIs after OS elicitation

We examined the transcript accumulation of JAR4 and TPIs as well as TPI activity levels in *Na*, *No* and synthetic polyploids, after plants were induced with OS, to investigate changes caused by synthetic polyploidy in the transcript accumulation of OS-induced resistance genes, and the consequences of these changes for the expression of an important resistance trait.

In *Na* and *No*, JAR4 transcript levels rapidly accumulated in response to OS elicitation; after 1h, this level had reached a maximum and was about twice as high in *Na* as in *No*. *Na*T and *No*T showed a 1.5-fold and 3-fold increase in the levels of JAR4 compared to the levels in their respective diploids. *N*×*o* lines accumulated *No*-JAR4, but not *Na*-JAR4. The maximum level of *No*-JAR4 transcripts in *N*×*o* lines was about two times that in *No*. Both auto- and allopolyploids showed a delay of 0.5h in attaining maximum levels of JAR4 transcripts compared to the diploid lines.

JAR4 activity mediates the conjugation of Ile with JA to form JA-Ile, which in turn interacts with the F-box protein, COI, to mediate JA-dependent defenses. In *Na* and *Na*T, JA-Ile increased after OS elicitation, attaining maximum at 1.5h and 3h, respectively. The maximum level of JA-Ile in *Na*T was 1.5 times that in *Na*. In *No*, *No*T and *N*×*o* lines, OS elicitation induced only attenuated levels of JA-Ile; the highest level was about 30% of the levels found in *Na*.

In *Na* and *No*, TPI transcripts increased after OS elicitation, reaching a maximum after 12h, this level was 1.5-times higher in *Na* as in *No*. TPI transcripts showed a 1.5-fold dosage-dependent increase in *Na*T compared to *Na*, whereas in *No*T the accumulation of TPI transcripts was 50% of the levels found in *No*. *N*×*o* lines accumulated *Na*-TPIs, but not *No*-TPIs. The maximum level of *Na*-TPI transcripts in the *N*×*o* lines was about 1.5-fold higher

than in *Na*. Both *No* and *N×o* lines showed a delay of 12h in reaching the maximum level of accumulated TPI transcripts compared to the diploid lines.

In *Na* and *No*, TPI activity increased after OS elicitation, reaching a maximum after 24h; this level was 2-fold higher in *Na* than in *No*. In *Na*T, but not in *No*T, TPI activity showed a 2-fold dosage-dependent increase compared to that in the diploids. TPI activity in lines 1 and 2 were comparable to that of *Na*, while in lines 3-5, TPI activity was at basal levels comparable to that in *No* (Fig. 4, Supplemental Fig. S3).
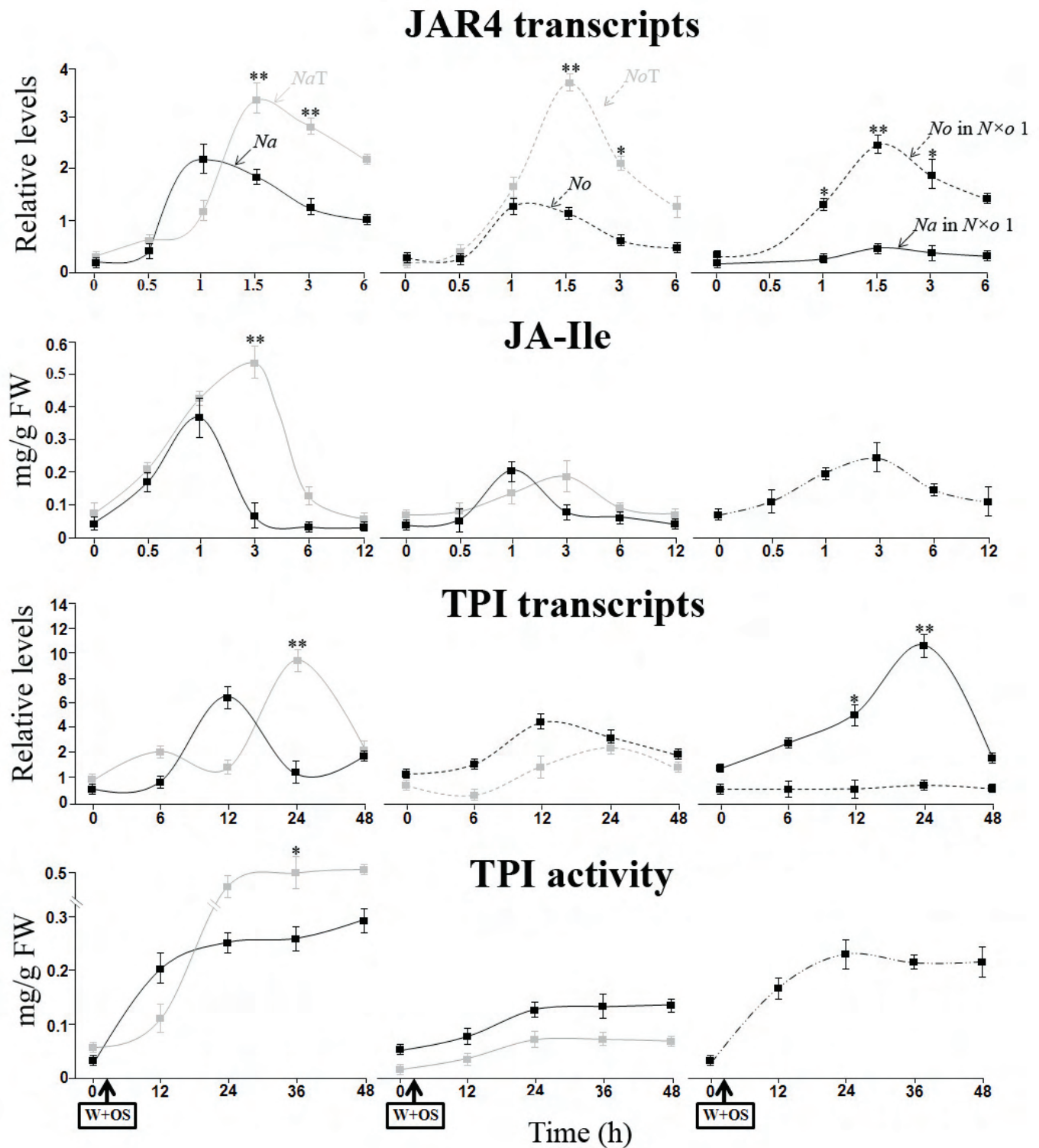
**Figure 4**. OS-elicitation enhances the accumulation of *No*-JAR4 and *Na*-TPI transcripts, and increases the levels of JA-Ile and TPI activity in the allopolyploid lines.

After elicitation with *M. sexta* oral secretions (OS), *N. attenuata* (*Na*) and *N. obtusifolia* (*No*) rapidly accumulate jasmonate-resistant 4 (JAR4) transcripts and jasmonic

acid–isoleucine (JA-Ile) levels, as well as transcript and levels of active trypsin protease inhibitors (TPIs). However, the levels of JAR4 and TPI transcripts, JA-Ile and TPI activity were higher in *Na* than in *No*. Autopolyploidy resulted in increased accumulations of OS-elicited JAR4 transcripts, JA-Ile levels, transcripts and TPI activity levels in *Na*, but not in *No* with the exception of *No*-JAR4 transcripts (which were more than double in theautopolyploid compared to the diploid *No*). *N. ×obtusiata* line1 (*N×o*1) accumulated only *No*-JAR4 and *Na*-TPI transcripts, but not *Na*-JAR4 and *No*-TPI transcripts. All polyploid lines showed a delay in the accumulation of JAR4, JA-Ile as well as transcripts and TPI activity. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (*P<0.05; **P<0.01).

### 4.3.5. Variations in phytohormone crosstalk (SA/JA) influence the accumulation of TPI activity in the allopolyploid lines.

Statistical comparisons among levels of JA, SA, JA-Ile and TPI transcripts accumulated in the allopolyploid lines, revealed that as in *Na*, SA was negatively correlated with JA (r= -0.608, P<0.001), and JA-Ile (r= -0.631, P=0.002), and JA-Ile was positively correlated with TPI transcript levels (r=0.746, P<0.001) (Fig. 5). However, comparisons among transcripts and TPI activity levels showed different linear distributions among the allopolyploid lines which clustered in three distinct groups: *N×o* lines 1-2, *N×o* lines 3-4 and *N×o* line 5. Comparisons among levels of TPI activity and SA revealed that differences in SA influence TPI activity; *N×o* lines 1-2 were distributed in distinct groups based on differences in SA levels. Finally, comparisons among levels of SA and NPR1 transcripts suggest that *N×o* 1-2 and *N×o* 3-5 may react differently to variation in NPR1 transcript levels; while in *N×o* lines 3-5, SA accumulation is down-regulated by NPR1, *N×o* lines 1- 2 seem to have adapted a different mechanism, probably involving ethylene.
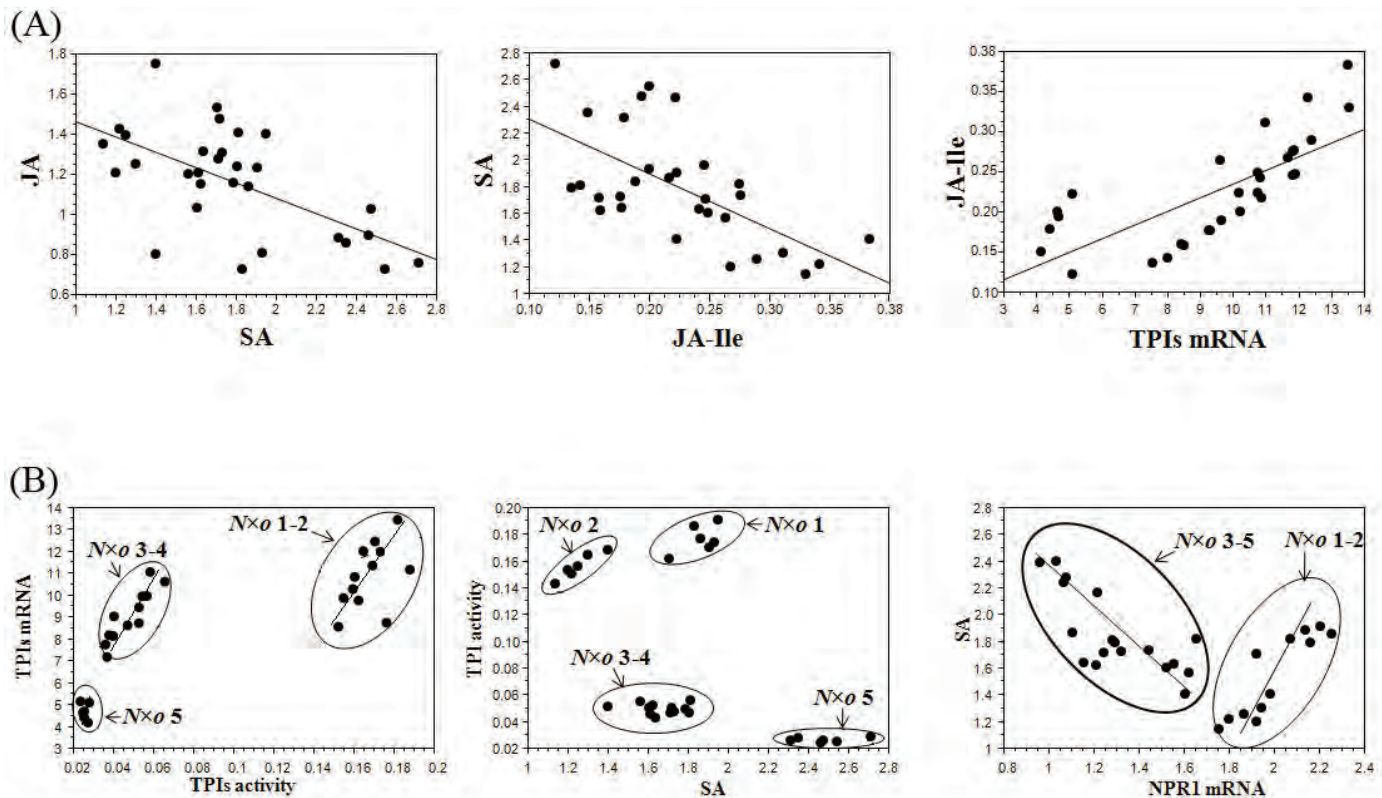
**Figure 5.** Variations in phytohormone crosstalk (SA/JA) influence the accumulation of TPI activity in the allopolyploid lines.

(A) Statistical comparisons among levels of JA, SA, JA-Ile and TPI transcripts accumulated in $N \times o$ lines revealed that SA was negatively correlated with JA (r= -0.608, P<0.001), and JA-Ile (r= -0.631, P=0.002), and JA-Ile was positively correlated with TPI transcript levels (r=0.746, P<0.001).

