Variation in Antiherbivore Defense Responses in Synthetic *Nicotiana* Allopolyploids Correlates with Changes in Uniparental Patterns of Gene Expression[1][W][OA]

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We examined the expression of *Nicotiana attenuata* (*Na*) and *Nicotiana obtusifolia* (*No*) herbivore-induced genes in synthetic autopolyploids (*Na*T and *No*T) and five independent allopolyploid *Nicotiana* × *obtusiata* (*N*×*o*) lines 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), nonexpressor of pathogenesis-related 1 (NPR1), and jasmonate-resistant 4 (JAR4) genes; these were correlated with increases in accumulation of jasmonic acid (JA), jasmonate-isoleucine, and trypsin protease inhibitors (TPIs). In *No*, OS elicitation reduced NPR1 transcripts and increased the level of salicylic acid (SA) that appeared to antagonize JA and JA-mediated defenses. OS elicited *N*×*o* lines, accumulated high levels of the uniparental transcript of WIPK, LOX3, JAR4, and TPI, but low levels of both parental NPR1 transcripts that in turn 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 at different levels among *N*×*o* lines; some lines expressed high levels comparable to *Na*, others low levels similar to *No*, suggesting that synthetic neoallopolyploids rapidly readjust the expression of their parental defensive genes to generate diverse antiherbivore responses. Changes in the expression of key genes and posttranscriptional events likely facilitate adaptive radiations during allopolyploid speciation events.

Polyploidy is an ongoing evolutionary process that generates new plant species. Estimates suggest that polyploidy is responsible for 2% to 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), for example, alfalfa (*Medicago sativa*) and potato (*Solanum tuberosum*), or from allopolyploidy (union of distinct genomes), for example, wheat (*Triticum aestivum*), oat (*Avena sativa*), cotton (*Gossypium hirsutum*), coffee (*Coffea arabica*), and canola (*Brassica napus*; Wendel, 2000). Other crops such as corn (*Zea mays*; Gaut and Doebley, 1997), soybean (*Glycine max*; Shoemaker et al., 1996), and cabbage (*Brassica capitata*; Lagercrantz and Lydiate, 1996) appear to have experienced polyploidization events in their evolutionary histories and are referred to as paleopolyploids. The model plant Arabidopsis (*Arabidopsis thaliana*), a typical diploid, is now considered as a paleopolyploid (Arabidopsis Genome Initiative, 2000). The widespread occurrence of polyploids presumably reflects their greater ability to evolve and adapt to new environments compared to their parental taxa. Allopolyploid lineages appear to undergo adaptive radiations more frequently than autopolyploid lineages. Indeed, by fusing two distinct genomes, allopolyploidy facilitates heterosis that offers a greater potential for adaptive diversification (Adams and Wendel, 2005; Flagel et al., 2008); nevertheless, the success of allopolyploids is unexpected given that the neospecies must be able to survive to the genomic shock associated with the fusion of two distinct genomes and to coherently express the parental polygenetic traits.

The last decade witnessed impressive advances in polyploidy research, with new results from Arabidopsis (Comai et al., 2000; Pontes et al., 2004), *Brassica* species (Lukens et al., 2006; Gaeta et al., 2007), cotton (Jiang et al., 1998), wheat (Han et al., 2003), and *Nicotiana* species (Lim et al., 2006; Pearse et al., 2006; Anssour et al., 2009). Most of these studies have focused on the genetic and genomic changes associated with the

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formation of polyploids, especially changes directly altering gene expression patterns, such as rapid and nonrandom changes including sequence elimination, changes in the DNA loci, and intergenomic cross talk, for example, transposon activation, chromosomal rearrangements, and chromosomal breaks (Kenton et al., 1993; Kitamura et al., 1997; Chase et al., 2003; Lim et al., 2004; Leitch et al., 2008). 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 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. *Nicotiana quadrivalvis* (*Nq*) and *Nicotiana clevelandii* (*Nc*), allopolyploids derived from amphidiploidy involving two diploid ancestors, *Nicotiana attenuata* (*Na*; as the paternal donor) and *Nicotiana obtusifolia* (*No*; as the maternal donor) approximately 2 million years ago (Chase et al., 2003), have been particularly useful for understanding how complex polygenic traits evolve. Lou and Baldwin (2003) 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 protease inhibitor (TPI) genes, which encode for protease inhibitors that reduce *M. sexta* performance, were retained in both *Nc* and *Nq*, whereas paternally inherited (*Na*) TPI genes were deleted. How-

