Chemoecology (2011) 21:161–169
DOI 10.1007/s00049-011-0080-8

CHEMOECOLOGY

RESEARCH PAPER

Leaf ontogeny interacts with Bt modification to affect innate resistance in GM aspens

E. Petter Axelsson · Joakim Hjältén · Thomas G. Whitham · Riitta Julkunen-Tiitto · Gilles Pilate · Anders Wennström

Received: 28 October 2010 / Accepted: 5 May 2011 / Published online: 20 May 2011

Abstract Bioassays with a non-target slug (*Deroceras* spp.) and chemical analyses were conducted using leaf tissue from already existing genetically modified insect-resistant aspen trees to examine whether genetic modifications to produce *Bacillus thuringiensis* (Bt) toxins could affect plant phytochemistry, which in turn might influence plant–herbivore interactions. Three major patterns emerged. First, two independent modifications for Bt resistance affected the phytochemical profiles of leaves such that both were different from the isogenic wild-type (Wt) control leaves, but also different from each other. Among the contributors to these differences are substances with a presumed involvement in resistance, such as salicortin and soluble condensed tannins. Second, bioassays with one Bt line suggest that the modification somehow affected innate resistance ("Innate" is used here in opposition to the "acquired" Bt resistance) in ways such that slugs preferred Bt over Wt leaves. Third, the preference test suggests that the innate resistance in Bt relative to Wt plants may not be uniformly expressed throughout the whole plant and that leaf ontogeny interacts with the modification to affect resistance. This was manifested through an ontogenetic determined increase in leaf consumption that was more than four times higher in Bt compared to Wt leaves. Our result are of principal importance, as these indicate that genetic modifications can affect innate resistance and thus non-target herbivores in ways that may have commercial and/or environmental consequences. The finding of a modification–ontogeny interaction effect on innate resistance may be especially important in assessments of GM plants with a long lifespan such as trees.

Keywords GM trees · Pleiotropic effect · Secondary substances · *Populus* · Slug · Ontogeny · Biotechnology

E. P. Axelsson (✉) · J. Hjältén
Department of Wildlife, Fish, and Environmental Studies, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden
e-mail: Petter.Axelsson@slu.se

T. G. Whitham
Department of Biological Science, Northern Arizona University, Flagstaff, AZ 86011, USA

R. Julkunen-Tiitto
Department of Biology, University of Eastern Finland, P.O. Box 111, 80101 Joensuu, Finland

G. Pilate
INRA, UR 0588 'Amélioration, Génétique et Physiologie Forestières', CS 40001 Ardon, 45075 Orléans Cedex 2, France

A. Wennström
Department of Ecology and Environmental Science, Umeå University, 901 87 Umeå, Sweden

Introduction

Although the effects of insect-resistant transgenic trees on non-target organisms have been in debate for a long time (see for example Raffa 1989), it is still a highly relevant topic for a safe deployment of such products. As genetic modifications for resistance are intended to increase yields through lowered herbivory without adverse effects on non-target organisms, a safe deployment of insect-resistant plant products relies on the effects on such organisms. In this respect, non-target herbivores are intricately important and are considered a key group of organisms in risk assessments of transgenic plants (Andow 2006). Herbivores are important as they may regulate host populations (Maron 1998; Mueller et al. 2005), determine community

structure and diversity (Carson and Root 2000), and drive ecological (Schweitzer et al. 2005) and evolutionary processes (Lankau 2007; Mauricio and Rausher 1997). In the case of insect-resistant transgenic plants, the non-target herbivores represent also a potential link between the plant and other possibly susceptible non-target organism at higher trophic levels (Alvarez-Alfageme et al. 2009; Zurbrugg and Nentwig 2009).

In the assessment of the effects on non-target herbivores, the innate resistance ("innate" is used here in opposition to the resistance "acquired" by the modification) of GM plants may be of particular importance as it plays a central role in the interaction among plants, their natural enemies and whole communities (Dickson and Whitham 1996; Kolehmainen et al. 1995; Rank et al. 1998). Some studies address this and confirm that resistance traits may exhibit unintended alterations following genetic modifications (Axelsson et al. 2010; Hjältén et al. 2007; Saxena and Stotzky 2001). For example, Saxena and Stotzky (2001) found that lignin concentrations in Bt corn were higher than in non-Bt corn. Unintended changes in such traits may affect non-target organisms (Hjältén et al. 2007), cause problems with secondary pest species (Balestrazzi et al. 2006) or affect ecosystem processes (Axelsson et al. 2010). Such findings validate further studies on how modifications in plants may affect resistance traits and further non-target herbivores.