(B) Statistical comparisons among levels of SA, transcripts and active TPIs revealed that the variation in TPI activity among $N \times o$ lines are caused by differences in accumulated SA (which negatively regulates JA-Ile). The levels of SA are differently regulated in $N \times o$ lines 1- 2 and 3-5; while in $N \times o$ 3-5, SA accumulation is down-regulated by NPR1, $N \times o$ lines 1- 2 seem to be using a different mechanism, which we speculate may involve ethylene production.

**4.3.6. MeJA elicitation of $N \times o$ lines reveals that OS-elicited uni-parental gene expression is not due to gene inactivation**

The accumulation of one or the other parental transcript -- namely, *Na*-LOX3, *No*-JAR4 and *Na*-TPIs– in *N×o* lines after OS elicitation was particularly noteworthy. To test the hypothesis that this pattern of transcript accumulation was due to inactivation of the non-expressed parental gene, we elicited plants with MeJA and measured transcript accumulation of both parental copies of the LOX3, JAR4 and TPI genes. In *Na* and *No*, MeJA treatment elicited changes in the timing and levels of accumulation of LOX3, NPR1, JAR4 and TPI transcripts that were comparable to those elicited by OS elicitation. In *Na*, autopolyploidy induced a gene dosage-dependent increase in the transcript accumulation levels of LOX3, NPR1and JAR4, but not in the levels of TPIs. In *No*T, JAR4 transcripts showed a gene dosage- dependent increase, but not those of TPIs and LOX3. Unlike OS elicitation, MeJA treatment of *N×o* lines induced the accumulation of *No*-LOX3, *Na*-JAR4 and *No*-TPI transcripts (Fig. 6, Supplemental Fig. S4), demonstrating that these parental gene copies are functional.
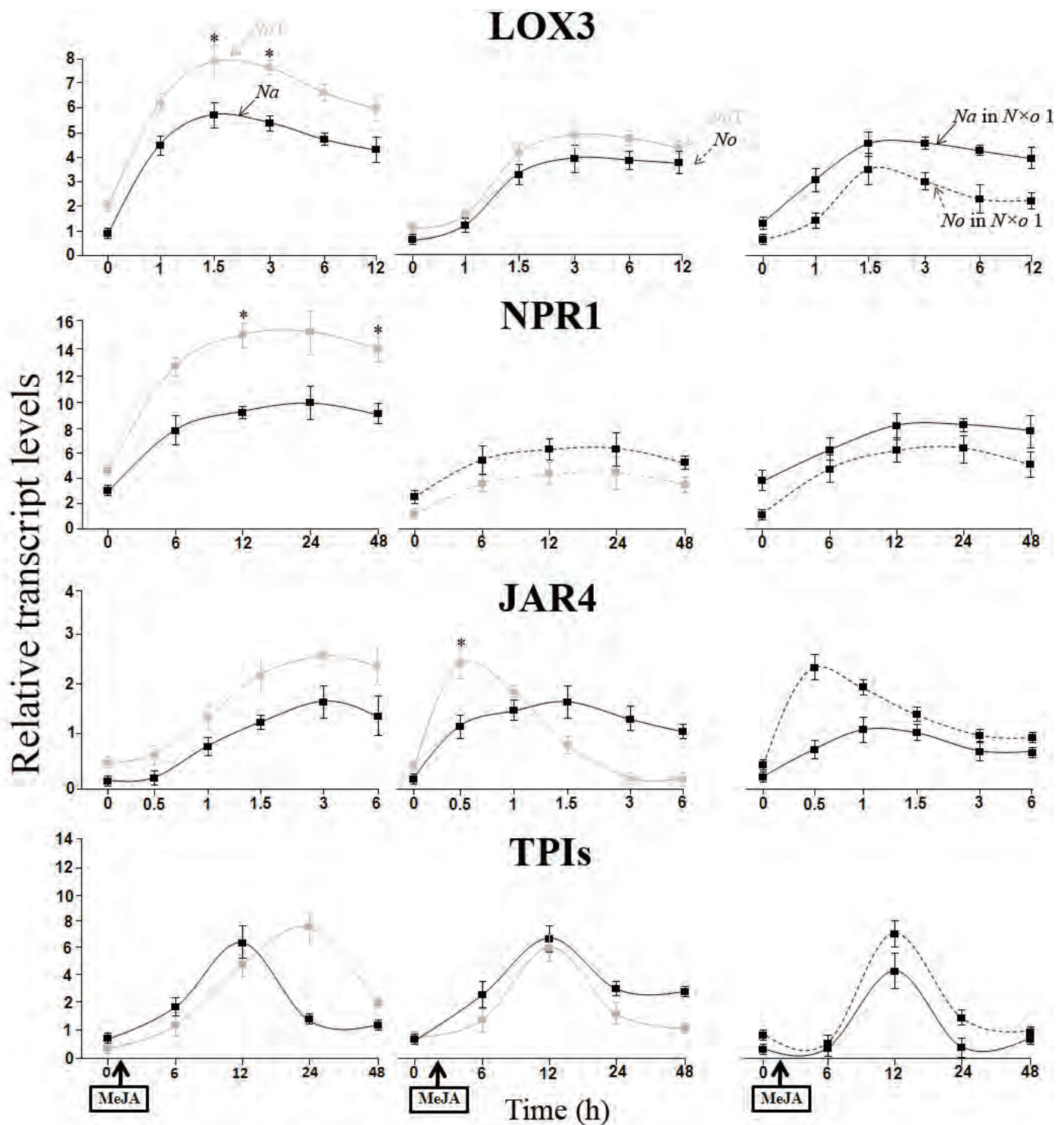
**Figure 6**. Methyl jasmonate treatment of allopolyploid lines elicits transcripts of both parental LOX3, JAR4 and TPIs, demonstrating that the uniparental pattern of transcript accumulation observed after OS elicitation is not due to gene inactivation.

After methyl jasmonate (MeJA) application, all *N×o* lines accumulated both parental lipoxygenase 3 (LOX3), jasmonate-resistant 4 (JAR4) and trypsin protease inhibitor (TPI)

transcripts with a similar pattern to that of the parental lines (Supplemental Fig. S3). Application of MeJA restored the delay in the accumulation of JAR4 and TPI transcripts observed after OS elicitation. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (*$P<0.05$; **$P<0.01$).

### 4.4. Discussion

Polyploidization is thought to provide evolutionary and ecological advantages to plant species over their parental taxa (Adams and Wendel, 2005). A commonly proposed explanation is that polyploidy, by increasing gene redundancy promotes novel functions that allow neo-species to adapt to wide range of habitats, and survive under unfavorable conditions (Otto and Whitton, 2000; Soltis and Soltis, 2000). Studies suggest that the occurrence of new traits in allopolyploids might be the consequence of flexible integration of parental components. Recently, Lou and Baldwin (2003) and Pearse et al. (2006), demonstrated that parental defensive components are reshuffled among allopolyploid lines to generate diverse defensive responses against *M. sexta* attack. However, both of these studies lacked information on the genetic causes of the diversity observed in the defensive responses of the allopolyploids. Here, we investigate how parental anti-herbivore gene expression is altered in synthetic autopolyploid of *N. attenuata* and *N. obtusifolia* (*Na*T and *No*T) and their allo-polyploids lines *N. ×obtusiata* (*N×o* lines 1 to 5) characterized by Anssour et al. (2009), and the consequences of the resulting alterations for plants' defensive responses. The results demonstrate that allopolyploids rapidly generate variability in their anti-herbivore defensive responses by altering the expression of particular parental components involved the herbivore recognition, phytohormone signaling and resistance responses.

In *Na*, the anti-herbivore defensive response is rapidly initiated after the introduction of fatty acid-amino acid conjugates present in *M. sexta* oral secretions (OS) to wounds. Wu et al. (2007) demonstrated that this initial recognition response is associated with a rapid accumulation of wound induced protein kinase (WIPK), lipoxygenase 3 (LOX3) and pathogenesis-related (NPR1) gene transcripts. The comparison of WIPK, LOX3 and NPR1 transcript levels in *Na* and *No* revealed that these two species respond differently to OS elicitation; while *Na* dramatically increases the level of WIPK, LOX3 and NPR1, *No* accumulates reduced levels of WIPK, LOX3 and does not induce the accumulation of NPR1 transcripts. Allopolyploidy induces variability in the accumulation of all parental transcripts in *N×o* lines; some transcripts show an increase in their accumulation (*Na*-WIPK and *No*-LOX3), others a reduction (*No*-WIPK, *Na*-LOX3, *Na* and *No* NPR1) (Fig. 1, Supplemental

Fig. S1). These variations in the transcript accumulation of WIPK, LOX3 and NPR1 genes in the allo-polyploids are probably a consequence of alterations in the regulatory network which controls the specific expression of these genes. Indeed, under OS elicitation, WIPK, LOX3 and NPR1 gene expression is under the control of *cis* and *trans* regulatory elements (Fig. 2), both of which are known to be preferentially associated with epigenetic elements that repress and activate gene expression (Zhang and Borevitz, 2009). These regulatory elements are also known to alter gene expression in other allopolyploid and interspecific hybrid systems (Wang et al., 2004; Wittkopp et al., 2004; de Meaux et al., 2006; Stupar and Springer, 2006; Wang et al., 2006; Chen, 2007)

Herbivore attack results in phytohormone bursts that spread throughout the attacked leaf to trigger defense responses. In *Na, M. sexta* OS elicits a JA burst, and a much more modest response in SA levels, which is known to be down-regulated by an OS-elicited ethylene burst (Diezel et al., 2009) and the activity of NPR1 (Rayapuram and Baldwin, 2007). However, in *No* and the synthetic allopolyploids, OS elicitation induce attenuated levels of JA and a dramatic increase in SA levels, which seems to antagonize JA (Fig. 3, Supplemental Fig. S2).

The elicited JA can be conjugated with various amino acids (Sembdner and Parthier, 1993; Sembdner et al., 1994). Recently, using *Na*, Kang et al. (2006) demonstrated that JA conjugation with amino acids is mediated by jasmonate-resistant 4 (JAR4) (the *Arabidopsis* JAR1 homolog) which adenylates JA before its conjugation. JA-Ile the most abundant of the JA conjugates (Staswick et al., 2002; Staswick and Tiryaki, 2004), is considered as the principle phytohormone elicitor of trypsin proteinase inhibitors (TPIs) production in *Na* (Wang et al., 2007), and its accumulation facilitates the physical interaction between jasmonate ZIM domain and coronatine insensitive proteins to increase downstream anti-herbivore resistance response. In *Na* and *No*, *M. sexta* OS elicits a rapid accumulation of JAR4 transcripts, JA-Ile as well as TPI (transcripts and activity) levels. This resistance response is more pronounced in *Na* than in *No* which accumulates low levels of JA. In the allopolyploid lines, OS elicitation induced the expression of only one parental transcript of JAR4 and TPIs. TPI activity (timing and levels) was variable among the allopolyploid lines; TPI activity in *N×o* lines 1 and 2 was comparable to that in *Na*, whereas TPI activity in lines 3-5 was comparable to that in *No* (Fig. 4, Supplemental Fig. S3).

It is not clear how the variability in the accumulated active TPIs is generated, but statistical comparisons among levels of JA, SA, JA-Ile transcripts, and TPI activity in the allopolyploid lines suggest that this variability is generated during the post-translational

modification of TPI expression. Indeed, comparisons among transcripts and TPI activity levels showed different linear distributions among allopolyploid lines that were separated in three distinct groups: *N×o* lines 1-2, *N×o* lines 3-4 and *N×o* line 5. Correlation analysis conducted on TPI activities and SA levels suggest that variations in TPI activity of *N×o* lines 1- 2 and 3-5 are caused by differences in accumulated SA (Fig. 5). These results agree with previous finding suggesting that SA might be involved in the processing and the maturation of PIs, by eliciting vacuolar proteases (Horn et al., 2005). The levels of SA appear to be differently regulated in *N×o* lines 1- 2 and 3-5; while in *N×o* 3-5, SA accumulation is down-regulated by NPR1, *N×o* lines 1- 2 seem to have adapted a different mechanism (Fig. 5), probably involving ethylene, known to down-regulate SA accumulation. Hence, variations in phytohormone crosstalk (SA/JA and likely ET/SA) can account for much of the expressed TPI activity in the allopolyploid lines.

The accumulation of one or the other parental transcript -- namely, *Na*-LOX3, *No*-JAR4 and *Na*-TPIs in *N×o* lines –in response to OS elicitation was of particular interest, since similar uni-parental expression pattern of homologous genes have been reported in several studies using *Arabidopsis* (Chen et al., 2007), or *Gossypium* allopolyploids (Adams et al., 2004). Here, to understand this transcript accumulation pattern, we initially assumed that the non-expressed parental gene copies in *N×o* lines had lost their functionality due either to chromosomal translocations (or transposition) or to DNA sequence elimination; all these genetic changes have frequently been reported in several allopolyploids (Song et al., 1995; Feldman et al., 1997; Shaked et al., 2001; Skalicka et al., 2005; Tate et al., 2006). However, in response to MeJA treatment, all *N×o* lines accumulated both parental LOX3, JAR4 and TPI transcripts (Fig. 6, Supplemental Fig. S4), suggesting that the uni-parental transcript accumulation pattern observed after OS elicitation was not due to gene inactivation.

