Genetic down-regulation of gibberellin results in semi-dwarf poplar but few non-target effects on chemical resistance and tolerance to defoliation

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Abstract

Aims

Plant stature can be strongly modified via regulation of endogenous levels and signalling of the plant hormone gibberellin (GA). Down-regulation of GA can produce semi-dwarf tree varieties with improved qualities such as reduced susceptibility to wind damage, enhanced root growth and more compact cultivation. However, these modifications may have unintended, non-target consequences for defence against herbivores, via either of two mechanisms: (i) reduced biomass production may cause trade-offs with chemical resistance traits, as predicted by the growth-differentiation balance hypothesis, and (ii) altered biomass allocation to either roots or photosynthetic tissues may affect regrowth potential and thus tolerance to defoliation.

Methods

We studied GA down-regulated (GE) and non-transgenic wild-type hybrid poplar (*Populus alba* × *P. tremula*) in an outdoor, aboveground common garden and defoliated half of all replicate trees to simulate defoliation. We then quantified the independent and interactive effects of genotype and defoliation on growth and chemical resistance-related traits, including phenolic glycosides (PGs), condensed tannin and nitrogen. We also calculated tolerance to defoliation as the differential in relative growth between undefoliated and defoliated trees.

Important Findings

Our results indicate that two of the four GA down-regulated genotypes had significantly reduced stem height, basal diameter, volume (d^2h), total biomass and increased allocation to leaves relative to the wild type. One of those two genotypes also had reduced allocation to roots. One and sometimes both of these same two genotypes also had at least 20% lower levels of condensed tannins and PGs and similar increases in lignin and nitrogen. Tolerance, as calculated by the differential in relative growth between undefoliated and defoliated trees, was similar among all experimental genotypes. However, two GE genotypes flushed fewer leaves in response to defoliation relative to the wild type. Our results indicate that GA down-regulation strongly alters biomass production and allocation in poplar but does not necessarily compromise the ability of these trees to tolerate damage. However, some of the modifications we observed do have the potential to alter non-target resistance traits over time, and warrant further research, especially under plantation conditions.

Keywords: genetic modification, gibberellic acid, *Populus*, pest susceptibility, tolerance, resistance

INTRODUCTION

Intraspecific variation in plant defence is an important driver of plant-pest interactions (Fritz and Simms 1992; Osier and Lindroth 2004). Variation in defence traits derives from genotypic variation, ontogeny, phenology, environment and interactions among these factors (Axelsson *et al.* 2011; Brodeur-Campbell *et al.* 2006; Coyle *et al.* 2003; Holeski *et al.* 2012). Defence traits may also be influenced, sometimes unintentionally, by genetic engineering (GE) and can greatly

influence plant-herbivore interactions (Hjältén *et al.* 2007). GE is an expeditious option for selective breeding of trees, allowing the introduction of novel traits as well as the modification of existing traits and physiological pathways. Target traits such as growth, pest resistance, drought and herbicide tolerance, photosynthesis, carbon sequestration and phytoremediation have been successfully modified in numerous plant species (Harfouche *et al.* 2011; Mannion and Morse 2012; Merkle and Nairn 2005; Tzfira *et al.* 1998).

Plant growth can be modified by genetic regulation of endogenous levels and signalling of gibberellin (GA). GA are naturally occurring plant hormones that are synthesised in plastids via the methylerythritol phosphate pathway. Most prominent among the many functions of GA is elongation of cells and regulation of flowering and fruiting (Ju 1996; Yildirim and Koyuncu 2010). Exogenous application of bio-active GAs has historically been used to stimulate growth of vegetative or reproductive tissues in agricultural crops (Morgan and Mees 1958; Williamson *et al.* 1996). More recently, endogenous levels of GA have been altered via up-regulation or down-regulation of associated biosynthetic, catabolic, or signalling genes (Ye *et al.* 2012). Down-regulation of these genes has been used to create dwarf poplar tree varieties often for utilization as biofuel feedstock. The expected benefits of dwarf tree varieties include reduced lodging and subsequent reaction wood formation, as well as increased capacity for dense cultivation (Busov *et al.* 2008; Klocko *et al.* 2013; Webster 2002). Down-regulation of GA levels typically reduces total biomass and stem height, but may also increase root:shoot biomass ratio and alters leaf morphology and canopy architecture (Busov *et al.* 2006; Han *et al.* 2011; Zawaski *et al.* 2011).

Altered biomass production and allocation may have consequences for defence against herbivores. According to the growth-differentiation balance hypothesis (Herms and Mattson 1992; Loomis 1932), reduction of biomass production can alter allocation of carbon to chemical resistance traits. This hypothesis states that if growth is reduced more than photosynthesis, then accumulated carbon may be allocated to defence traits such as secondary compounds. This trade-off has been observed in transgenic semi-dwarf poplar, which showed higher levels of phenolic resistance compounds in semi-dwarf genotypes (Busov *et al.* 2006).

The principal chemical resistance traits in poplar are condensed tannins and phenolic glycosides (PGs). Condensed tannins (CTs) deter feeding by vertebrates and reduce larval growth rate, pupal mass and survival of some invertebrates, particularly for chrysomelid beetles (Bryant *et al.* 1991, 1993; Donaldson and Lindroth 2004; Salminen and Karonen 2011). PGs function as inhibitors of growth, development and fecundity for many other invertebrates, particularly Lepidoptera (Boeckler *et al.* 2011; Lindroth and St. Clair 2013; Roth *et al.* 1997). In the case of poplar, some chrysomelids are specialists that are attracted to higher levels of PGs and even sequester them for their own defence (Ikonen 2002). Because condensed tannins and PGs have contrasting effects on the performance of different types of herbivores, changes in either can result in complex changes in host plant susceptibility to pests. Lignin and nitrogen are also determinants of host plant suitability and may be influenced, along with tannins and PGs, by resource trade-offs prompted by modification (Ikonen 2002; Scheirs *et al.* 2003). Lignin is a mechanical barrier against pests and inhibits digestion and absorption of nitrogen (Ohkuma 2003; Zehnder *et al.* 2009). Nitrogen, an index of protein, is not a resistance factor per se, but is often limiting for insect development (Hemming and Lindroth 1995; Mattson 1980). Here, we use the terms "chemical resistance" or "resistance traits" to collectively describe CTs, PGs, lignin and nitrogen, all of which are known to affect pest preference (e.g., host choice) and performance (e.g., growth, survival, reproduction).