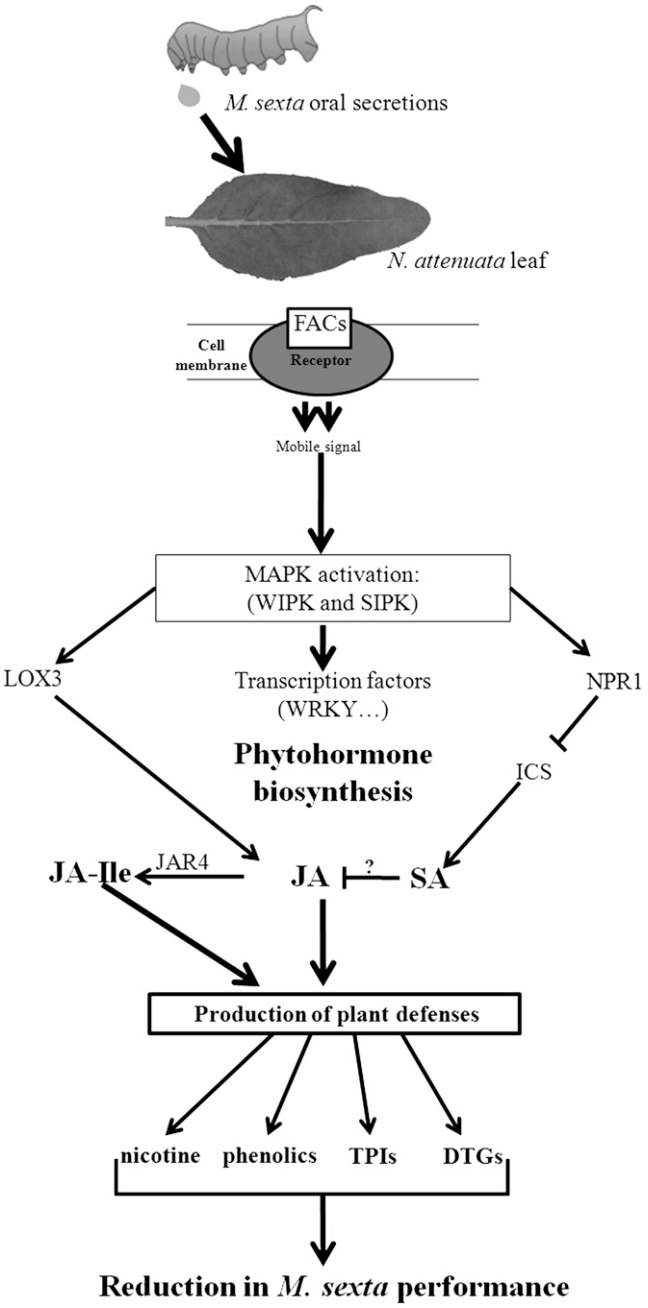

Figure 1. An overview of the OS-elicited signaling cascade that activates direct defenses in *Na*'s leaves depicting the elements that were studied in the synthesized tetraploid and allopolyploid *Nicotiana* species. During herbivore attack by *M. sexta* larvae, FACs from larval OS bind to hypothetical receptors in the cell membrane at the attack site and activate unknown short-distance mobile signals. These signals activate mitogen-activated protein kinases that include SA-induced protein kinases and WIPKs that phosphorylate transcription factors (such as WRKYs), which in turn activate phytohormone signaling such as JA, SA, and JA-Ile and their associated biosynthetic genes such as LOX3, isochorismate synthase (ICS), and JAR4. By inhibiting ICS, NPR1 negatively regulates SA production and thereby SA/JA antagonism, allowing the expression of JA-mediated direct defenses such as nicotine, phenolics, TPIs, and diterpene glycosides (DTGs) that diminish the performance of *M. sexta* larvae feeding on elicited plants.

ever, when these changes occurred, either directly after polyploidization or during the intervening 2 million years in either parents or allopolyploids, remains a mystery. Answering this question requires an understanding of the changes that occur rapidly after neopolypoloidization 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 *Nicotiana miersii* as a maternal surrogate for *No* (the species most phylogenetically related to *No*) to create the synthetic neoallopolyploid, *Nicotiana* × *mierata*. By eliciting plants with OS and methyl jasmonate (MeJA) and comparing the changes in TPI activity, secondary metabolites, and released volatile organic compounds 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* antiherbivore gene expression, phytohormone accumulation, and TPI activity in five independent lines of the allotetraploid *Nicotiana* × *obtusiata* (*N*×*o*) (*Na* [as the paternal donor] × *No* [as the maternal donor]), and autotetraploids of *Na* (*Na*T) and *No* (*No*T) characterized by Anssour et al. (2009). We first dissect the specific transcript accumulation (levels and timing) of some of the early antiherbivore responsive genes in the OS-elicited signaling cascade (Fig. 1), namely, wound-induced protein kinase (WIPK), lipoxygenase 3 (LOX3), nonexpressor of pathogenesis-related 1 (NPR1), and jasmonate-resistant 4 (JAR4) of *Na* and *No*. Then, we measure the phytohormone levels of JA, jasmonate-Ile (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 antiherbivore signaling and resistance genes and in the levels of phytohormones and TPI activity among the synthetic polyploid lines are compared to their parental lines.

RESULTS

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

Na's antiherbivore 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 mitogen-activated protein kinases, WIPK and SA-induced protein kinase, which in turn stimulate the expression of NPR1 and JA biosynthetic genes, such as LOX3 (Fig. 1). To understand how the expression of these early antiherbivore responsive genes are altered after synthetic auto- and allopolyploidy, 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.

Na and *No* responded differently to OS elicitation; *Na* dramatically increased the transcript levels of WIPK (reaching a maximum at 1 h after OS elicitation), LOX3 (a maximum level at 1.5 h), and NPR1 (a maximum level at 2.5 h), whereas *No* accumulated much lower levels of these transcripts with similar patterns as in *Na*. The autotetraploids, *Na*T and *No*T, followed a similar pattern of WIPK, LOX3, and NPR1 transcript accumulations and did not differ significantly compared to their respective diploids. After OS elicitation, the allopolyploid *N*×*o* lines induced both parental WIPK and NPR1 transcripts, but showed uniparental accumulation for LOX3 transcripts; in this concern all *N*×*o* lines expressed *No*-LOX3 transcripts, but did not induce any *Na*-LOX3 transcripts.