Interestingly, unlike OS elicitation, MeJA treatment of the autopolyploids resulted in a gene dosage-dependent increase in the accumulation of LOX3 (in *Na*T and *No*T), NPR1 (only in *Na*T) and JAR4 transcripts (only in *No*T). This increase in transcript accumulation in the autopolyploids might also reflect an increase in expressed gene copies; suggesting that under OS elicitation, the expression of gene homologs in polyploids might be subjected to a selective mechanism which restricts gene expression to specific parental copies. Whether this regulatory mechanism is achieved by specific elements that differentially target one or the other parental copy, or via epigenetic modifications (such as histone methylation or acetylation) is still not clear. However, the ability to recover the expression of the silenced

gene copies under MeJA treatment, strongly suggest that the regulatory mechanism is located upstream of JA production.

Synthetic auto- and allo-polyploidy induced a reshuffling in the accumulation of parental defensive gene transcripts, phytohormones and active TPI levels that mediate the anti-herbivore resistance responses. Allopolyploidy seems to have integrated both the defensive components of *Na* and *No*. However, in response to OS elicitation, *N×o* lines accumulated only one or the other parental defensive transcripts and generated variability in expressed active TPI levels (Fig. 7).
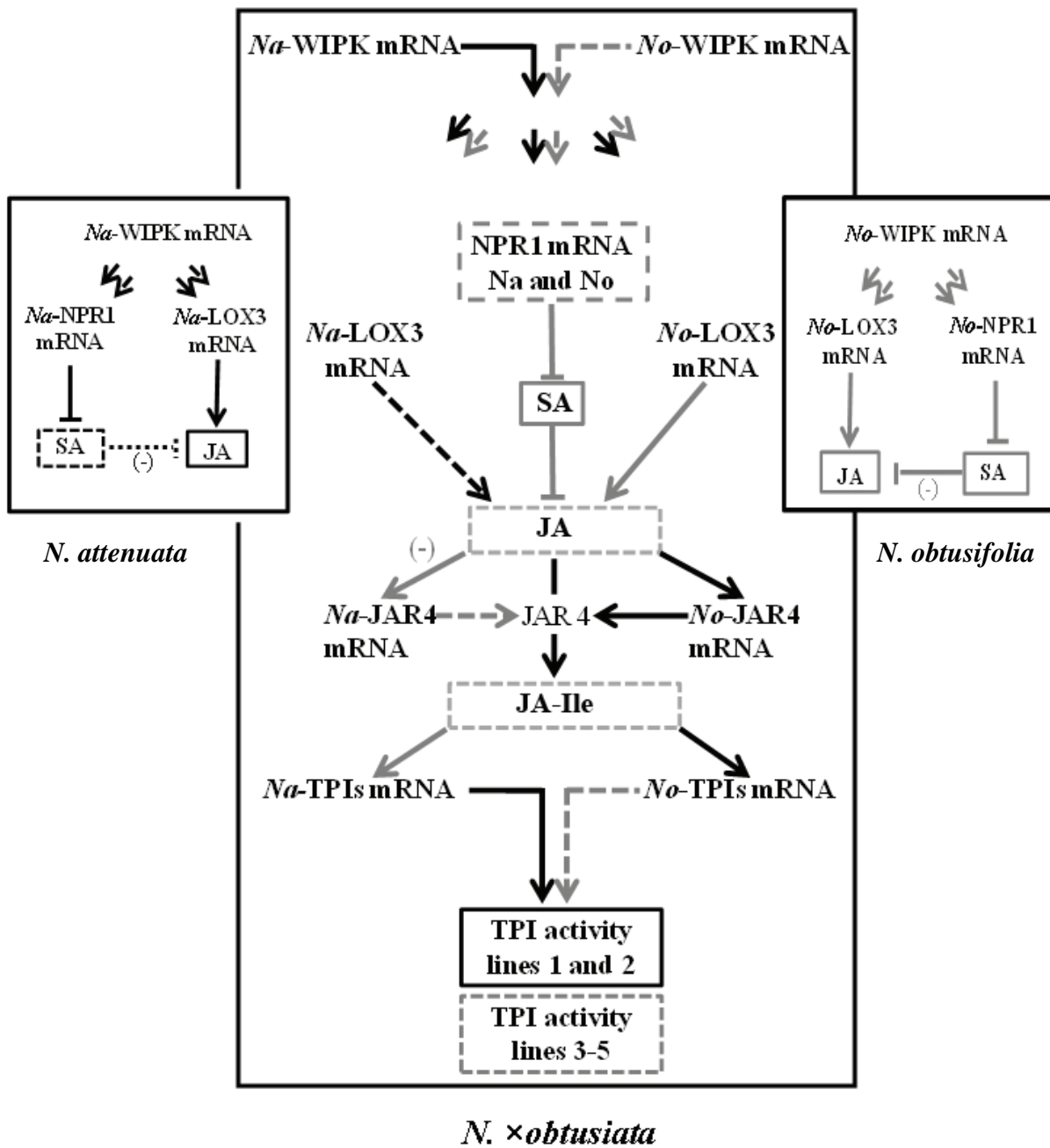
**Figure 7.** Schematic summary of the OS-elicited components of the signal cascade that elicits anti-herbivore defense responses in the synthetic allopolyploid lines.

After elicitation with *M. sexta* oral secretions (OS), *N. ×obtusiata* (*N×o*) lines accumulate high levels of *N. attenuata*-wound induced protein kinase (*Na*-WIPK), *N. obtusifolia*-lipoxygenase 3 (*No*-LOX3), *No*-jasmonate-resistant 4 (*No*-JAR4) and *Na*-trypsin protease inhibitors (*Na*-TPIs), but low levels of *Na*- and *No*-non-expressor of pathogenesis-related (NPR1) transcripts. The low accumulated transcript levels of NPR1 were correlated with an increase in SA and a decrease in JA levels, suggesting SA/JA antagonism in *N×o*

lines. TPI levels were variable among *N×o* lines; the patterns of TPIs accumulation in lines 1 and 2 were similar to that in *Na*, whereas that of lines (3-5) were comparable to that in *No*. This model suggests a rapid readjustment of the expression of *Na* and *No* defensive genes to generate a diversity of anti-herbivore responses. Transcripts or metabolites that accumulated at low levels after OS-elicitation are represented by dashed arrows and lines, while those that accumulated at high levels are represented by solid arrow and lines.

# Summary

## 5. Summary

Polyploidy is a common phenomenon in the plant kingdom. It is estimated that about 30 to 80% of extant species are polyploids (Meyers and Levin, 2006; Rieseberg and Willis, 2007). Large-scale chromosome duplication also shaped the evolutionary histories of several diploid species (Sidow, 1996; Kellis et al., 2004). A remarkable number of what were classically considered as typical diploid plants, such as *Arabidopsis* and maize, are now regarded as ancient polyploids (The Arabidopsis Genome Initiative, 2000; Wolfe, 2001; Cui et al., 2006). Recent evidence suggests that polyploidy can lead to changes in gene expression [through gene dosage effects, altered regulatory interactions, and genetic and epigenetic changes], that frequently results in novel phenotypic variations, enhancing adaptation to a wide range of habitats and unfavorable conditions. This phenotypic diversity often provides polyploids with evolutionary, and ecological advantages over their parental taxa.

The genus *Nicotiana* has many advantages for the study of polyploidization, not only because of its robust phylogenetic framework -- it contains 75 species (Chase et al., 2003; Clarkson et al., 2004), 35 of which are recognized as allopolyploids (Clarkson et al., 2004; Leitch et al., 2008) -- but also for its well-known ecology. *N. quadrivalvis* (*Nq*) and *N. clevelandii* (*Nc*), allopolyploids derived from amphidiploidy involving two diploid ancestors, *N. attenuata* (as the paternal donor) and *N. obtusifolia* (as the maternal donor) approx. 2 Myrs ago (Chase et al., 2003), have been of a particular use in several studies to understand the evolution of polygenic defenses (Lou and Baldwin, 2003; Qu et al., 2004; Wu et al., 2006), and the variation in genome size (Leitch et al., 2008), during polyploidy. These studies revealed that the evolution of *Nq* and *Nc* have been accompanied by an increase in genome size [*N. clevelandii* (*Nc*) and N. *quadrivalvis* (*Nq*) have underwent a genome upsizing (increase in genome size) of 2.5 % and 7.5 %, respectively] and a massive rearrangement of the parental polygenetic defensive components [*Na*'s recognition response were retained with modifications in *Nq*, but many have been lost in *Nc*, whereas the paternal trypsin protease inhibitors (TPIs) gene was completely lost in both *Nq* and *Nc*]. However, when these changes occurred, either directly after polyploidization, or during the intervening 2 Myrs in either parents or allopolyploids remains a mystery. For this, we simulated *Nc* and *Nq* allopolyploid systems, by synthesizing five independent lines of the allotetraploid *N. ×obtusiata* (*N ×o*) [*N. attenuata* (*Na*) (as the paternal donor) × *N. obtusifolia* (*No*) (as the maternal donor)], and autotetraploids of *Na* (*Na*T) and *No* (*No*T).

In the first part of my thesis **(Chapter I),** we examined genetic, genomic and phenotypic changes of the synthetic auto and allopolyploids, and compared them with those of the parental diploid species (*Na* and *No*) as well as to the natural allotetraploids, Nq and *Nc*. 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 *Na*T), but not on stalk height at first flower. *Nc* showed paternal (*Na*) cellular phenotypes but inherited maternal (*No*) biomass and seed mass, whereas *Nq* showed maternal (*No*) 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 clustered *Na*T closer to *Nq* and *N×o* lines than to *Na*. In this thesis part we showed that both allo- and autotetraploidy induce considerable morphological, genetic and genomic changes, many of which are retained by at least one of the natural polyploids. We proposed that both natural and synthetic polyploids are well suited for studying the evolution of adaptive responses.

In the second part of my thesis **(Chapter II),** we examined the expression of *Na* and *No* herbivore-induced genes in synthetic auto and allo- polyploids to understand how the expression of genes regulating complex polygenetic defense traits is altered in the early stages of allopolyploid hybridization. In *Na*, applying *Manduca sexta* oral secretions (OS) to wounds rapidly increased the transcript accumulation of wound-induced protein kinase (WIPK), lipoxygenase 3 (LOX3), non-expressor of pathogenesis-related 1 (NPR1), and jasmonate-resistant 4 (JAR4) genes; these were correlated with increases in accumulation of JA, JA-Ile and TPIs. In *No*, OS elicitation reduced NPR1 transcripts and increased the level of SA which appeared to antagonize JA and JA-mediated defenses. OS elicited *N×o* lines accumulated high levels of the uni-parental transcript of *Na*-WIPK, *No*-LOX3, *No*-JAR4 and *Na*-TPI, but low levels of *Na*- and *No*-NPR1 transcripts. *Na*- and *No*-NPR1 transcript levels were correlated with an increase in SA and a decrease in JA levels, suggesting SA/JA antagonism in the allopolyploid crosses. Methyl jasmonate treatment of *N×o* lines elicited transcripts of both parental LOX3, JAR4 and TPIs, demonstrating that the uniparental pattern observed after OS elicitation was not due to gene inactivation. TPIs were induced in *N×o* lines 1 and 2 at levels

similar to that in *Na*, whereas TPI levels in lines (3-5) were comparable to that in *No*, suggesting that synthetic neo-allopolyploids rapidly readjust the expression of their parental defensive genes to generate diverse anti-herbivore responses. We propose that changes in the expression of key genes and post-transcriptional events likely facilitate adaptive radiations during allopolyploid speciation events.

## 6. Zusammenfassung

Polyploidie ist ein häufiges Phänomen im Pflanzenreich. Geschätzte 30 bis 80% der bekannten Arten sind polyploid (Meyers and Levin, 2006; Rieseberg and Willis 2007). Desweiteren haben auch Duplikationen einzelner Chromosomen die Evolution diploider Spezies beeinflusst (Sidow 1996; Kellis et al. 2004). Eine beträchtliche Anzahl an Pflanzen, die als klassische diploide Arten galten, wie z.B. *Arabidopsis* und Mais, werden heute als ursprünglich polyploid betrachtet (The Arabidopsis Genome Initiative, 2000; Wolfe, 2001; Cui et al., 2006). Jüngste Ergebnisse deuten darauf hin, dass Polyploidie zu Änderungen in der Genexpression führen kann (durch Gen-Dosis-Effekte, Beeinflussung regulatorischer Wechselwirkungen und durch genetische und epigenetische Veränderungen), was oft zu neuen phänotypischen Variationen führt und eine Anpassung an ein breites Habitatsspektrum und an ungünstige Umgebungsbedingungen erlaubt. Diese phänotypische Vielfalt verleiht polyploiden Organismen evolutionäre und ökologische Vorteile gegenüber ihren Elternspezies.