Another major herbivore defence strategy utilized by plants is tolerance. Tolerance enables growth and reproduction following damage and can be measured as the differential in fitness (or traits correlated with fitness) between plants in undamaged and damaged states (Strauss and Agrawal 1999). Mechanisms of tolerance include changes in biomass allocation and/or photosynthetic rates to increase carbon stores that aid in regrowth (Tiffin 2000; Stamp 2003; Strauss and Agrawal 1999). Genetic modification of GA levels can alter biomass allocation among photosynthetic and storage tissues (Lu *et al.* 2015) that support regrowth following defoliation. Root biomass has been positively correlated with tolerance in herbaceous species, whereas stem biomass has been positively correlated with tolerance in trees (Pratt *et al.* 2005; Stevens *et al.* 2008). Increased root:shoot ratios have been reported in some GA down-regulated poplar (Etherington *et al.* 2007). Increased root development may provide an enhanced source of carbon for shoot regrowth.

Although non-target effects of GA modification have been investigated in previous research, few studies have explored how these modifications affect both growth and plant defence syndromes (resistance and tolerance). In this study, we evaluated expression of biomass production and allocation patterns, chemical resistance and tolerance in GA down-regulated poplar. We predicted that:

(i) Down-regulation of GA would reduce height and total biomass production, and shift biomass allocation from shoots to roots,

(ii) GE genotypes with reduced height growth would also express increased levels of chemical resistance traits, in accordance with the growth-differentiation balance hypothesis and

(iii) Modified genotypes with shifts in biomass allocation from stems to roots would also express modified tolerance, possibly due to reduced carbon stores in shoots to support regrowth, or enhanced carbon stores in roots to support shoot regrowth.

MATERIALS AND METHODS

We established GA down-regulated and wild-type (WT) trees in an aboveground (potted) common garden and defoliated half of all replicate trees. We evaluated the independent and interactive effects of genotype and defoliation on biomass production and allocation. We also evaluated the independent and interactive effects of GA down-regulation and defoliation on chemical resistance traits in both June and August. We calculated tolerance from measurements of relative growth differentials between undefoliated and defoliated trees.

Plant materials

Hybrid poplar (*Populus alba* × *P. tremula*) clone 717-IB4 was the experimental model system utilized in this study. Poplar is an ideal model for this type of research as it is one of few taxa that have been genetically modified for reduced GA levels (Etherington *et al.* 2007; Jansson and Douglas 2007). Additionally, the primary defence strategies of *Populus*, chemical resistance and tolerance, have been well studied (Chen *et al.* 2009; Philippe and Bohlmann 2007; Stevens *et al.* 2007). Hybrid poplar trees were transformed to create four separate GA down-regulated genotypes; control trees were non-transgenic WTs (Table 1). Genes encoding *gai* (MTG and XG genotypes), *rgl* (RGL) and GA2-oxidase (C17) proteins were transformed by a method similar to that described by Busov *et al.* (2006). The *gai* protein acts as a constitutive repressor of GA signalling and is expressed as moderate dwarfing (Busov *et al.* 2006; Peng *et al.* 1997). The function of *rgl* proteins is similar to that of *gai* but with a stronger dwarfing effect (Busov *et al.* 2006). GA2-oxidase catabolises active GA and causes more severe dwarfing than either *gai* or *rgl* (Busov *et al.* 2006; Zawaski *et al.* 2011). Each allele was combined with a 35S promoter except for one *gai* allele (MTG), which was combined with a native *Arabidopsis* promoter. Expression is often stronger in transformations utilizing the 35S promoter rather than the native *Arabidopsis* promoter (Elias *et al.* 2012; Etherington *et al.* 2007) although this promoter may also contribute to gene silencing (Mishiba *et al.* 2005). We analysed single transformation events (i.e., gene insertions) from each construct based on previous growth data showing mild but statistically significant semi-dwarfism and modification of GA levels (Elias *et al.* 2012).

In spring 2011, rooted greenwood cuttings were planted outside in 15-liter pots containing a 50/50 mixture of silt-loam field soil and sand. Nutricote 3–4 month slow release fertiliser (13:13:13 N-P-K + micronutrients) was added to each pot at a rate of 4.5 g/l of soil and pots were hand-watered daily. We arranged potted trees in a randomized block design in a common garden at the University of Wisconsin-Madison. Each experimental block contained one replicate tree for each tree genotype and each defoliation treatment. Genotypes (subplot) were grouped by defoliation treatment (whole plot). Treatments consisted of five hybrid poplar genotypes (four modified and one wild type; *n* = 5–10 replicate trees per treatment) and two defoliation treatments (0% and 75%). Number of replicates varied due to loss of trees during the project (e.g., rodent damage) and culling of trees that produced an additional stem (as that would confound biomass assessments; see Materials and Methods-Statistical Analysis).

Defoliation

In June 2011, we applied 75% defoliation to half of the replicate trees, using first insect herbivory, then artificial defoliation to ensure uniform damage rates. A high level of defoliation was used to approximate insect pest outbreak conditions (Mattson *et al.* 1991). We obtained third instar gypsy moth larvae from USDA-APHIS Otis Air National Guard Base (Buzzards Bay, Massachusetts) and applied them to defoliation treatment trees at a rate of approximately one larva per five leaves. Larvae were enclosed in fine mesh nylon bags at the top third portion of each tree. We also fitted mesh cages onto undefoliated treatment trees to standardize caging effects. All larvae and cages were removed after one week and additional tissue was removed using pinking shears to effect a 75% defoliation. Pinking shears were used to cut leaves perpendicularly to the midrib in a manner that simulated uneven perforation by insect herbivory. The two complementary methods of defoliation were used to replicate natural pest defoliation while ensuring a consistent 75% removal of leaf tissue (Havill and Raffa 1999; Stevens *et al.* 2007; Stowe *et al.* 2000). We attached a single twist-tie to the top of each stem to mark the starting point of new shoot growth and leaf flush following defoliation.

Table 1: *Populus alba* × *P. tremula* gibberellic acid down-regulated and WT genotypes produced at Oregon State University

Genotype	Promoter	Transgene (origin)	Predicted phenotype[a]	Replicates (U,D)
WT	None	None	Normal	5,7
MTG	Native (*A. thaliana*)	*gai* (*Arabidopsis thaliana*)	Some dwarf	5,5
XG	35S	*gai* (*A. thaliana*)	Some dwarf	7,6
RGL	35S	*rgl* (*A. thaliana*)	Strong dwarf	10,9
C17	35S	GA2-oxidase (*Populus* sp.)	Strong dwarf	5,8

The 'replicates' column indicates number of undefoliated (U) and defoliated (D) replicate trees.
[a]Per Busov *et al.*, 2006.

Biomass production and allocation

We quantified biomass production and allocation to assess response to the independent and interactive effects of genotype and defoliation. Biomass 'production' variables consisted of stem height (h), basal diameter (d) and aboveground volume as well as leaf, stem, root and total mass. Biomass 'allocation' variables consisted of leaf, stem and root mass ratios as well as root:shoot ratio. Biomass allocation to leaves included quantity, area per leaf (cm^2), total leaf area (cm^2) and leaf mass per unit area (LMA; mg/cm^2) of leaves flushed post-defoliation.