To get an insight into the genetic interactions acting on the expression of LOX3, WIPK, and NPR1 genes, 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 (Supplemental Fig. S2).

In short, *Na* and *No* responded differently to OS elicitation; *Na* dramatically increased the transcript levels of WIPK, LOX3, and NPR1, whereas *No* accumulated much lower levels.

In the autopolyploids, the transcript accumulation of WIPK, LOX3, and NPR1 followed a pattern similar to that observed in the parental lines. All allopolyploid lines favored the accumulation of *Na*-WIPK and *No*-LOX3 transcripts. Finally, we suggest that both cis- and trans-regulatory elements act on the expression of WIPK, LOX3, and NPR1 gene in the allopolyploid lines.

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 among 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 3 h, 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 and Baldwin, 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 1 h after elicitation. The highest level of SA in *No*, *No*T, and *N*×*o* lines was about 4 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. S3).

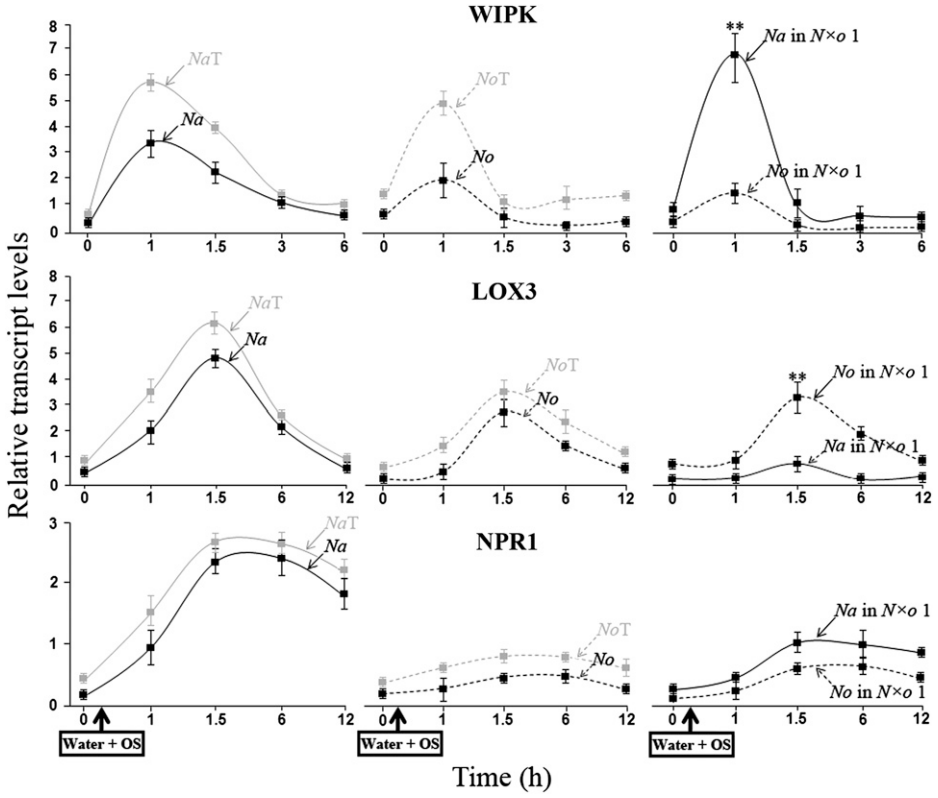

Figure 2. OS elicitation induces uniparental transcript accumulation of WIPK and LOX3, but not NPR1, in the synthetic allopolyploids. After elicitation with *M. sexta* OS, *Na* dramatically increases the levels of WIPK, LOX3, and NPR1 transcripts, whereas *No* accumulates comparatively lower levels of 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*×*o* 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 SYBR Green PCR. For this, single leaves from seven to eight 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 (*$P < 0.05$; **$P < 0.01$).

In short, OS elicitation resulted in a JA burst in *Na* but an SA burst in *No*, and the allopolyploids followed a pattern similar to that observed in *No*. Only *Na* autopolyploids show a dosage-dependent increase in OS-elicited JA. Correlations among levels of JA and SA in *Na*, *No*, and *N*×*o* lines suggest SA/JA antagonism in the allopolyploid crosses.

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 1 h, this level had reached a maximum and was higher in *Na* than in *No*. *Na*T and *No*T showed a dosage-dependent increase in the levels of JAR4 compared to the levels in their respective diploids. *N*×*o* lines accumulated *No*-JAR4, but not *Na*-JAR4. Both auto- and allopolyploids showed a delay of 0.5 h 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 1.5 and 3 h, 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 12 h. TPI transcripts showed a dosage-dependent increase in *Na*T compared to *Na*, whereas in *No*T the accumulation of TPI transcripts was reduced. *N*×*o* lines accumulated *Na*-TPIs, but not *No*-TPIs. Both *No* and *N*×*o* lines showed a delay of 12 h 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 24 h; this level was 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