Die Gattung *Nicotiana* bietet viele Vorteile für die Untersuchung von Polyploidie, nicht nur wegen des gut beschriebenen phylogenetischen Stammbaums [die Gattung umfaßt 75 Arten von denen 35 als allopolyploid beschrieben sind (Chase et al., 2003; Clarkson et al., 2004)] sondern auch wegen ihrer umfassend untersuchten Ökologie. *N. quadrivalvis* (*Nq*) und *N. clevelandii* (*Nc*) sind allopolyploide Arten, die durch Amphidiploidie aus den zwei diploiden Vorfahren *N. attenuata* (Vater) und *N. obtusifolia* (Mutter) vor etwa zwei Mio. Jahren entstanden sind (Chase et al., 2003). *Nq* und *Nc* wurden bereits in mehreren Studien verwendet um den Einfluß von Polyploidie auf die Evolution polygener Verteidungsstrategien (Lou and Baldwin, 2003; Qu et al., 2004; Wu et al., 2006) und auf Veränderungen der Genomgröße zu untersuchen (Leitch et al.,2008). Dabei kamen die Autoren zu dem Schluss, dass die Evolution von *Nq* und *Nc* mit einer Vergrößerung des Genoms von 2.5 bzw. 7.5% und einer massiven Umgestaltung der elterlichen polygenetischen Verteidigungsmechanismen einherging (die spezifische Signalantwort von *Na* auf bestimmte Herbivoren wurde in abgeänderter Form in *Nq* erhalten, während sie in *Nc* größtenteils verloren ging; das väterliche TPI-Gen hingegen ging sowohl in *Nq* als auch in *Nc* verloren). Ob diese Veränderungen jedoch direkt nach der Polyploidisierung oder erst im Verlauf der folgenden zwei Mio. Jahre aufgetreten waren blieb ungewiß. Aus diesem Grund simulierten wir die Allopolyploidie von *Nc* und *Nq* durch die Herstellung synthetischer

polyploider Linien: der allotetraploiden *N. ×obtusiata* (*N ×o*) [*N. attenuata* (*Na*) (als Vater) × *N. obtusifolia* (*No*) (als Mutter)] und der jeweils autotetraploiden Nachkommen von *Na* (*Na*T) und *No* (*No*T).

Im ersten Teil meiner Arbeit (Kapitel I) untersuchten wir die genetischen, genomischen und phänotypischen Veränderungen der synthetischen auto- und allopolyploiden im Vergleich zu den diploiden Elternarten (*Na* und *No*) und den natürlichen Allotetraploiden *Nq* und *Nc*. DNA-„Fingerabdruck"-Profile (durch UP-PCR) ergaben, dass die fünf *N×o*-Linien ähnliche aber nicht identische Profile aufwiesen. Synthetische und natürliche Polyploidie hatte einen Dosis-abhängigen Effekt auf die Genomgröße (gemessen in den Samen), wobei allerdings nur *Nq* mit der Genomvergrößerung in Beziehung stand. Analysen auf zellulärer Ebene ergaben, dass die *N×o* -Linien intermediäre Phänotypen ihrer Elternspezies aufwiesen. Sowohl Allo- als auch Autotetraploidisierung hatten einen Dosis-Effekt auf Samenmasse und Trockenbiomasse (außer bei *Na*T), allerdings hatten sie keinen Einfluß auf die Stammhöhe zur Zeit der ersten Blüte. *Nc* wies väterliche (*Na*) zelluläre Phänotypen auf aber vererbte mütterliche (*No*) Biomasse und Samenmasse, während hingegen *Nq* mütterliche (*No*) zelluläre Phänotypen aber vererbte väterliche (*Na*) Muster von Biomasse und Samenmasse zeigte. In einer Hauptkomponenten-Analyse gruppierte *Nq* aufgrund der ähnlichen Samenmasse, der Stammlänge und der Genomgröße mit den *N×o* -Linien. Dieselben Eigenschaften unterschieden *Nc*, *No* und *Na* von *Nq* und den *N×o* -Linien, wobei die Biomasse *Na* von *N×o* - und den *Nq*-Linien trennte und *Na*T näher mit *Nq* und den *N×o* -Linien gruppierte als mit *Na*. In diesem Kapitel konnten wir zeigen, dass sowohl Allo- als auch Autotetraploidie beträchtliche morphologische, genetische und genomische Veränderungen hervorrufen, von denen viele zumindest in einer der natürlichen Polyploiden Arten erhalten wurden. Wir betrachten daher sowohl natürliche als auch synthetische Polyploide als gut geeignet um die Evolution adaptiver Merkmale zu untersuchen.

Im zweiten Teil meiner Arbeit (Kapitel II) untersuchten wir die Expression Herbivorie-induzierter Gene von *Na* und *No* in den synthetischen Auto- und Allopolyploiden um allgemein zu verstehen wie die Expression von Genen, die komplexe polygene Merkmale regulieren, durch Polyploidisierung beeinflußt wird. Die Applikation oraler Sekrete (OS) von *Manduca sexta* auf mechanisch verwundete Blätter von *Na* führt u.a. zu einer schnellen Akkumulation von Transkripten der Gene „wound-induced protein kinase" (WIPK), „lipoxygenase 3" (LOX3), „non-expressor of pathogenesis-related 1" (NPR1) und

jasmonate-resistant 4" (JAR4). Diese tran skriptionelle Antwort korreliert mit einer Akkumulation von Jasmonsäure (JA), Jasmonsäure -Isoleucin (JA-Ile) und Trypsin-Protease-Inhibitoren (TPIs). In *No* hingegen reduzierten die oralen Sekrete NPR1-Transkripte und erhöhten die Mengen an Salicylsäure (SA) , welche der Wirkung von JA und der JA-abhägigen Verteidigung entgegenzu wirken schien. OS-induzierte *N×o*-Linien produzierten groß Mengen der uniparentalen Transkripte *Na*-WIPK, *No*-LOX3, *No*-JAR4 und *Na*-TPI aber nur geringe Mengen von *Na*- und *No*-NPR1. *Na*- und *No*-NPR1 Transkripte korrelierten mit einer Zunahme von SA und einer Abnahme von JA, was erneut auf einen SA/JA-Antagonismus in den allopolyploiden Kreuzungen hinweist. Eine Behandlung der *N×o*-Linien mit Methyljasmonat induzierte die Transkripte von LOX3, JAR4 und TPI von beiden Eltern in ähnlichem Ausmaß was darauf hi ndeutet, dass das beobachtete uniparentale Expressionsmuster nach OS-Induktion nicht auf eine spezifische Inaktivierung von Genen zurückzuführen ist. Bezüglich der TPI-Aktivitä entsprachen die *N×o*-Linien 1 und 2 *Na*, wärend die Linien 3 bis 5 eher mit *No* vergleichbar waren. Dies legt nahe, dass synthetische Neo-Allopolyploide die Expression der elterlichen und für die Verteidigung relevanten Gene rasch neu anpassen um diverse Verteidigungsantworten gegen Herbivoren hervorzubringen. Wir folgern daraus, dass solche Expressionsäderungen von Genen mit Schlüsselfunktionen und post-transkriptionelle Vorgäge eine adap tive Radiation wärend der allopolyploiden Artbildung begünstigen.

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## 9. Declaration of independent work

I declare in accordance with the conferral of the doctoral degree from School of Biology and Pharmacy of Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

The people who assisted in the experiments, data analysis and writing the manuscripts are listed as coauthors of the manuscript and acknowledged in the thesis. I was not assisted by any consultant for doctoral theses.

The thesis has not been previously submitted either to the Friedrich Schiller University or to any other University.

Jena, April 1, 2010

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Samir Anssour

## 11. Supplementary material

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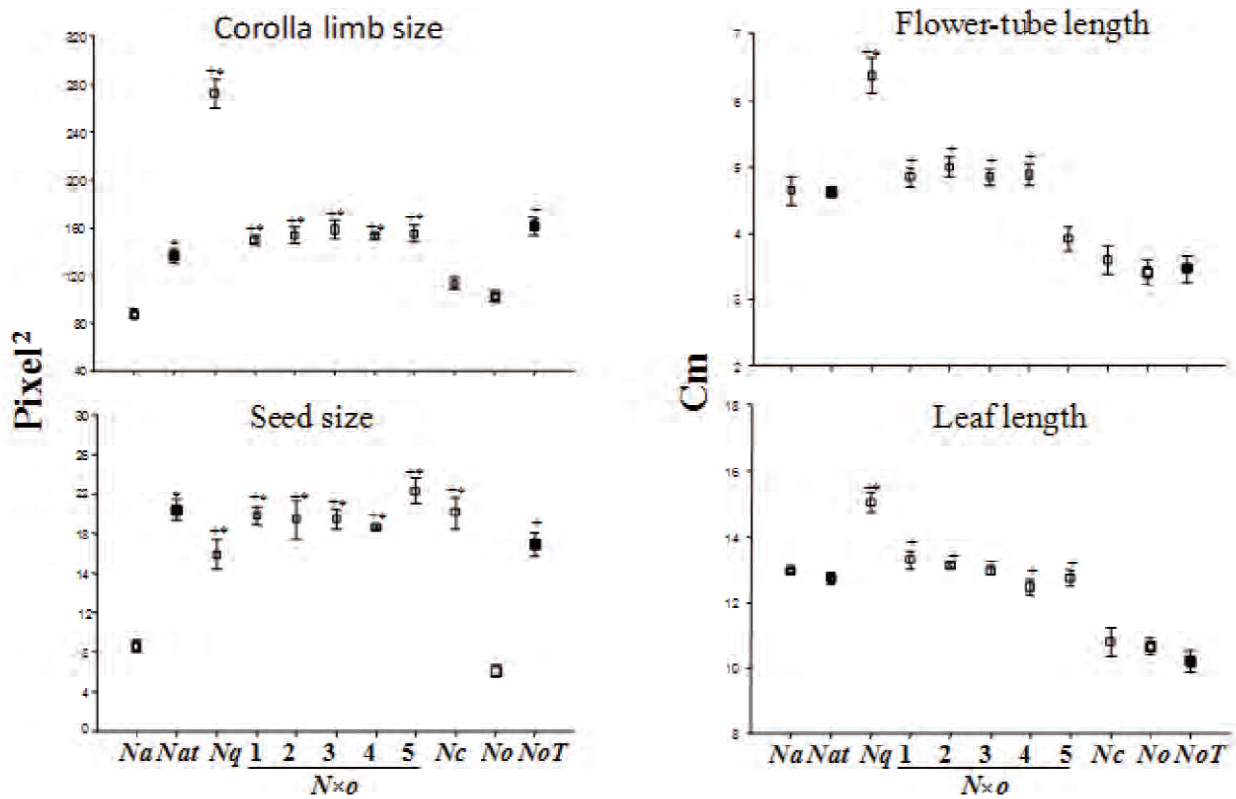