In contrast to trees, the potential non-target effects of insect-resistant transgenic crops have been extensively studied. Most studies focus on lethal and sublethal effects of the introduced toxins (de Vaufleury et al. 2007; Rose and Dively 2007; Rosi-Marshall et al. 2007; Sisterson et al. 2007), but see also Saxena and Stotzky (2001). Some of these studies suggest that genetic modification for pest resistance may be an environmentally benign alternative to insecticide spraying (Rose and Dively 2007; Sisterson et al. 2007), whereas others indicate that they may indeed affect non-target organisms (Rosi-Marshall et al. 2007). However, in a recent review of 41 papers published on the non-target effects of Bt corn in 2008 and 2009, it is suggested that serious effects on non-target organisms are uncommon and only two articles found a minor effect (Ricroch et al. 2010).

Nevertheless, with the development of insect-resistant transgenic trees, a totally new set of interactions different from the ones in agriculture may be affected, and with that new concerns have been raised (van Frankenhuyzen and Beardmore 2004). This reappraisal is based on the unique characters of trees. Trees have a long life span, are essentially undomesticated, and represent foundation species that structure their ecosystems by creating locally stable conditions and provide specific resources for diverse organisms (Ellison et al. 2005). The genetics of these species as "community drivers" are especially important to understand, as they are likely to have cascading ecological and evolutionary effects throughout an ecosystem (Whitham 2006). Information on risks from GM crops is thus not necessarily valid or sufficient when considering GM trees (Bradshaw and Strauss 2001).

We used two lines of an isogenic hybrid tree (*Populus tremula* × *Populus tremuloides*) modified to express Bt toxins (cry3Aa) and one unmodified wild type (Wt), to explore the effects of genetic modification on innate resistance traits and the potential for effects on non-target herbivores. The effect of two independent modifications for Bt resistance on the phytochemical profile of leaves was explored during leaf ontogeny. We further examined how leaf ontogeny and modification of one Bt line affected the preference of a non-target herbivore. Preference of non-target generalist slugs (*Deroceras reticulatum* and *D. agreste*) for Bt-expressing and Wt leaves was tested in bioassays and a 10-day no-choice feeding trial was also conducted.

Materials and methods

Plant material

Two independent modifications of an isogenic *P. tremula* × *P. tremuloides* hybrid clone (INRA # 353-38) and one unmodified wild-type line provided the plant material used in these experiments. The genetically modified lines, Bt17 and Bt27, have previously been described by Genissel et al. (2003b), and have been modified for resistance with a synthetic cry3Aa gene, which make the plants produce Bt toxin. This toxin targets coleopteran beetles and is highly effective against the leaf beetle *Chrysomela tremulae*. Cry3Aa protein quantity has previously been estimated to be approximately 0.05 and 0.0025% of the total soluble protein in Bt17 and Bt27 plants, respectively (Genissel et al. 2003b). Plants were propagated in the laboratory and later relocated to a greenhouse with one individual of each line making up a block. In total, 15 blocks were available and 5 of these were randomly assigned to be used in the bioassays. The other ten sets of plants provided leaves for the chemical analyses and were used to determine stem biomass. The plants were harvested after 10 weeks in the greenhouse at an approximate mean size of 160, 130 and 125 cm for Wt, Bt27 and Bt17, respectively. Stems were collected and air dried at room temperature (~22°C) for 3 months, dry mass was determined and one-way analyses of variance (ANOVA) was used to test for significant differences.