We measured stem height (h) and basal diameter (d) before defoliation and again 8 weeks after defoliation during the destructive harvest. From these measurements, we calculated volume of aboveground tissues (d^2h) and relative growth (ln[final d^2h]-ln[initial d^2h]) (Abrahamson *et al.* 1990; Stevens *et al.* 2007). During the destructive harvest we counted, weighed and scanned (via Li-Cor 3100 area meter) all leaves growing above the twist-tie on each tree. From these measurements, we determined biomass allocation to leaves flushed after defoliation. Total biomass of each tree was divided into leaves, stems and roots, which were dried (five days at 60°C) and weighed separately to determine the mass of each tissue. Total mass was calculated as the sum of leaf, stem and root mass. Mass ratios were calculated as the mass of each tissue divided by total mass for each tree. Root:shoot ratios (R:S ratio) were calculated from the masses of roots and shoots.

Chemical resistance

We collected and chemically analysed leaves from each tree 1 week and again 8 weeks after defoliation (June and August, respectively) to assess immediate and delayed chemical induction. Mass of leaves collected in June and August for chemical analyses were accounted for in final calculations of leaf mass. In each collection, we divided tree canopies into vertical quadrants and gathered approximately the same number of leaves from each quadrant to accurately represent the chemical profile of each tree. In the first collection, we gathered 10 leaves (both whole and previously damaged) and in the second collection we gathered 25 leaves (whole and previously damaged) from each tree. Leaves were clipped at the petiole, vacuum-dried and ground to a coarse particle size in a Wiley Mill (mesh size #20). Coarse-ground leaves were weighed for lignin analyses and remaining tissue was ground to a fine particle size by ball milling, and allocated to other analyses. We quantified CTs spectrophotometrically via a modified acid-butanol method (Porter *et al.* 1986). Standards used in analyses of CTs were purified via adsorption chromatography (Hagerman and Butler 1980) from WT *P. alba* × *P. tremula* leaves. Qualitative and quantitative analysis of PGs was performed via an ultra high performance liquid chromatography method (method modified from Abreu *et al.* 2011) with standards purified from *Populus* and *Salix* spp. WT leaves. We report individual PGs (i.e., salicin, salicortin, tremuloidin, hydroxycyclohexen-on-oyl salicortin [HCH-salicortin],

tremulacin and 2′-cinnamoyl-salicortin) found at concentrations > 0.5% dry mass and the sum of these values as total PGs. We quantified lignin levels gravimetrically via sequential extraction in a hot acid-detergent solution in an Ankom 200 digestor and incubation in 72% sulfuric acid bath (Rowland and Roberts 1994). Nitrogen levels were quantified via combustion analysis using a Flash EA1112 C/N analyser. Levels of all chemical compounds are reported as concentrations (percent dry mass).

Tolerance

Tolerance was determined using measurements of relative growth between undefoliated and defoliated trees. Aboveground volume of each tree was measured before and 2 months after defoliation (i.e., June and August, respectively) to calculate relative growth (ln[final d^2h]-ln[initial d^2h]). Tolerance was then calculated as the differential of relative growth between undefoliated and defoliated trees within each genotype. Differences in tolerance among genotypes may also be ascertained by any significant growth × defoliation interactions for biomass production and allocation (Figs. 1–4).

Statistical analysis

During the time period between initial and final tree measurements, ~30% of the trees produced an additional stem. Production of secondary stems did not differ among genotypes or between defoliation treatments ($F_4 = 4.75$, $P = 0.316$ and $F_1 = 0.02$, $P = 0.883$, respectively). Because large secondary stems could compromise estimates of stem height and biomass allocation, we excluded double-stemmed trees from statistical analyses.

We tested the independent and interactive effects of genotype and defoliation on biomass production and allocation, and on chemical resistance in June and August, using a fixed effects model, split-plot analysis of variance (ANOVA). Chemical concentration data were arcsine square root-transformed $\left(\arcsine\left[\sqrt{(\% \text{ dry mass}/100)}\right]\right)$ to adjust for non-normality before running ANOVA. We tested the independent and interactive effects of genotype and defoliation on stem height and volume using a fixed effects model analysis of covariance (ANCOVA), with initial stem height as a covariate. Preceding statistical analyses, we log-transformed volume data to adjust for non-normality. Satterthwaite approximation was used to calculate degrees of freedom for all ANOVAs and ANCOVAs. An alpha level of 0.05 was considered significant and 0.05 < 0.10 was considered marginally significant for all statistical analyses. For each significant ANOVA and ANCOVA result, we used Tukey's honestly significant difference post hoc tests to determine which modified genotypes differed from the wild type.

We used Pearson correlations to test for relationships between biomass production and levels of chemical resistance in August. (Chemical resistance traits that were measured in June were not included in correlations because only a few biomass production variables could be measured in June.)

Both undefoliated and defoliated trees were included in these correlations. Pearson correlations were also used to test relationships between means of biomass allocation and tolerance. Means, rather than raw values for each tree, were used in this correlation because tolerance is calculated for pairs of undefoliated and defoliated trees. All statistical analyses were performed using JMP Pro 9 (SAS Institute, Cary, NC).

RESULTS

Biomass production and allocation

Modification and defoliation each independently influenced biomass production (Table 2). Two genotypes in particular, XG and RGL, produced less biomass than did the wild type. Post hoc tests among genotypes indicated an average of 32% less stem height in XG and RGL relative to the wild type (Fig. 1). Additionally, basal diameter was 27% lower in RGL and volume was 63% lower in XG and RGL relative to the wild type. Post hoc tests indicated 54% lower leaf mass in RGL relative to wild type (Fig. 2), and stem, root and total mass were each at least 50% lower in XG and RGL. Defoliation significantly affected basal diameter, volume and also leaf, stem and total mass but not root mass (Table 2, Figs. 1 and 2). Post hoc tests between defoliation treatments indicated that basal diameter was reduced by 14% and volume was reduced by 23% due to defoliation. Additionally leaf, stem and root mass were each reduced by ~20% due to defoliation.

Biomass allocation patterns were influenced only by modification (Table 3), although two genotypes responded to defoliation by producing fewer leaves relative to the other genotypes (G × D interaction, Table 4). Post hoc tests among genotypes indicated that leaf and stem mass ratios were 44% and 11% lower in XG and RGL, respectively, relative to the wild type (Fig. 3). Root mass ratio and R:S ratio were 24% and 33% lower, respectively, in RGL (Fig. 3). Biomass allocation to leaves also varied among genotypes (Table 4). Results showed that XG and RGL flushed fewer leaves after defoliation than other genotypes. Leaves in XG tended to be more broad and thin than those produced by RGL. Post hoc tests indicated that XG and RGL flushed an average of 45% fewer leaves after defoliation than did wild type (Fig. 4). Additionally, area per leaf was 48% higher in XG, while total area was 45% lower in RGL Leaf mass per unit area was 24% lower in XG. Post

hoc tests indicated 63% larger area in defoliated XG relative to leaf area in all other trees.