 **Figure. S1.** Corolla limb size, seed size, flower tube and leaf length were measured in *N. attenuata* (*Na*), *N. attenuata* autotetraploid (*Na*T) ($F_5$), *N. obtusifolia* (*No*), *N. obtusifolia* autotetraploid (*No*T) ($F_4$), *N × obtusiata* (*N×o*) (lines 1–5, $F_5$), *N. clevelandii* (*Nc*) and *N. quadrivalvis* (*Nq*). Digital pictures of corolla limbs and seeds were taken from each studied species, and sizes were measured using the Axio Vision LE software and expressed in Pixel$^2$ (1 pixel = 0.26 mm)
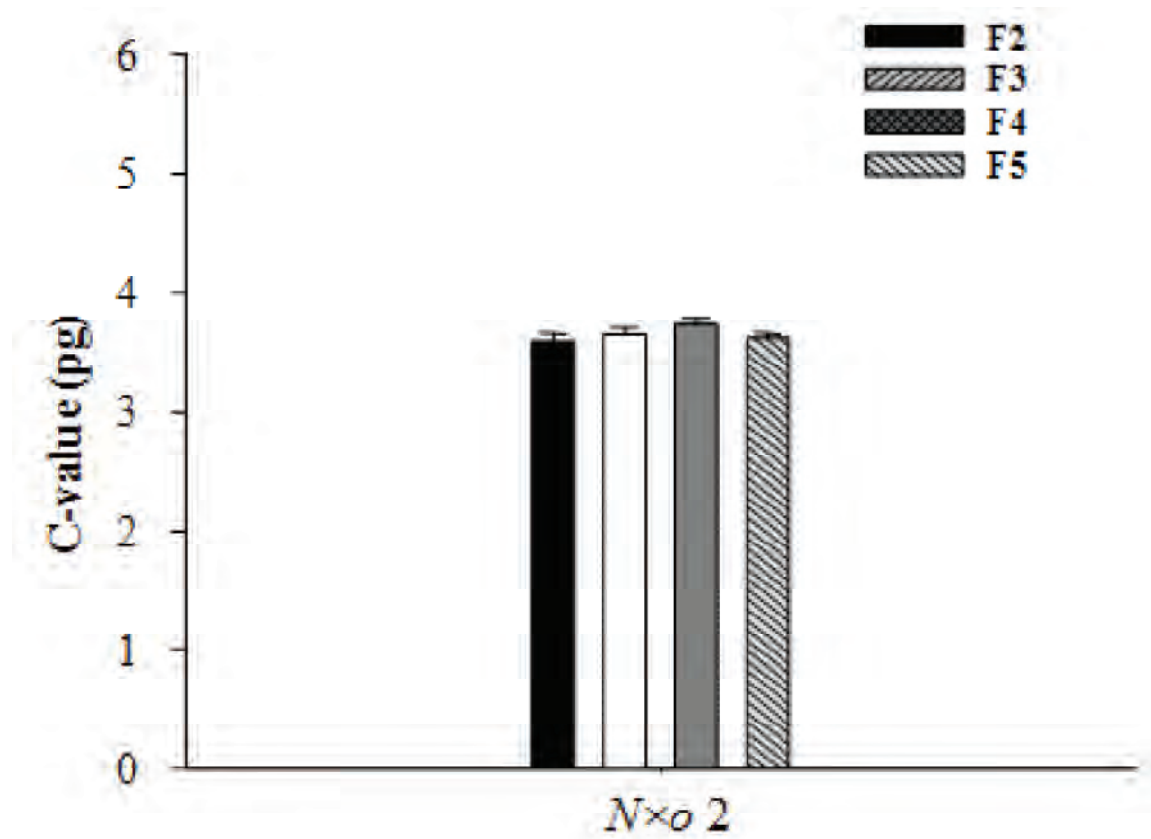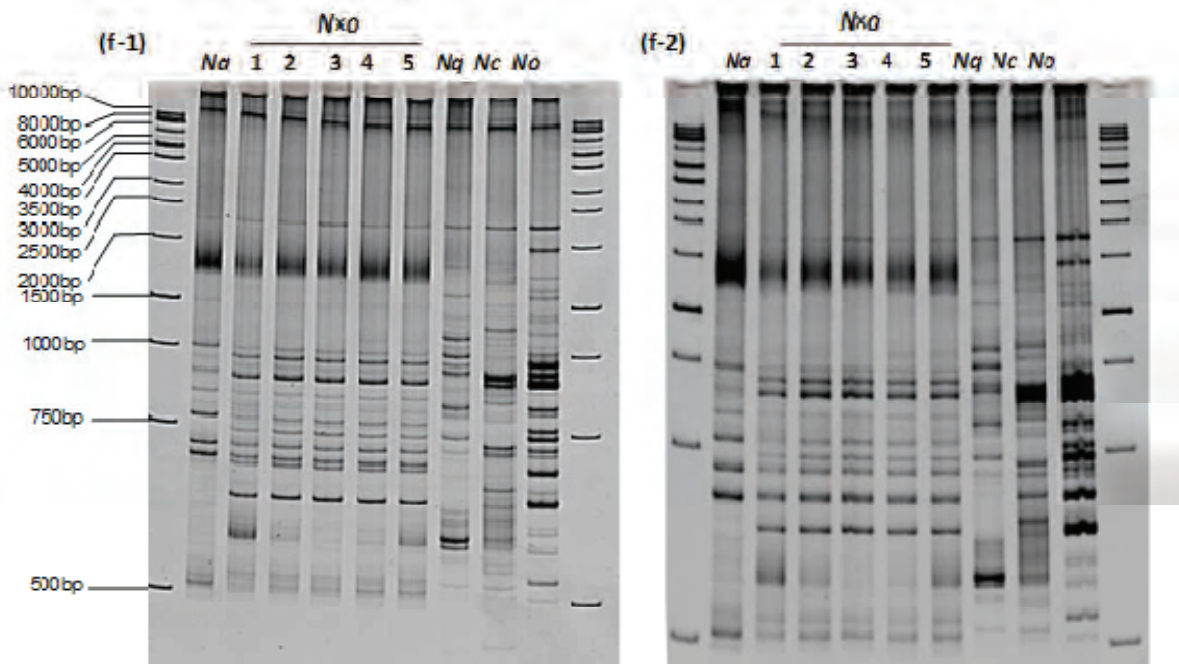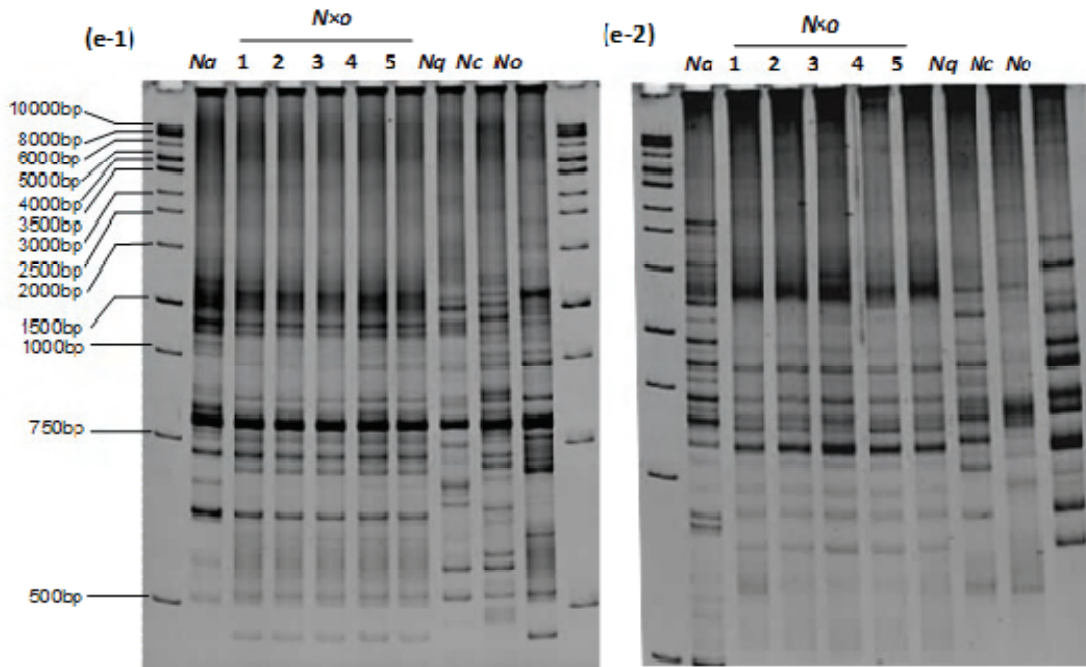