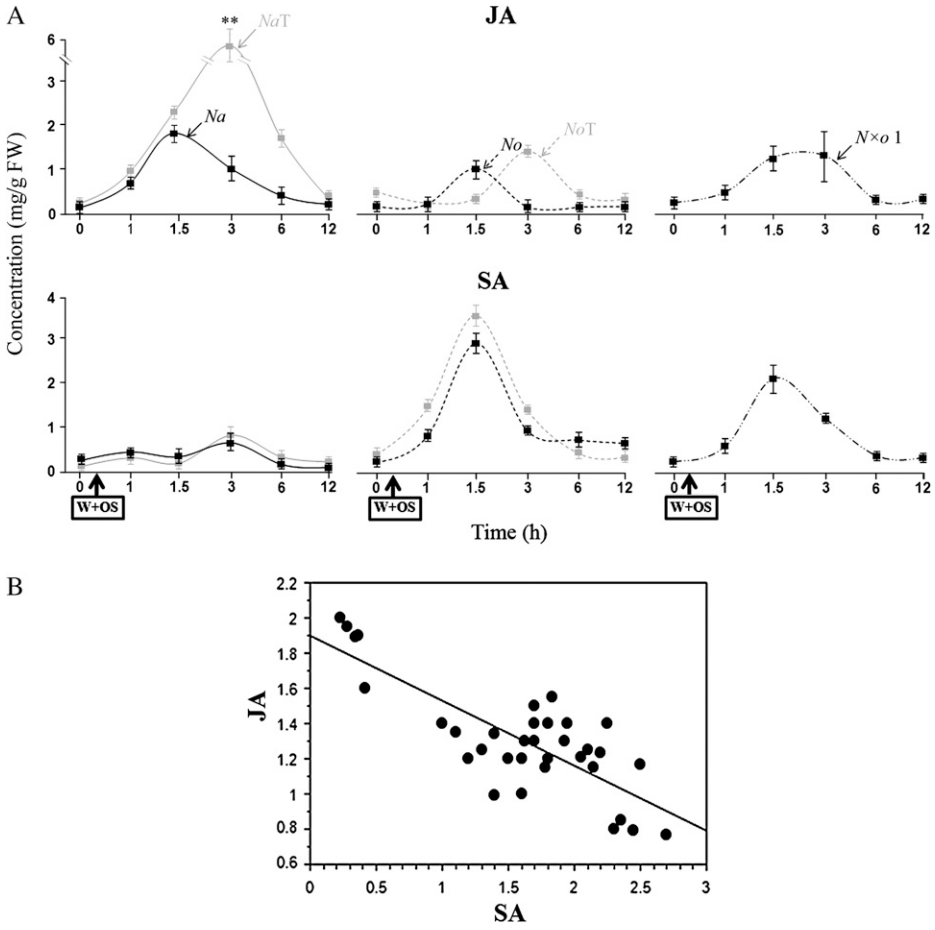

Figure 3. Patterns of OS-elicited SA and JA accumulation reveal SA/JA antagonism in the allopolyploid lines. A, OS elicitation in *Na* and *Na* tetraploid (*Na*T: left sections) elicited strong JA bursts and attenuated SA bursts. In contrast, OS elicitation of *No*, *No* tetraploid (*No*T; center sections), and *N*×*o* line 1 (*N*×*o* 1; right sections) 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).

to 5, TPI activity was at basal levels comparable to that in *No* (Fig. 4; Supplemental Fig. S4).

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 that clustered in three distinct groups: $N\times o$ lines 1 and 2, $N\times o$ lines 3 and 4, and $N\times o$ line 5. Comparisons among levels of TPI activity and SA revealed that differences in SA influence TPI activity; $N\times o$ lines 1 and 2 were distributed in distinct groups based on differences in SA levels. Finally, comparisons among levels of SA and NPR1 transcripts suggest that $N\times o$ 1 and 2 and $N\times o$ 3 and 5 may react differently to variation in NPR1 transcript levels; while in $N\times o$ lines 3 to 5, SA accumulation is down-regulated by NPR1, $N\times o$ lines 1 and 2 seem to have adapted a different mechanism, probably involving ethylene.

In short, after OS elicitation, autopolyploidy induced a dosage-dependent increase in the accumulation of JAR4 transcripts in both *Na* and *No*; in *Na*, this dosage effect was correlated with an increase in JA-Ile levels, TPI transcripts, and TPI activity. Whereas in *No*, there was no gene dosage effect beyond the accumulation of JAR4 transcripts. The accumulation patterns of JA-Ile, TPI transcripts, and active TPIs were similar between diploid and autotetraploid lines. All allopolyploid lines accumulated *No*-JAR4 and *Na*-TPI transcripts and increased levels of JA-Ile and TPI activity in response to OS elicitation. In the allopolyploid lines, SA was negatively correlated with JA and JA-Ile, and JA-Ile was positively correlated with TPI transcript levels. The allopolyploids accumulated variable levels of active TPIs due to differences in SA levels.