Chemical resistance

Chemical defence traits (measured in June) were not influenced by modification or defoliation (Table 5), although the composition of PGs was affected (see supplementary Fig. S1). Post hoc tests revealed that levels of salicortin and tremulacin were 55% ($F_{4,56}$ = 6.04, P < 0.001) and 66% ($F_{4,56}$ = 13.84, P < 0.001) lower, respectively, and HCH-salicortin was 40% higher ($F_{4,56}$ = 4.13, P < 0.005) in RGL relative to the wild type. Defoliation reduced salicin by 47% ($F_{1,56}$ = 54.21, P < 0.001), salicortin by 60% ($F_{1,56}$ = 52.48, P < 0.001) and tremulacin by 24% ($F_{1,56}$ = 17.21, P < 0.001).

Chemical defence traits measured in August were affected by modification but not by defoliation or the interaction of these two factors (Table 5, Fig. 5). Post hoc tests among genotypes indicated an average of 23% lower CT levels in XG and RGL, and 19% lower PG levels in RGL, relative to the wild type. The composition of PGs was also influenced by modification (see supplementary Fig. S1). Tremulacin was 25% lower in RGL relative to the wild type ($F_{4,58}$ = 4.35, P = 0.004). Post hoc tests also indicated that lignin levels were 24% higher in RGL, and nitrogen levels were an average of 14% higher in XG and RGL, than in the wild type.

Correlation analyses revealed that biomass production was positively related to CTs and PGs and negatively related to lignin and nitrogen (Table 6). Relationships were strongest between stem height and mass with CTs and between stem height and root mass with PGs. Root mass and total mass were most strongly correlated with lignin and nitrogen.

Tolerance

Tolerance (as the differential in relative growth between undefoliated and defoliated trees) did not vary among genotypes ($F_{4,25}$=1.14, P = 0.361, Fig. 6). Results indicated that regrowth in all genotypes undercompensated for tissue lost to defoliation. Tolerance as a function of all other growth metrics, except for area per leaf, also did not vary significantly among genotypes. Area per leaf of leaves flushed post-defoliation was higher in defoliated XG trees relative to all undefoliated trees from all genotypes (including XG). Correlation analyses revealed that biomass allocation was not related to

Table 2: summary of ANOVA and ANCOVA (for stem height and volume) examining the independent and interactive effects of genotype and defoliation on biomass production

|	$df_{n,d}$	Stem height		Basal diameter		Volume		Leaf mass		Stem mass		Root mass		Total mass	
		F	P	F	P	F	P	F	P	F	P	F	P	F	P
Genotype	4,57	24.79	**<0.001**	17.81	**<0.001**	22.47	**<0.001**	13.48	**<0.001**	22.83	**<0.001**	14.55	**<0.001**	18.99	**<0.001**
Defoliation	1,57	0.57	0.454	11.65	**0.001**	6.33	**0.015**	7.16	**0.010**	5.74	**0.020**	1.27	0.265	4.52	**0.038**
G × D	4,57	0.30	0.877	0.60	0.668	0.89	0.477	0.09	0.984	0.27	0.898	0.26	0.903	0.13	0.972

Statistically significant values are in bold.
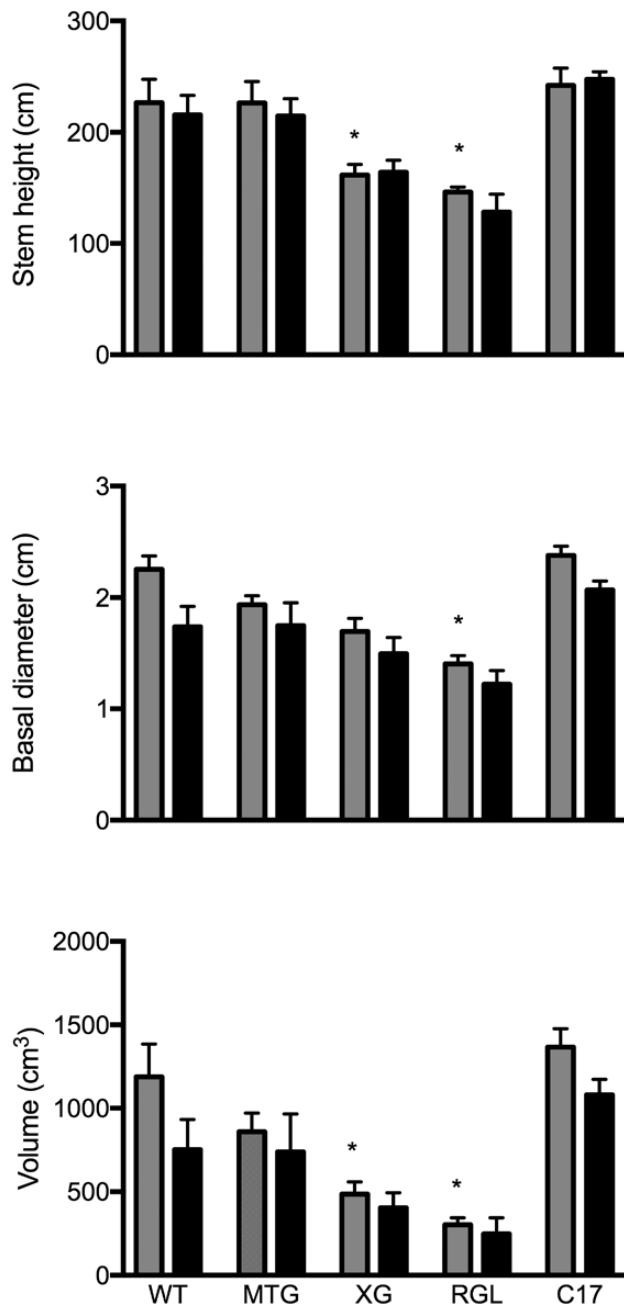
Figure 1: biomass production (height, basal area, volume) of genetically modified and wild-type (WT) genotypes. Bars represent mean response (n = 5–10 replicate trees, error bars represent +1 SE). Grey bars represent undefoliated trees and black bars represent defoliated trees. Asterisks denote significant differences ($P < 0.05$) between undefoliated and defoliated trees for each modified genotype when compared against the wild type, as indicated by post hoc tests.

tolerance, although total leaf area shared a marginally significant, positive relationship with tolerance (Table 7).