**Figure. S2.** Genome sizes (n = 10) measured in seeds of *N. × obtusiata* (*N×o*) lines 2 ($F_2$ to $F_5$) using flow cytometry for single seeds (FCSS).
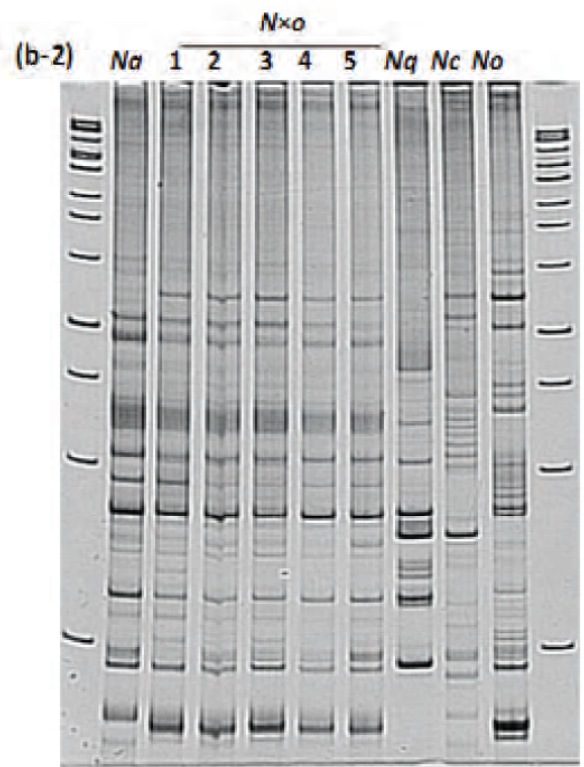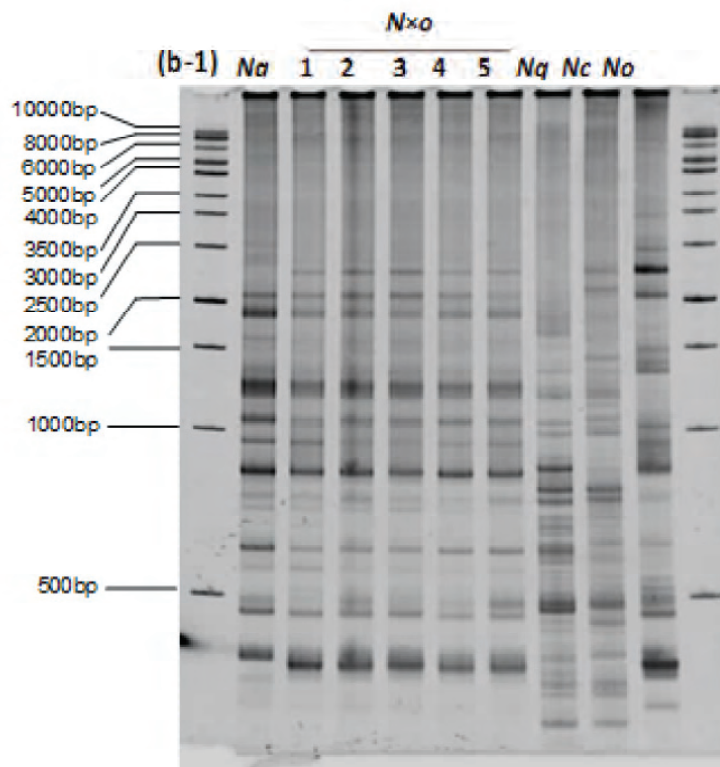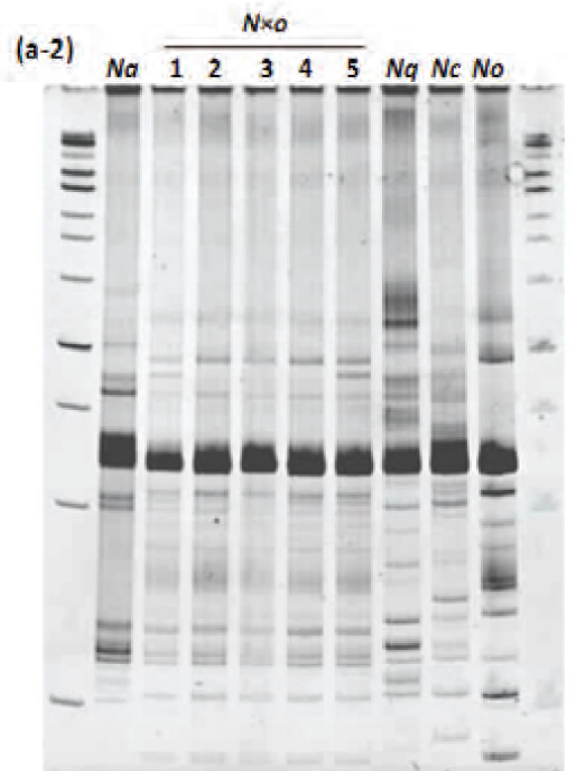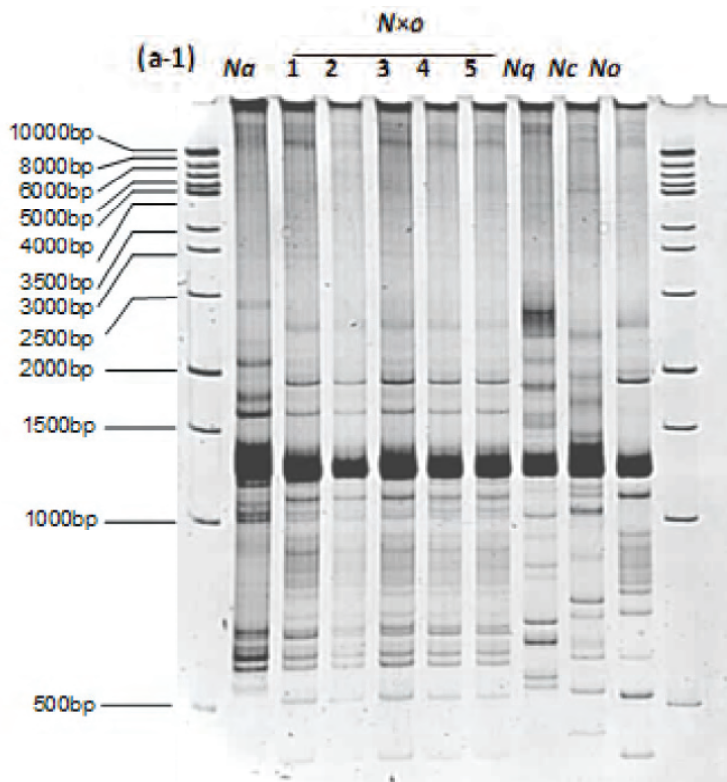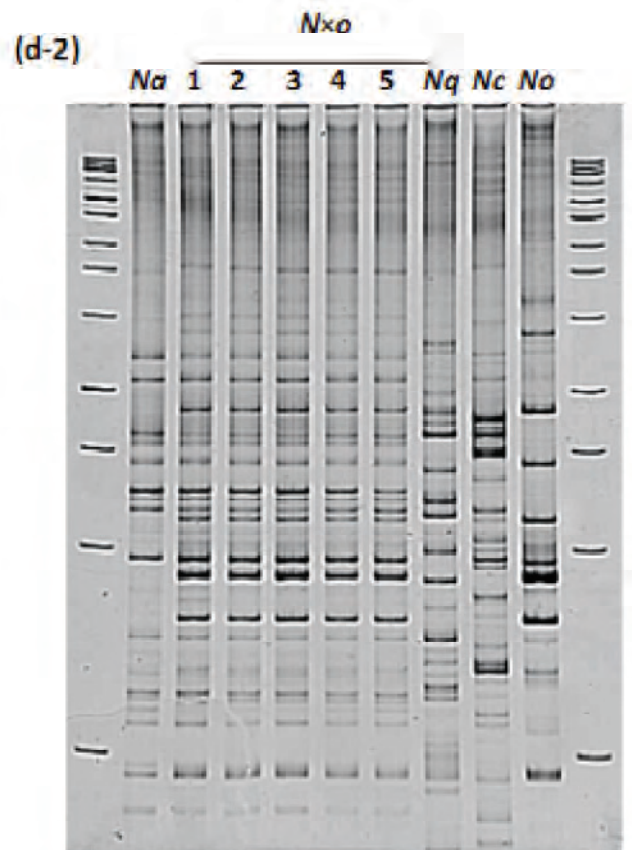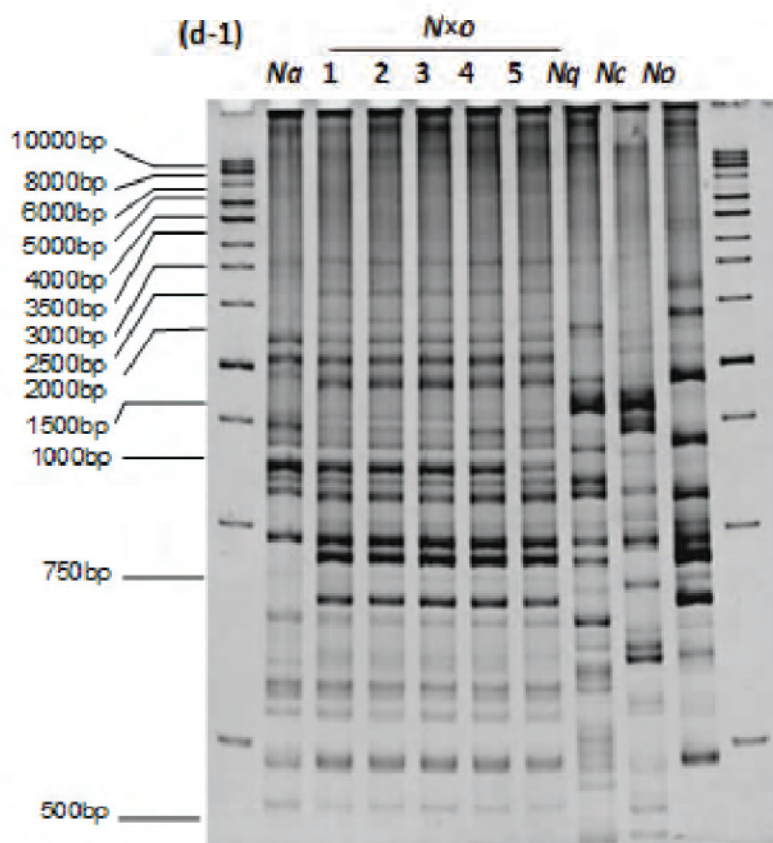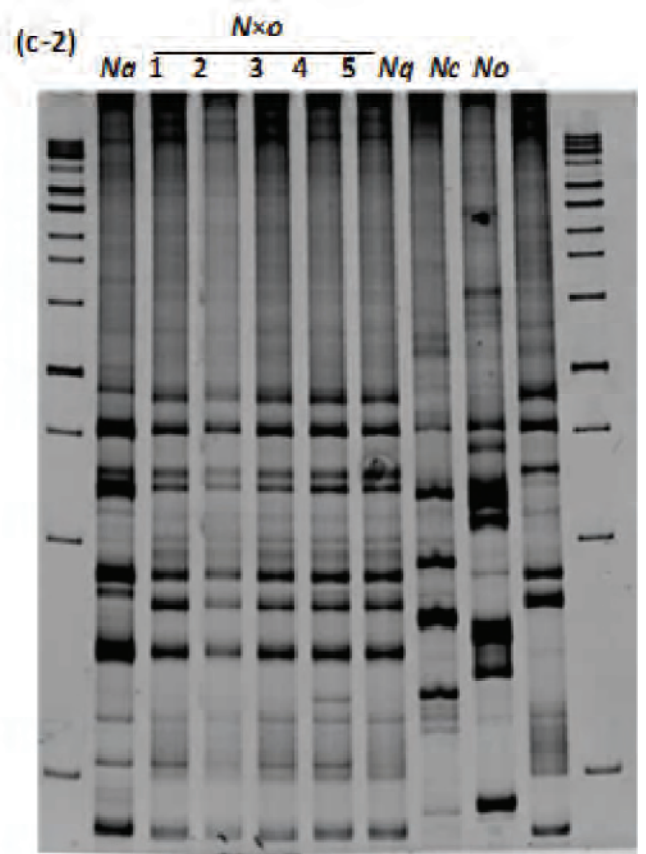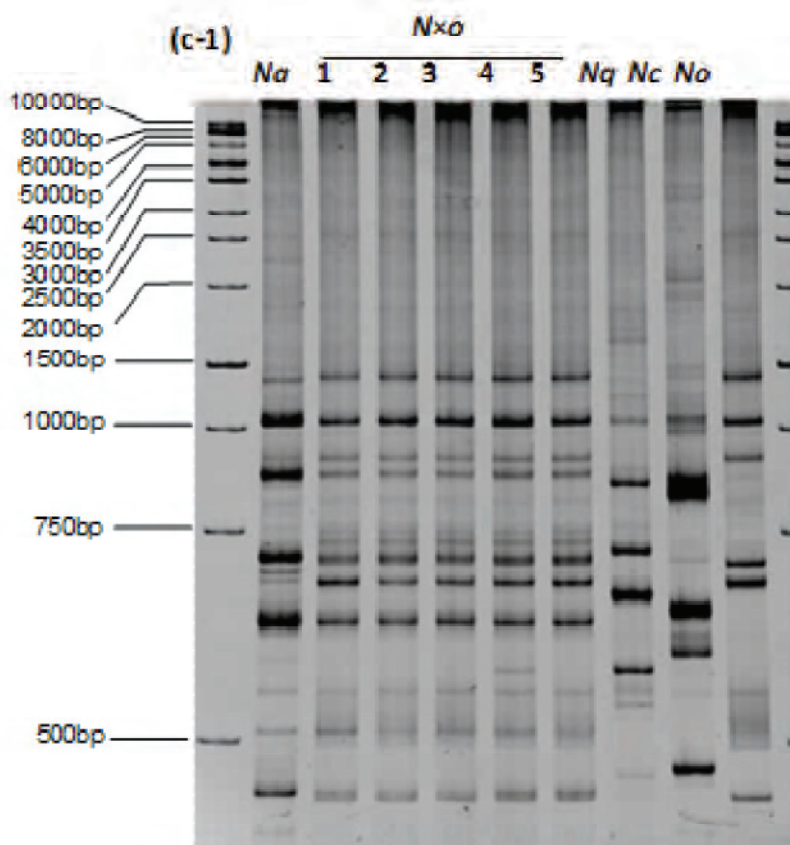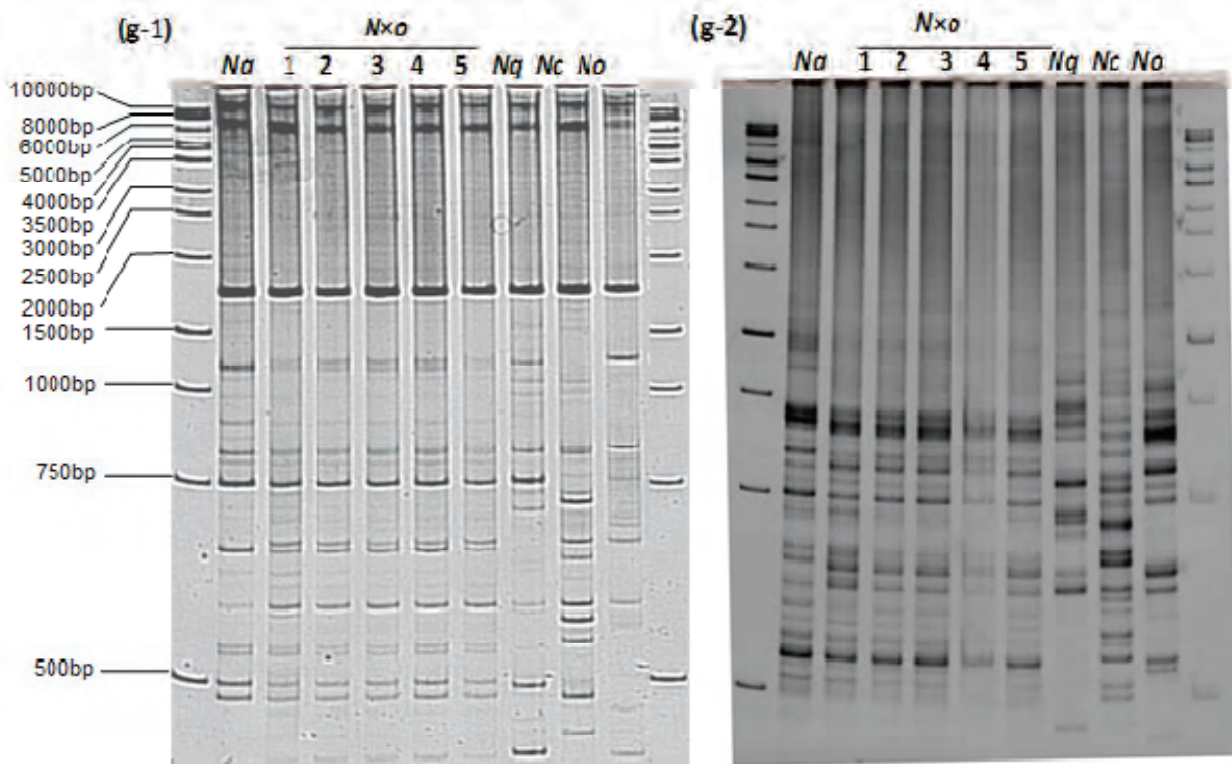
**Figure. S3.** UP-PCR DNA fingerprinting profiles of *N. attenuata*, *N. × obtusiata* lines 1–5, *N. quadrivalvis*, *N. clevelandii*, *N. attenuata* and *N. obtusifolia*. The figures represent UP-PCR DNA fingerprinting profiles of two biological replicates of the above-cited species using seven universal primers (see Table S1).