MeJA Elicitation of $N\times o$ Lines Reveals That OS-Elicited Uniparental 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

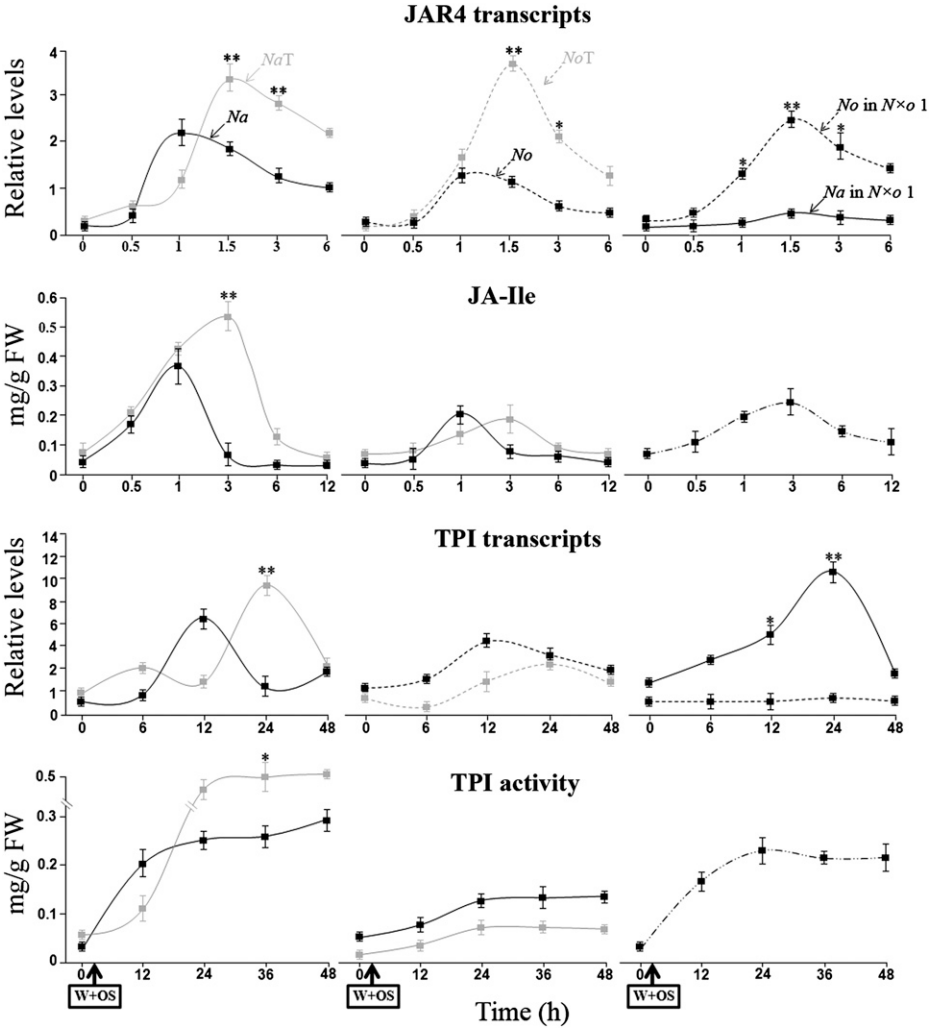

Figure 4. OS elicitation enhances the accumulation of *No*-JAR4 and *Na*-TPI transcripts, and increases the levels of JA-Ile and TPI activity in the allopolyploid lines. After elicitation with *M. sexta* OS, *Na* and *No* rapidly accumulate JAR4 transcripts and JA-Ile levels, as well as transcripts and levels of active 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 the autopolyploid compared to the diploid *No*). $N\times o$ line 1 ($N\times o$ 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 (*$P < 0.05$; **$P < 0.01$).

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 nonexpressed 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, NPR1, and 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. S5), demonstrating that these parental gene copies are functional.

In short, MeJA treatment resulted in a gene dosage-dependent increase in the accumulation of LOX3, NPR1, and JAR4 in *Na*T and in the accumulation of only JAR4 transcripts in *No*T. Treatment of allopolyploid lines with MeJA elicited both *Na* and *No* LOX3, JAR4, and TPI transcripts, demonstrating that the uniparental pattern of these genes observed after OS elicitation was not due to gene inactivation.

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 neospecies 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 antiherbivore gene expression is altered in synthetic autopolyploid of *Na* and *No* (*Na*T and *No*T) and their allopolyploid lines *N*×*o* (lines 1–5) 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 antiherbivore defensive responses by altering the expression of particular