DISCUSSION

In this study, we explored how different types of GA down-regulation affect poplar biomass production, growth

allocation, chemical defences and tolerance of herbivory. The independent effects of genotype and defoliation significantly influenced many aspects of biomass production and allocation, although the interaction of these factors affected only leaf area. Chemical defences measured in June were influenced only by genotype, in the form of altered PG composition. Traits measured in August were, however, influenced by genotype. Tolerance also did not vary significantly among genotypes, although allocation to leaves in response to defoliation may play some role in promoting photosynthetic capability following damage. GA down-regulation therefore had strong effects on poplar biomass production and allocation and moderate effects on resistance traits. Even moderate changes in levels of resistance traits may be biologically significant for herbivores that are sensitive to specific defence chemicals.

Suppression of GA levels, via down-regulation of genes controlling *gai*, *rgl* or GA2-oxidase proteins, is reported to reduce stem height and total biomass production (Busov *et al*. 2006, 2008; Zawalski *et al*. 2011). We also observed reduced stem height and total mass but only in XG and RGL. In other studies, GA down-regulated genotypes also had decreased stem height and increased diameter and number of branches (via shorter internodes) (Busov *et al*. 2003; Etherington *et al*. 2007). Experimental genotypes utilized in this study, however, exhibited positive relationships between stem height and both basal diameter (R^2 = 0.57, $P \leq 0.001$) and aboveground biomass (R^2 = 0.82, $P \leq 0.001$). Down-regulation of GA has also been reported to increase allocation of biomass to roots (Elias *et al*. 2012; Etherington *et al*. 2007; Farquharson 2010), and conversely up-regulation has increased allocation to shoots (Lu *et al*. 2015). Our results on above versus belowground allocation were mixed. One GA down-regulated genotype allocated more to roots (RGL) and another allocated more to shoots (C17). These results indicate that different types of modification of GA levels can influence tree growth allocation strategies differently.

XG and RGL were the only genotypes that exhibited strong phenotypic expression of GA down-regulation. Both XG and RGL exhibited significantly lower biomass production relative to the wild type. These genotypes also allocated more biomass to leaves and flushed fewer leaves after defoliation relative to the other experimental genotypes. Biomass allocation strategies, however, differed between XG and RGL. Leaf area was larger and LMA was smaller in leaves flushed post-defoliation in XG relative to RGL. Other studies have also reported changes in leaf area due to modification of GA levels (Eriksson *et al*. 2000; Zawaski *et al*. 2011). The other experimental genotypes (MTG and C17) had biomass production and allocation similar to that of the wild type, signifying weak expression of targeted genes. Pleiotropic effects in transgenic tissues can result from methods of gene transfer or somaclonal variation in tissue culture (Cellini *et al*. 2004; Strauss *et al*. 2001), number of copies of an inserted gene and insertion loci or origin of an inserted gene (e.g., native versus transgenic; Han *et al*. 2011; Käppeli and Auberson
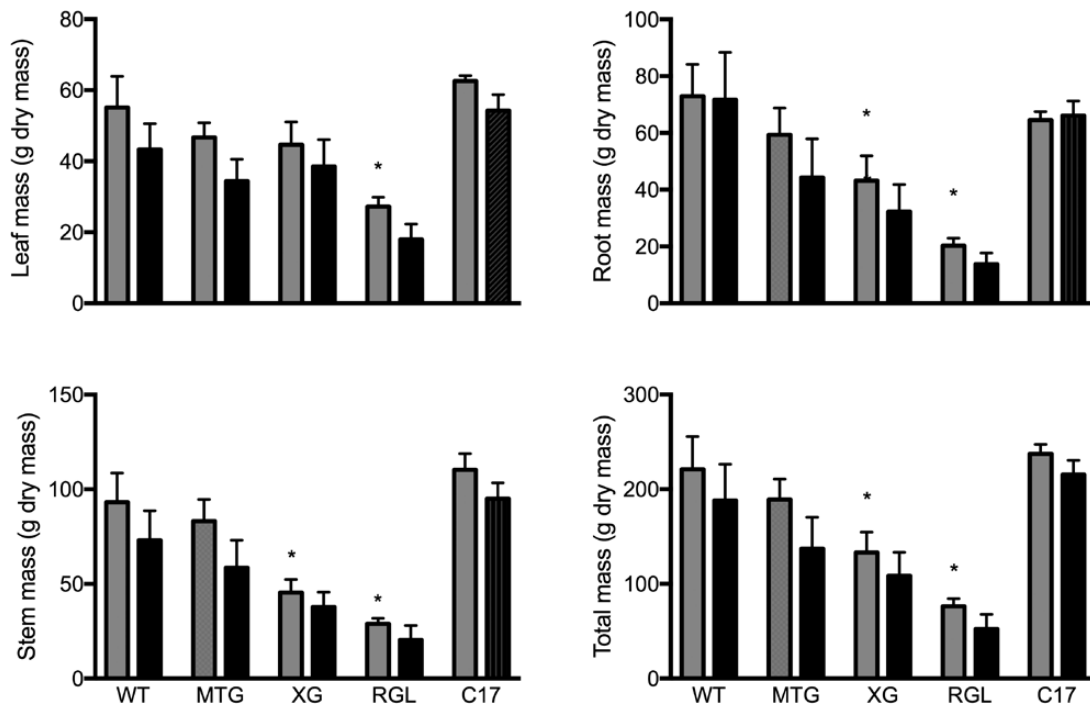
Figure 2: biomass production (individual and total tissue mass) of genetically modified and wild-type (WT) genotypes. Bars represent mean response (*n* = 5–10 replicate trees, error bars represent +1 SE). Grey bars represent undefoliated trees and black bars represent defoliated trees. Asterisks denote significant differences (*P* < 0.05) between undefoliated and defoliated trees for each modified genotype when compared against the wild type, as indicated by post hoc tests.

Table 3: summary of ANOVA examining the independent and interactive effects of genotype and defoliation on biomass allocation

	$df_{fn,d}$	Leaf mass ratio		Stem mass ratio		Root mass ratio		Root:shoot ratio	
		F	P	F	P	F	P	F	P
Genotype	4,57	30.42	**<0.001**	14.14	**<0.001**	3.45	**0.014**	3.47	**0.013**
Defoliation	1,57	0.88	0.353	1.77	0.188	0.08	0.780	0.23	0.636
G × D	4,57	0.49	0.742	0.59	0.672	0.85	0.502	0.86	0.494

Statistically significant values are in bold.

Table 4: summary of ANOVA examining the independent and interactive effects of genotype and defoliation on biomass allocation to leaves

	$df_{fn,d}$	No. of leaves		Area per leaf		Total leaf area		Leaf mass per unit area	
		F	P	F	P	F	P	F	P
Genotype	4,57	15.14	**<0.001**	5.57	**<0.001**	9.98	**<0.001**	11.00	**<0.001**
Defoliation	1,57	0.52	0.474	1.64	0.205	1.89	0.174	0.04	0.851
G × D	4,57	3.40	**0.014**	0.18	0.947	0.14	0.967	0.43	0.790

Biomass allocation was measured only in leaves flushed after defoliation. Statistically significant values are in bold.