| Primer | Sequence |
|--------|----------|
| (a) L21 | 5'-GGA GAG GGT GGC GGT TCT-3' |
| (b) L21i | 5'-GGA TCC GAG GGT GGA TCT -3' |
| (c) 2M2 | 5'-CTG CGG ACC CAG ACC CAG AGC GG-3' |
| (d) AS15 | 5'-CAT TGC TGG CGA ATC -3' |
| (e) AS15i | 5'-GGC TAA GCG GTC CTT AC-3' |
| (f) AS4 | 5'-TGT GGG CGC TCG ACA-3' |
| (g)M13i | 5'-GAG GGT GGT GGA TCT-3' |

**Table S1.** Sequences of universal primers used in UP-PCR DNA fingerprinting profiles of *N. attenuata*, *N. × obtusiata* lines 1–5, *N. quadrivalvis*, *N. clevelandii*, *N. attenuata* and *N. obtusifolia*.

**Chapter II. Variation in anti-herbivore defense responses in synthetic *Nicotiana* allopolyploids correlates with changes in uni-parental patterns of gene expression**
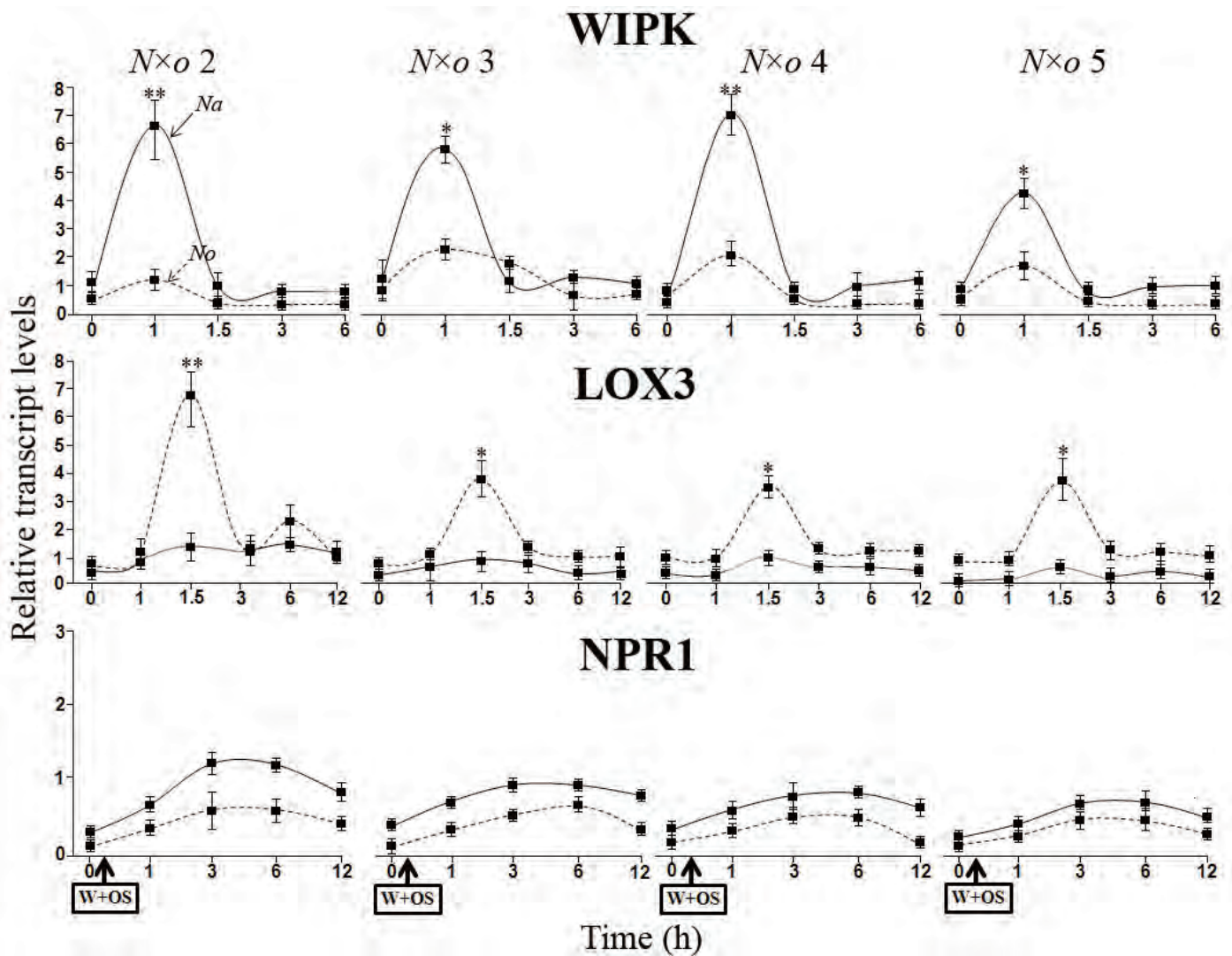


**Supplemental Figure S1**. OS elicitation enhances the accumulation of uni-parental transcript patterns of WIPK and LOX3, but not NPR1 in *N×o* lines (2-5).
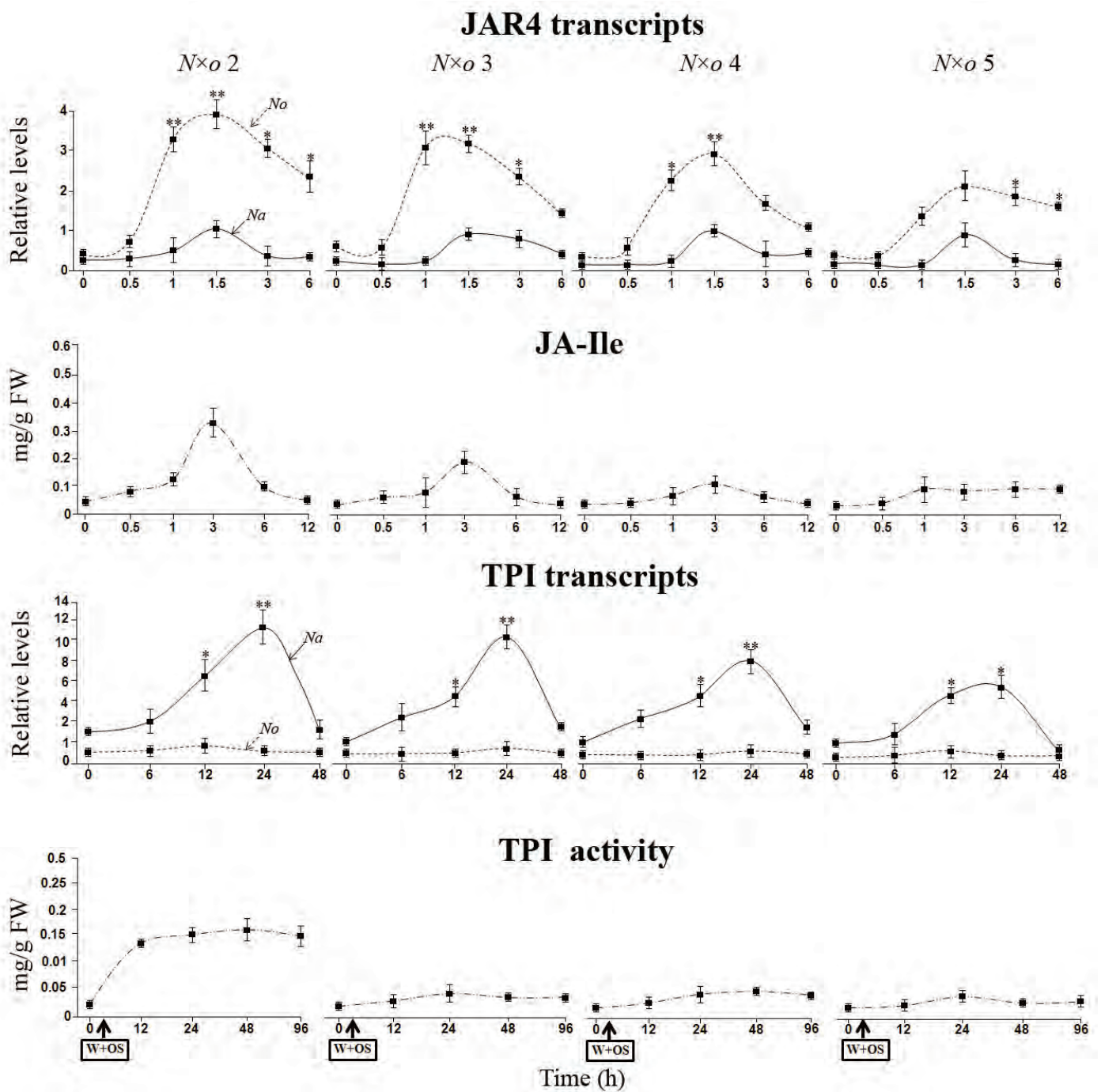
After elicitation with *M. sexta* oral secretions (OS), *N. ×obtusiata* (*N×o*) lines 2-5 induced an increase in the accumulation of *N. attenuata*-wound induced protein kinase (*Na*-WIPK), and *N. obtusifolia*- lipoxygenase 3 (*No*-LOX3), and attenuated levels of *Na*-LOX3, *Na* and *No*- non-expressor of pathogenesis-related (NPR1) transcripts. Asterisks indicate

levels of significant difference between the maximum values of the pairs plotted together on the same graph (*$P<0.05$; **$P<0.01$).
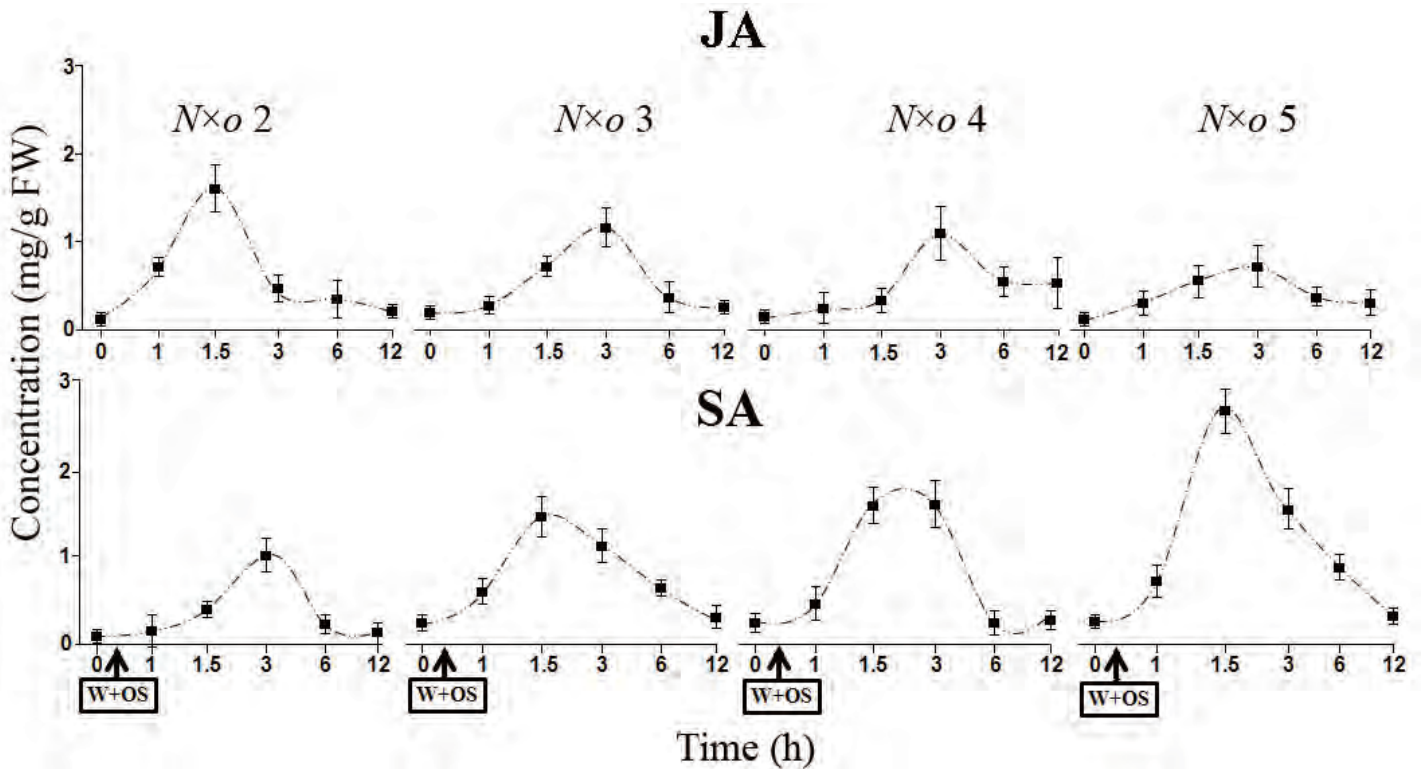


**Supplemental Figure S2**. Patterns of OS-elicited SA and JA accumulation in $N\times o$ 2-5 after OS elicitation.

After elicitation with *M. sexta* oral secretions (OS), *N. $\times$obtusiata* ($N\times o$) lines 2-5 accumulated high levels of *salicylic acid* (SA) and low levels of jasmonic acid (JA). Only the accumulation of JA was associated with a delay in the synthetic polyploids compared to their parental lines.

**Supplemental Figure S3**. *N×o* lines (2-5) enhance the accumulation of only one parental transcript of JAR4 and TPI, and accumulated different levels of JA-Ile and TPI activity after *M. sexta* OS elicitation.