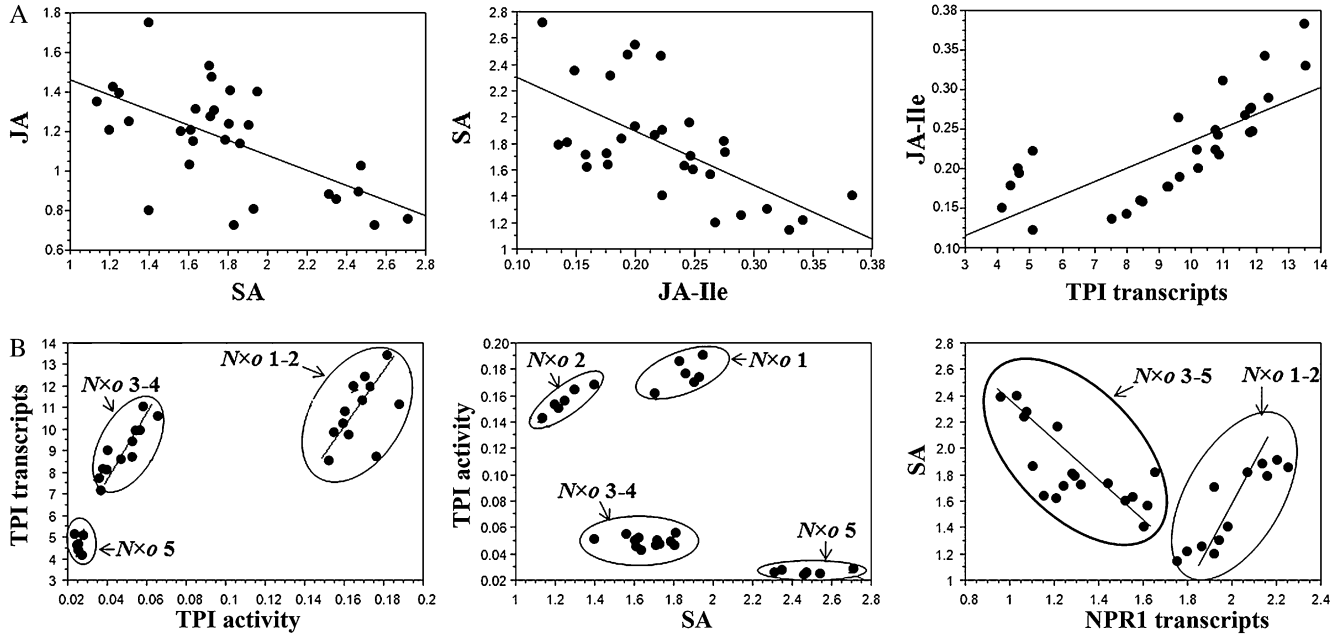

Figure 5. Variations in phytohormone cross talk (SA/JA) influence the accumulation of TPI activity in the allopolyploid lines. A, Statistical comparisons among levels of JA, SA, JA-Ile, and TPI transcripts accumulated in *N*×*o* lines revealed that SA was negatively correlated with JA ($r = -0.608$, $P < 0.001$) 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*×*o* lines are caused by differences in accumulated SA (which negatively regulates JA-Ile). The levels of SA are differently regulated in *N*×*o* lines 1 and 2 and 3 to 5; while in *N*×*o* 3 to 5, SA accumulation is downregulated by NPR1, *N*×*o* lines 1 and 2 seem to be using a different mechanism, which we speculate may involve ethylene production.

parental components involved the herbivore recognition, phytohormone signaling, and resistance responses.

In *Na*, the antiherbivore defensive response is rapidly initiated after the introduction of FACs present in *M. sexta* OS to wounds. Wu et al. (2007) demonstrated that this initial recognition response is associated with a rapid accumulation of WIPK, 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. 2; Supplemental Fig. S1). These variations in the transcript accumulation of WIPK, LOX3, and NPR1 genes in the allopolyploids are probably a consequence of alterations in the regulatory network that 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 (Supplemental Fig. S2), 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, 2006; Wittkopp et al., 2004; de Meaux et al., 2006; Stupar and Springer, 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 induced attenuated levels of JA and a dramatic increase in SA levels, which seems to antagonize JA (Fig. 3; Supplemental Fig. S3). The elicited JA can be con-