1998; Li *et al*. 2009), promoters (Elias *et al*. 2012; Etherington *et al*. 2007) or environment and plant cultivation methods (Kumar and Fladung 2001; Lu *et al*. 2015). Environmental stressors such as herbivore damage may have the potential to further influence modified traits by eliciting changes in resource allocation. We expected less aboveground biomass production in defoliated versus undefoliated trees due to the inherent negative effects of removal of photosynthetic tissues that support growth (Kulman 1971; Reichenbacker *et al*. 1996). Defoliation had a negative influence on most biomass production variables, but for the most part genotype did not affect the influence of defoliation.
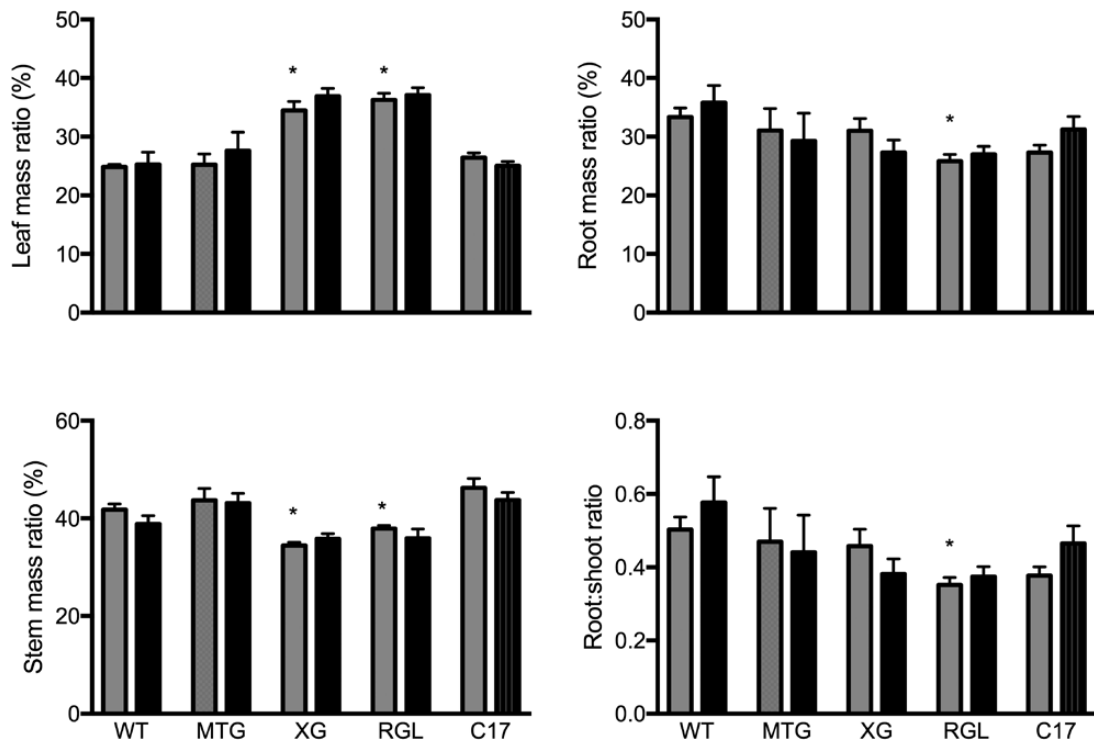
Figure 3: biomass allocation of genetically modified and wild-type (WT) genotypes. Bars represent mean response (n = 5–10 replicate trees, error bars represent +1 SE). Grey bars represent undefoliated trees and black bars represent defoliated trees. Asterisks denote significant differences ($P < 0.05$) between undefoliated and defoliated trees for each modified genotype when compared against the wild type, as indicated by post hoc tests.
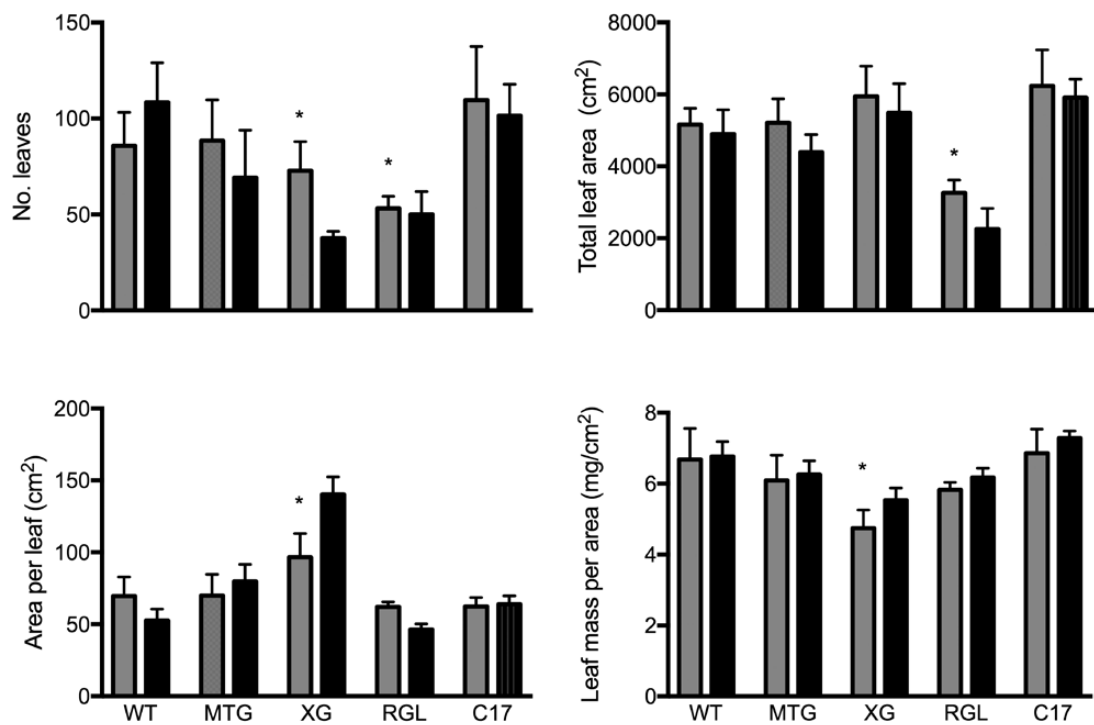

Figure 4: biomass allocation to leaves flushed after defoliation of genetically modified and wild-type (WT) genotypes. Bars represent mean response (n = 5–10 replicate trees, error bars represent +1 SE). Grey bars represent undefoliated trees and black bars represent defoliated trees. Asterisks denote significant differences ($P < 0.05$) between undefoliated and defoliated trees for each modified genotype when compared against the wild type, as indicated by post hoc tests.