After elicitation with *M. sexta* oral secretions (OS), *N. ×obtusiata* (*N×o*) lines 2-5 accumulated only *N. obtusifolia*-jasmonate-resistant4 (*No*-JAR4) transcripts and *Na*-trypsin

protease inhibitor (*Na*-TPI) transcripts, but not that of *Na*-JAR4 and *No*-TPIs. The maximum levels of accumulated jasmonic acid–isoleucine (JA-Ile) and active TPIs were different among *N×o* lines. All polyploid lines showed a delay in the accumulation of JAR4, JA-Ile as well as transcript and active TPI levels compared to their parental lines. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (**P<0.01).
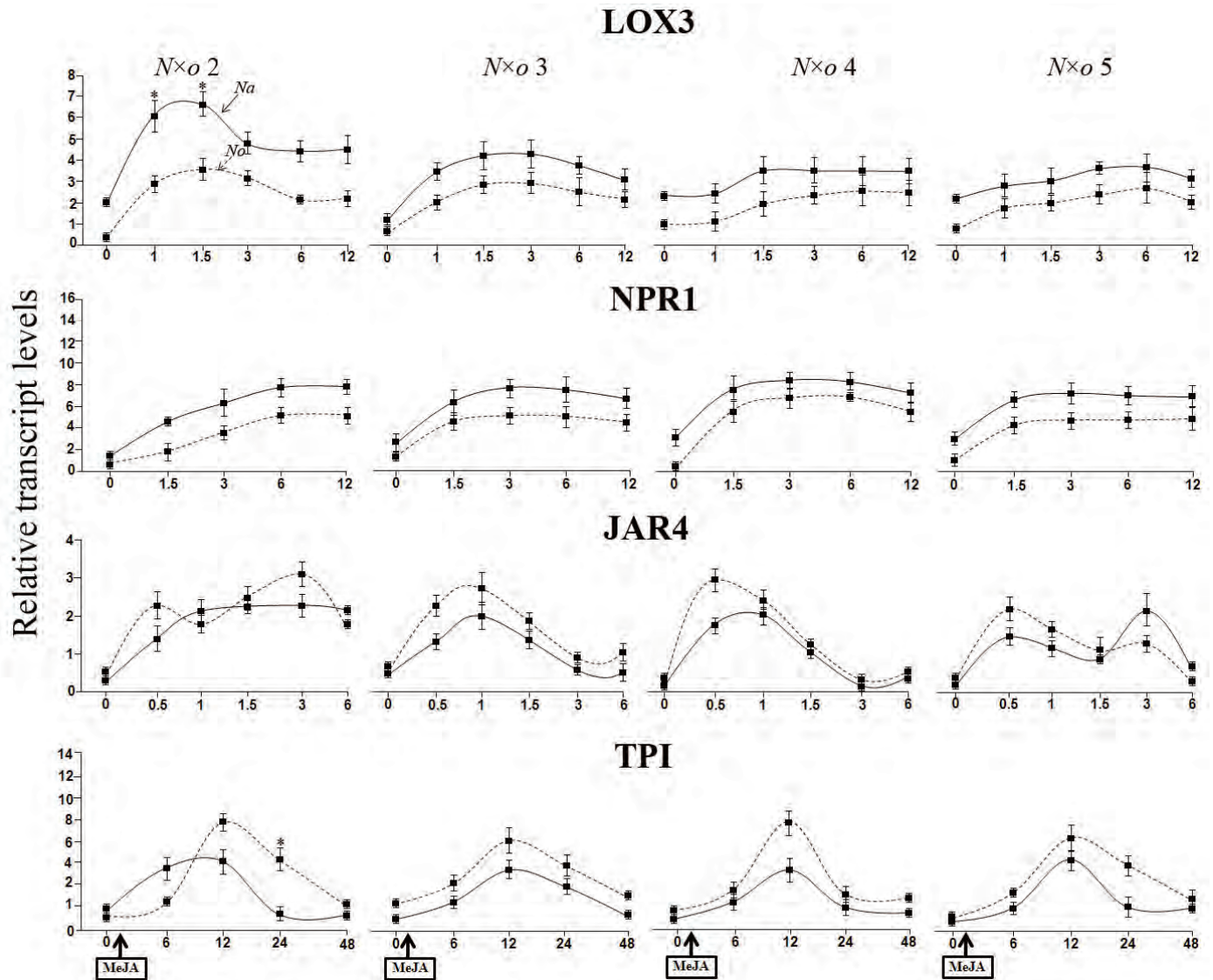


**Supplemental Figure S4**. Methyl jasmonate treatment of *Na*, *No* and their respective autotetraploids elicit gene dosage-dependent increase in the accumulation of LOX3 (in *Na*T and *No*T) and JAR4 (in only *Na*T) transcripts.

Applying methyl jasmonate (MeJA) to *N. attenuata* (*Na*) and *N. obtusifolia* (*No*) did not induce changes in the timing and levels of the accumulation of wound induced protein kinase (WIPK), lipoxygenase (LOX3) and non-expressor of pathogenesis-related (NPR1)

transcripts compared to what was observed for these genes in OS elicited plants. Autopolyploidy induced dosage-dependent increase in the accumulation of LOX3 (in *Na*T and *No*T) and JAR4 (in only *Na*T) transcripts. All values were normalized with actin as an internal standard. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (**P<0.01).
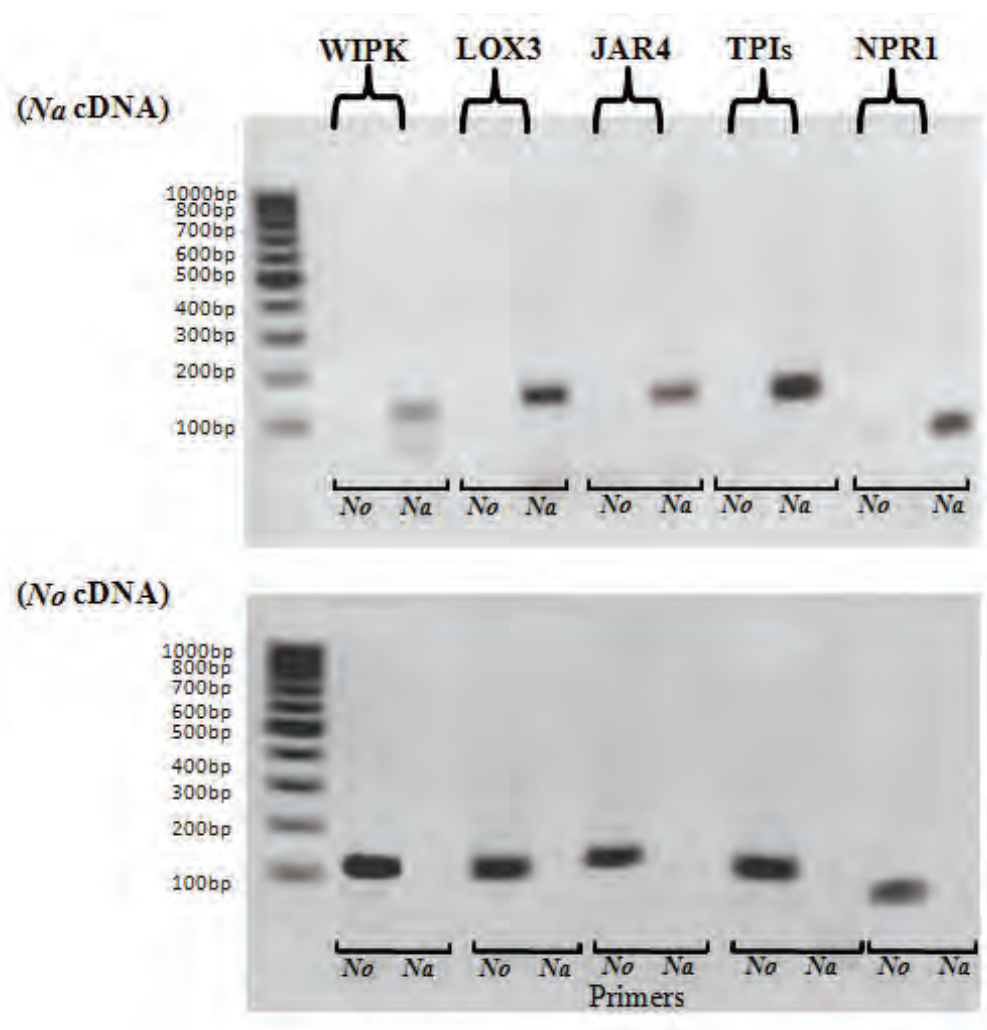


**Supplemental Figure S5.** PCR products amplified in *Na* and *No* cDNA using RT primer pairs specific for *Na* and *No*-WIPK, LOX3, JAR4, TPIs and NPR1.

PCR products (10 µL/well) were run in a 1.5% agarose gel and visualized with ethidium bromide staining.

| | *N. obtusifolia* partial sequences |
|---|---|
| **WIPK** | 5′-gatcactgtcagtacttcatgtatcagcttctccgtggcctaaagtacattcattccgcgaatgtccttcatagagatct caaaccgagcaacctttgttaaatgcaaattgtgatcttaagatatgtgactttggtcttgctaggccaaacataga gaatgagaatatgacagaatatgttgtaaccagatggtacagggcaccagagctttgttgaactcttcggattaca ctgctgccatagacgtttggtctgtggtgtatcttcatggagcttatgaatagaaaacccttgtttgctggaaaaga tcatgtacatcaaatacgcttgctaactgagcttctcggcaccccaacagaatctgatcttgacttcctccaaaatga agacgcaaagagatacatcaggcaactcccgcaacatcctcgccagcagttagcagaagtttccctcatgtgaat ccattggccattgatcttgtagataaaatgttaacattcaatcctactagaagaattacagttgaggaagcattagct catccctaccttgcaaagctccacgatgcaggtgacgaac -3′ |
| **LOX3** | 5′-ctggatccgcatatgagatacacactagaaattaacgcattggctcgccagagcttgatcagtgccgatggtgtaatc gaggcttgttttactcctggtcgttattgcatggagatgagtgctgctgcctacaagaatttttggcgttttgatttggaa ggcctccctgctgacccttatcagaagagggatggcagtaccggacccaacacagcctcatggactgaaacttctaat agaggactatccctatgcggcagatggactcatgatatgggccgcaatagagggctgggttcgcagctatgtaaatc tctactaccctgactcggcccgagtttgcaatgacagagaactccaagcctggtatgccgagtccattaacgtgggcc acgcagacctccgaaatgcagaatggtggcccacgttagctactccagaggatctcatttcgatcctcaccactctcat ctggctagcttcagcgcaacatgcttcgctgaatttcggccagtacccatacggtggctacgtcccgaaccggccacc tctcatgcg- -3′ |
| **JAR4** | 5′-cataggctccagaccgtctaaattttccatgagaggaatgaattcgaaataaccaatattaggaagtactgcatatgta acaagctcgggaggcaactcggggttaatgtttgctccaatccacccttcagaagatccatagtctgcacttagcagag gtaactcgcccgcataatgcctcagtttcttcaagtaaggttccatggatccagtcatgataccatatatgtacctagtgtt ggggaaaagttcaggtatcaagccataccaattgctcaatcggctgcacttgttaaaaatagtatcagcaagctcggg atcaggcttgagtaatttcgacattactgctctcatagaaggaaccgtcacacggctagaaaggattccttctcttatgtt ggtaacaagttcttgccatacttgttcgaaatttcgaaaagcatggacaatactatgggcaaatgtagaggagacaact tgaacctcatcgcgaaaaatgaggccgcacaaaagatgacagtacaaagattgttggaaatcagggccaaatatcac ttcatcagggctacaacaaggggtctgcatttccctcattgtcttcttgaattgtatgtttcggtacacattggtagtggca gttccag -3′ |
| **NPR1** | 5′cagatgttgctaagagagggacatagtactctagatgatgcatatgctctccattatgctgtagcatattgcgatgcaa agactacagcagaacttctagatcttgcacttgctgacattaatcatcaaaattcaagaggatacacggtgctgcatgtt gcagctatgaggaaagagcctaaaattatagtgtcccttttaaccaagggagctaaaccttctgatctgacatccgatgg cagaaaagcacttcaa |
| **Actin** | 5′gtattttaactcttaaatacccaattgagcatggaattgtcagcaactgggatgatatggagaagatctggcatcatacttt ctacaatgagcttcgtgttgcgcccgaggagcatccagtcctcttaactgaggcgcctcttaacccaaaggctaatcgtgaaa agatgacccagattatgtttgagactttaatacccagctatgtatgttgctattcaggctgtcctctcacgtatgccagtggt cgtaccaccggtattgtgttggactctggtgatggtgtcagccacaccgtcccaatttatgaggggtatgccctcccacatgcc attctccgtcttgacttggcaggtcgtgacctcactgatagtttgatgaagatccttaccgagcgtggttacatgttcaccacct cagctgagcgggaaattgtcagggacgtgaaagaaaagcttgcttacatagctcttgactatgaacaggaactcgagactg caaagaccagctcttctgtagagaagaactatgagctcccggatgggcaggtgatcaccattggtgctgagcgtttccgttgt cctgaggtcctttttccaaccatcaatgattggaatggaagctgc3′ |

**Supplemental Table S** Partial sequences of *N. obtusifolia* WIPK, LOX3, JAR4, NPR1 and actin cDNA.