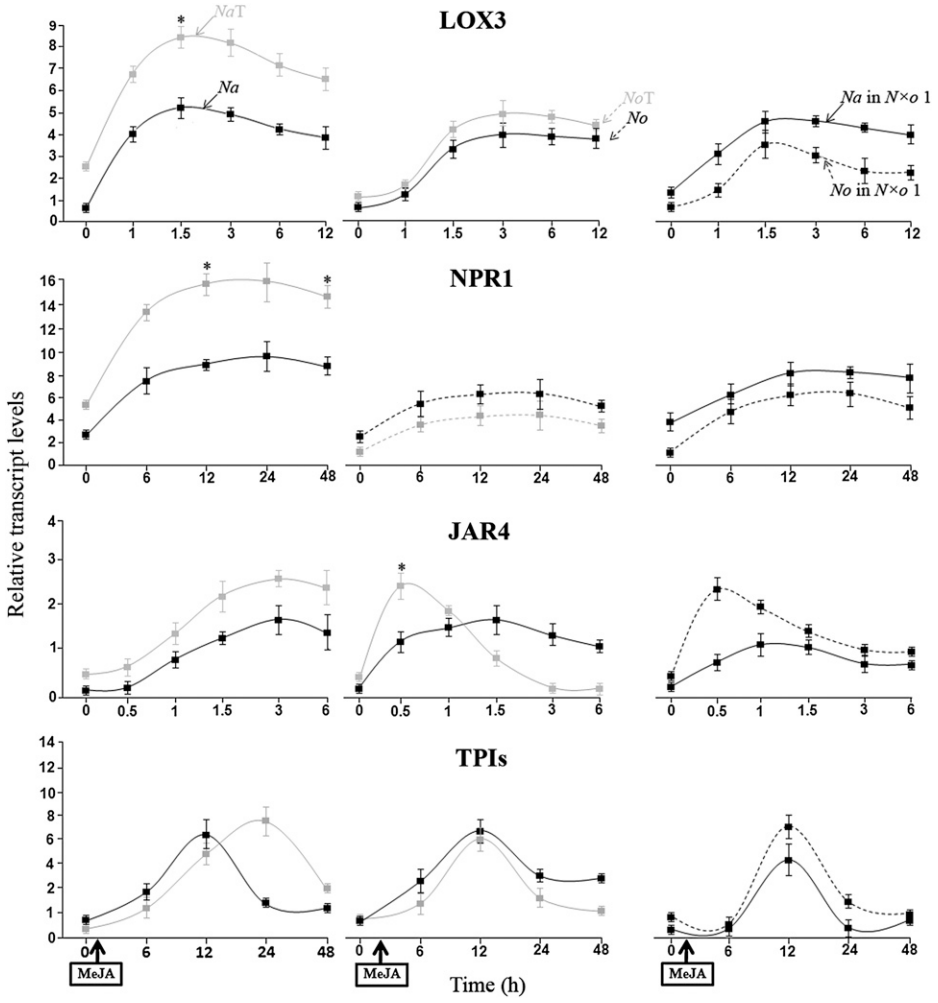

Figure 6. MeJA treatment of *N*×*o* line 1 (*N*×*o* 1) elicits transcripts of both parental LOX3, JAR4, and TPIs, demonstrating that the uniparental pattern of transcript accumulation observed after OS elicitation is not due to gene inactivation. After MeJA application, *N*×*o* line 1 accumulated both parental LOX3, JAR4, and 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 *M. sexta* OS elicitation. Asterisks indicate levels of significant difference between the maximum values of the pairs plotted together on the same graph (*$P < 0.05$; **$P < 0.01$).

jugated with various amino acids (Sembdner and Parthier, 1993; Sembdner et al., 1994). Recently, using *Na*, Kang and Baldwin (2006) demonstrated that JA conjugation with amino acids is mediated by 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 TPI 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 antiherbivore 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* that 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 $N\times o$ lines 1 and 2 was comparable to that in *Na*, whereas TPI activity in lines 3 to 5 was comparable to that in *No* (Fig. 4; Supplemental Fig. S4).

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 posttranslational 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: $N\times o$ lines 1 and 2, $N\times o$ lines 3 and 4, and $N\times o$ line 5. Correlation analysis conducted on TPI activities and SA levels suggest that variations in TPI activity of $N\times o$ lines 1 and 2 and 3 to 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 protease inhibitors, by eliciting vacuolar proteases (Horn et al., 2005). The levels of SA appear to be differently regulated in $N\times o$ lines 1 and 2 and 3 to 5; while in $N\times o$ 3 to 5, SA accumulation is down-regulated by NPR1, $N\times o$ lines 1 and 2 seem to have adapted a different mechanism (Fig. 5), probably involving ethylene, known to down-regulate SA accumulation. Hence, variations in phytohormone cross talk (SA/JA and likely ethylene/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

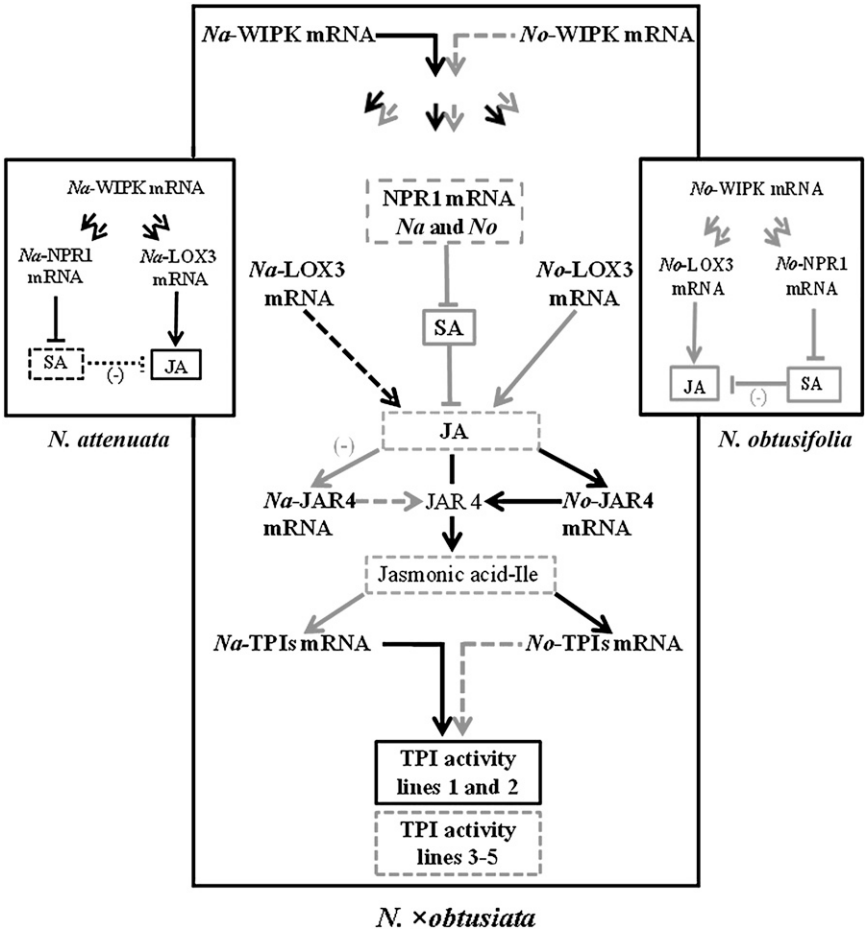

Figure 7. Schematic summary of the OS-elicited components of the signal cascade that elicits antiherbivore defense responses in the synthetic allopolyploid lines. After elicitation with *M. sexta* OS, $N\times o$ lines accumulate high levels of *Na*-WIPK, *No*-LOX3, *No*-JAR4, and *Na*-TPIs, but low levels of *Na*- and *No*-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 $N\times o$ lines. TPI levels were variable among $N\times o$ 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 antiherbivore 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.