Table 5: summary of ANOVA examining the independent and interactive effects of genotype and defoliation on levels of chemical resistance in June (top table) and August (bottom table)

	Condensed tannin			Phenolic glycoside			Lignin			Nitrogen		
	$df_{n,d}$	F	P	$df_{n,d}$	F	P	$df_{n,d}$	F	P	$df_{n,d}$	F	P
June												
Genotype	4,35	1.66	0.180	4,56	1.96	0.114	4,55	2.18	0.083	4,55	1.10	0.367
Defoliation	1,35	1.27	0.268	1,56	0.39	0.533	1,55	3.91	0.053	1,55	2.78	0.101
G × D	4,35	0.63	0.643	4,56	0.71	0.589	4,55	1.67	0.171	4,55	0.77	0.552
August												
Genotype	4,63	10.33	**<0.001**	4,64	4.98	**0.002**	4,64	5.79	**0.001**	4,64	23.21	**<0.001**
Defoliation	1,63	3.35	0.072	1,64	2.17	0.146	1,64	0.55	0.460	1,64	0.72	0.400
G × D	4,63	0.50	0.733	4,64	0.36	0.834	4,64	0.11	0.977	4,64	0.63	0.641

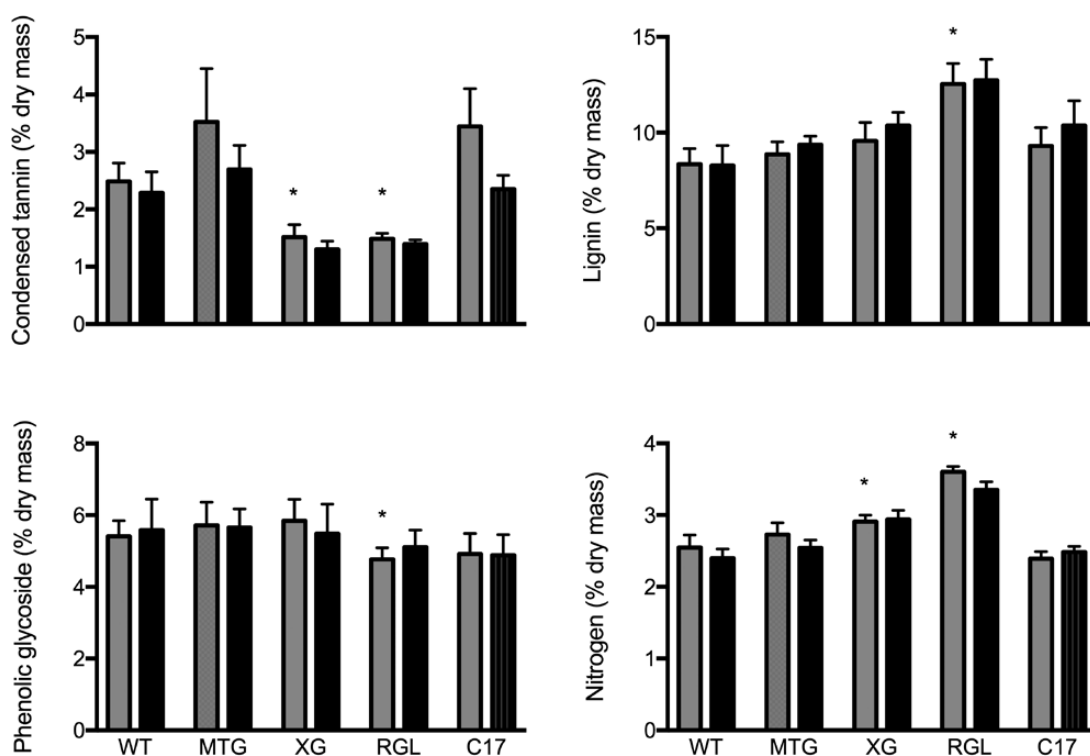Statistically significant values are in bold.

Figure 5: chemical resistance traits (measured in August) in genetically modified and wild-type (WT) genotypes. Bars represent mean response (n = 5–10 replicate trees, error bars represent +1 SE). Grey bars represent undefoliated trees and black bars represent defoliated trees. Asterisks denote significant differences ($P < 0.05$) between undefoliated and defoliated trees for each modified genotype when compared against the wild type, as indicated by post hoc tests.

We predicted that GA down-regulated genotypes with reduced biomass production would also express increased levels of chemical resistance, per the growth-differentiation balance hypothesis. Our observations did not support this prediction. Because XG and RGL each had significantly reduced biomass production, we expected higher levels of chemical resistance. In contrast to our prediction, however, we observed lower levels of CTs in both of these genotypes as well as lower levels of PGs in RGL, relative to the wild type. Pleiotropic effects of genetic modification on non-target plant chemistry have been identified in other studies (Biemelt *et al.*

2004; Busov *et al.* 2006; Hjältén *et al.* 2008). Of those studies, trade-offs between growth and chemical resistance have been demonstrated by the accumulation of the PGs salicin and tremulacin in *gai* and *rgl* down-regulated poplar (Busov *et al.* 2006). We observed, however, a 'decrease' in tremulacin in our *gai* down-regulated genotype, RGL. Both XG and RGL had higher levels of nitrogen than those found in the wild type. Higher nitrogen paired with the low levels of CTs and PGs identified in these genotypes may elevate their pest susceptibility. Results from bioassay experiments conducted on these same experimental genotypes in another study (Buhl

Table 6: Pearson correlation analyses between biomass production and chemical resistance traits that were measured in August

	Condensed tannin		Phenolic glycoside		Lignin		Nitrogen	
	R	P	R	P	R	P	R	P
Stem height	0.67	<0.001	0.36	0.003	−0.43	<0.001	−0.79	<0.001
Basal diameter	0.53	<0.001	0.26	0.032	−0.39	0.001	−0.65	<0.001
Volume	0.63	<0.001	0.31	0.012	−0.35	0.004	−0.73	<0.001
Leaf mass	0.51	<0.001	0.28	0.023	−0.42	0.001	−0.73	<0.001
Stem mass	0.67	<0.001	0.34	0.004	−0.41	0.001	−0.77	<0.001
Root mass	0.58	<0.001	0.36	0.002	−0.48	<0.001	−0.80	<0.001
Total mass	0.63	<0.001	0.35	0.004	−0.46	<0.001	−0.80	<0.001
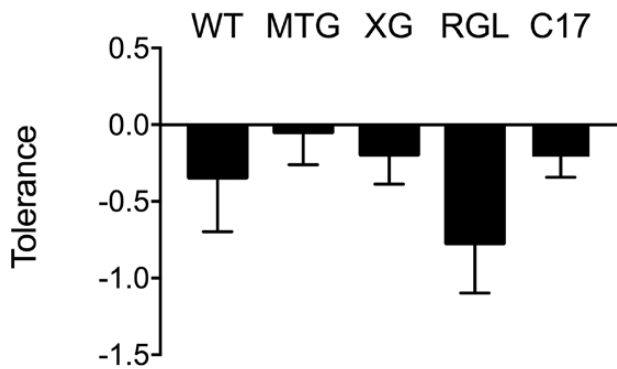
Figure 6: tolerance in experimental genotypes. Bars represent mean tolerance in each genetically modified or wild-type (WT) genotype ($n = 5$–9 replicate pairs of undefoliated and defoliated trees in each genotype, error bars represent +1 SE). Tolerance was calculated as the difference in relative growth (ln[final d^2h]-ln[initial d^2h]) between undefoliated and defoliated trees. Negative values indicate lower relative growth (under-compensation) in defoliated trees than in undefoliated trees within a genotype.

Table 7: Pearson correlation analyses between means of biomass allocation and tolerance to defoliation

	Tolerance	
	R	P
Leaf mass ratio	−0.57	0.312
Stem mass ratio	0.48	0.408
Root mass ratio	0.43	0.466
Root:shoot ratio	0.45	0.442
Leaf area per leaf	0.49	0.406
Total leaf area	0.82	0.086
Leaf mass per unit area	0.05	0.938

et al. 2015), however, did not indicate better performance in pests reared on XG and RGL relative to those reared on the other experimental genotypes. Correlation analyses also confirmed results indicating that biomass production did not share an inverse relationship with all chemical resistance traits.

In addition to chemical resistance, we predicted that tolerance would also be affected by GA down-regulation. Tolerance as a measure of relative growth was not, however, significantly affected by down-regulation of GA. Studies of other modification targets in poplar have also reported null effects on tolerance (Axelsson and Hjältén 2012). Tolerance was negative (i.e., under-compensation) for all of our experimental genotypes, i.e., relative growth was higher in undefoliated versus defoliated trees within each genotype. A similar result was also found by Axelsson and Hjältén (2012). We found no statistically significant variation in tolerance among modified and WT genotypes, although we did observe trends indicating lower tolerance in RGL than all other genotypes. RGL exhibited the strongest reduction in biomass production and more allocation to root versus photosynthetic tissues, which may explain low tolerance to defoliation. For example, tolerance was most strongly correlated with total leaf area of leaves flushed post-defoliation, which was significantly lower in RGL relative to all other genotypes. Results from this study indicate that mechanisms of tolerance may vary depending on biomass allocation strategies. Our third prediction was that allocation to stem or root biomass may have a correlation with tolerance to defoliation. This prediction was based on evidence of a positive relationship between tolerance and stem biomass in *Populus* (Axelsson and Hjältén 2012; Stevens *et al.* 2008). Biomass allocation in favour of photosynthetic or storage tissues may support regrowth and ultimately facilitate tolerance (Hochwender *et al.* 2012; Tiffin 2000; Tschaplinksi and Blake 1989a, b). Stem biomass has been positively correlated to tolerance in trees, whereas photosynthetic rate and root biomass have been positively correlated to tolerance in forbs (Hochwender *et al.* 2000; Stowe *et al.* 2000; Stevens *et al.* 2007). We found that stem biomass production did not correlate with tolerance ($R = 0.480$, $P = 0.408$). C17 trended toward taller stems and exhibited the highest allocation of biomass to stems, which facilitate competition for sunlight and provide additional photosynthetic and storage tissues and therefore support regrowth and tolerance; however, tolerance was only moderate in this genotype. Although positive correlations between stem biomass (rather than root biomass) and tolerance have been reported in trees, roots, which provide storage for nitrogen and carbohydrates, may also play a role in tolerance to defoliation in trees. Trends in this experiment indicate that MTG had the largest reduction in root mass (but not root mass ratio) due to defoliation. This

genotype also trended toward higher tolerance relative to all other genotypes. Stem and root allocation paired with tolerance trends for RGL, C17 and MTG demonstrate that production of root biomass may also support regrowth in trees, just as it does in herbaceous plants. Longer-term studies may yield stronger evidence for tolerance responses.

We also found suggestive evidence that allocation of biomass to leaf area was a mechanism of tolerance. First, total leaf area was positively correlated with tolerance, although marginally so. And second, although XG and RGL exhibited similar biomass production and allocation, they differed in biomass allocation response to defoliation and tolerance (not significant). XG flushed leaves with larger area in response to defoliation and exhibited marginally higher tolerance relative to RGL. RGL also flushed leaves post-defoliation with more area, relative to all other genotypes. Increased leaf area may act as a mechanism of tolerance by increasing net photosynthesis to support regrowth (Lambers and Poorter 1992; Ryser and Lambers 1995). Defoliation has been reported to increase leaf area, possibly as an alternative to increasing photosynthesis rates in remaining tissues (Bassman and Zwier 1993). Despite these findings our results show that genetic modification of GA levels may affect 'mechanisms' of tolerance but not enough to significantly affect tolerance itself. However, evidence of tolerance to defoliation may not have been manifested strongly within the limited duration of this study, and may be expressed more strongly in future reproductive tissues or with successive defoliation (Trumble *et al.* 1993; Stevens *et al.* 2012; Strauss and Agrawal 1999).

In contrast to previous research, we comprehensively assessed the influence of genetic modification of GA by evaluating multiple modification strategies and their impacts on both chemical resistance and tolerance to defoliation. Our research determined that modification of GA levels alters biomass production and allocation and may affect chemical resistance, but not tolerance. Despite the fact that levels of chemical resistance were lower in some of our experimental genotypes, down-regulation of GA may not always substantially alter subsequent plant-pest interactions. Although the effects of down-regulated GA levels on defence against herbivores appear moderate, they highlight the potential for unanticipated side-effects when modifying even a single gene. Impacts such as increases in specific defence traits can make a particular modified genotype more or less susceptible to generalist versus specialist pests. Results obtained within the limited duration of this experiment suggest that genetic modification of growth traits in *Populus* has some non-target effects on chemical resistance traits but has no statistically significant effect on tolerance. Similar to results from growth studies of GA up-regulation and enhanced growth in GE trees (Lu *et al.* 2015), the physiological effects of GA modification have variable effects that change over time and are not readily predictable from greenhouse or short-term field studies. Larger and longer-term studies are needed to fully understand GE consequences for growth and herbivore defence. Stringent regulations and market restrictions on GE trees, however, make such studies difficult or impossible to conduct, hindering both science and their potential development for forestry applications (Strauss *et al.* 2015).

SUPPLEMENTARY DATA

Supplementary material is available at *Journal of Plant Ecology* online.

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