in $N\times o$ lines—in response to OS elicitation was of particular interest, since similar uniparental expression pattern of homologous genes have been reported in several studies using Arabidopsis (Chen, 2007), or *Gossypium* allopolyploids (Adams et al., 2004). Here, to understand this transcript accumulation pattern, we initially assumed that the nonexpressed parental gene copies in $N\times o$ 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 $N\times o$ lines accumulated both parental LOX3, JAR4, and TPI transcripts (Fig. 6; Supplemental Fig. S5), suggesting that the uniparental transcript accumulation pattern observed after OS elicitation was not due to inactivation of LOX3, JAR4, and TPIs gene expression. An alternative hypothesis would be that some OS-elicited regulatory elements, influencing the expression of these genes, were lost through homologous recombinations during polyploidy.

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 that 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 (e.g miRNA, histone methylation, or acetylation) is still not clear. Particularly, in $N\times o$ lines, the recovery of *No*-LOX3 transcript accumulations after MeJA elicitation is consistent with a regulatory block that discriminates between the parental LOX3 transcripts elicited by OS. Such a regulatory mechanism is probably located upstream of JA signaling.

Synthetic auto- and allopolyploidy induced a reshuffling in the accumulation of parental defensive gene transcripts, phytohormones, and active TPI levels that mediate the antiherbivore resistance responses. Allopolyploidy seems to have integrated both the defensive components of *Na* and *No*. However, in response to OS elicitation, $N\times o$ lines accumulated only one or the other parental defensive transcripts and generated variability in expressed active TPI levels (Fig. 7). Here, we demonstrated that the variation in the level of active TPIs among the allopolyploid lines is probably a consequence of differences in phytohormone cross talk (SA/JA and likely ethylene/SA). We propose that the alterations in parental gene transcripts accumulated in $N\times o$ lines is the consequence of epigenetic and regulatory network changes that may facilitate adaptive radiations during allopolyploidy. More detailed molecular studies are required to understand the timing and frequency of these changes among natural polyploids.

MATERIALS AND METHODS

Plant Material

Plant Breeding

Na's seeds originated from a native population in Utah (Baldwin et al., 1994) and were inbred for 17 generations. *No*'s seeds were collected in 2004 at the Lytle ranch preserve (Santa Clara, UT) and inbred for one generation. The polyploids' seeds, inbred for five generations, were produced from synthetic polyploids described by 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 d of growth, seedlings were transferred to soil-based growth medium in Teku pots and, after an additional 10 d, transplanted to soil in 1-L individual pots and grown in a glasshouse at 26°C to 28°C under 16 h of light supplied by Philips Son-T Agro 400-W sodium lights. 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 (1:1 diluted with deionized water) from fourth- to fifth-instar *Manduca 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.

Protein Extraction and TPI Activity Assay

Leaf tissues 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 (0.1 M 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 bovine serum albumin as a standard. TPI activity was determined by radial diffusion activity as described by Van Dam et al. (2001).

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 lineage was 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 standard), 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 (16,000*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, http://www.varianinc.com). Fifteen microliters of each sample was injected onto a ProntoSIL column (C18; 5 mm, 50 × 2 mm; Bischoff, www.bischoff-chrom.com) attached to a precolumn (C18, 4 × 2 mm; Phenomenex, www.phenomenex.com). The mobile phase consists 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 mass-to-charge ratio (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.

Molecular Cloning

cDNA molecular cloning and sequencing was performed as described by Wu et al. (2006). Briefly, total RNA was extracted from seven to 10 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, NPR1, and JAR4 DNA fragments using PCR primers designed on *Na*'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 have been deposited in the GenBank database under the following accession numbers: HM362911 (WIPK), HM362912 (LOX3), HM362910 (NPR1), HM362913 (JAR4), and HM362914 (Actin). TPI sequence was published by Wu et al. (2006) under the following accession: DQ158201.

SYBR Green Real-Time PCR Assay (Quantitative PCR)

Quantitative PCR (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 that 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. Two microliters 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. S6). 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.

Statistical Analysis

Transcript and metabolic data, obtained at specific time points per treatment (wounding, OS elicitations, and MeJA), were analyzed with unpaired *t* tests using StatView statistical software (SAS Institute). We considered as significant only differences in transcript levels that are correlated with significant variations in metabolite levels. In other transcripts, e.g WIPK and NPR1, only comparisons that show more than 2-fold differences are considered as significant.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HM362910 to HM362914.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. OS elicitation enhances the accumulation of uniparental transcript patterns of WIPK and LOX3, but not NPR1, in *N×o* lines (2–5).

Supplemental Figure S2. Both cis- and trans-regulatory elements act on the expression of WIPK, LOX3, and NPR1 genes elicited by *M. sexta* OS.

Supplemental Figure S3. Patterns of OS-elicited SA and JA accumulation in *N×o* 2 to 5 after OS elicitation.

Supplemental Figure S4. *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.

Supplemental Figure S5. MeJA treatment of *N×o* 1 to 5 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.

Supplemental Figure S6. PCR products amplified in *Na* and *No* cDNA using reverse transcription primer pairs specific for *Na* and *No* WIPK, LOX3, JAR4, TPI, and NPR1.

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