Slugs

Two slug species (*Deroceras reticulatum* and *D. agreste*: family Limacidae) were used as representatives of non-target herbivores. This polyphagous genus may feed on

various agricultural crops and woodland plant species (Jennings and Barkham 1975). They can be found in various habitats such as forests (Beyer and Saari 1977), agroforestry systems and agricultural fields (Griffiths et al. 1998). Because *D. reticulatum* and *D. agreste* can only be distinguished from each other using internal characters (Kerney and Cameron 1979), they were pooled for our bioassays. Slugs were collected from one free-living population 2 days before the start of the bioassays and were fed on dandelion (*Taráxacum officinale*) leaves until 24 h prior to the preference test.

Phytochemistry

Leaves for the chemical analyses from all three lines (Bt17, Bt27 and Wt) were picked from the same positions as in the preference test and in synchrony with the start of the bioassays (see "Bioassay"). All leaf materials were air dried (2 weeks at ~22°C) and milled before the analyses. To quantify the concentrations of individual secondary compounds, we used high-performance liquid chromatography (HPLC) for low molecular weight phenolics. Agilent's Series1100 high-pressure liquid chromatography (HPLC) system (Agilent Technologies, Germany) equipped with Agilent's G1315B diode array detector (DAD) and a reversed-phase (RP) octadecyl carbon chain (C18) column (Agilent Technologies, USA) was used. Leaf material (5 mg) was homogenized in 0.6 ml of methanol for 30 s with an Ultra-Turrax homogenizer. The samples were then left in an ice bath for 15 min and then re-homogenized and centrifuged at 16,000×*g* for 3 min. The supernatant was collected, while the residue was washed three more times with 0.6 ml of methanol, homogenized for 30 s and centrifuged. All supernatants were combined, and methanol was evaporated off in a vacuum centrifuge. The dried samples were dissolved in 150 µl of methanol, 150 µl of water was added and the samples were analyzed using HPLC for low molecular weight phenolics. The compounds were separated on a 60 mm × 4.6 mm column (HP Hypersil ODS II, 3 µm). The elution solvents were aqueous 1.5% tetrahydrofuran plus 0.25% orthophosphoric acid and methanol. The gradients used have previously been described by Julkunen-Tiitto and Sorsa (2001). The flow rate was 2 ml/min and the injection volume was 20 µl. Individual compounds were identified by comparing their UV–visible spectra and retention time to those of known compounds. The quantification of salicin, chlorogenic acid, hyperin, kaemferol 3-glucoside, apigenin and tremulacin were based on commercial standards. The quantification of salicortin and tremuloidin was based on purified compounds from the leaves of *Salix* sp. The quantification of other compounds was based as follows: neochlorgenic acid based on chlorogenic acid; quercetins based on hyperin; isorhamnetin glycoside based on isorhamnetin 3-glucoside; apigenin derivative based on apigenin; monocoumaroyl-astragalin derivatives based on kaempferol 3-glucoside; HCH-tremulacin and tremulacin derivatives based on tremulacin. Soluble polymeric condensed tannins were measured from HPLC samples with the butanol–HCl assay (Waterman and Mole 1994).

Phytochemistry profiles of leaves were explored with PERMANOVAs (Anderson 2001) using PRIMER (PRIMER-E, 2007) testing for the effect of line (Wt, Bt17 and Bt27) and position (high and low). In these analyses, we used Bray–Curtis distances in distance matrix construction and 4999 permutations. Chemical profiles of leaves from the different lines and leaf positions were visualized with MDS plot, using Bray–Curtis dissimilarities. To clarify which substances contributed most to the observed differences in chemistry, we used similarity percentage analysis (SIMPER), also on fourth-root transformed data. This is not a test of statistical probabilities per se, but a way of conceptualizing what differs between two sets of data: SIMPER calculates the overall percentage contribution that each substance makes to the average dissimilarity between two groups and lists the substances in decreasing order of their importance in discriminating the two sets of samples (Clarke and Gorley 2001).

Bioassay

The preference assay was conducted on individual slugs ($N = 100$) given the choice between leaf discs from the Wt line and the Bt17 line. The leaf discs were cut from newly picked leaves with a 13-mm Ø core borer. The leaf material was derived from one "high" and one "low" position on source aspens. The high and low leaf positions in this study are equivalent to leaves of different plastochron indexes. This was performed to reflect the different leaf ontogenetic stages that can differ markedly in phytochemistry and in resistance to pests (Donaldson 2006; Hjältén et al. 2007; Holeski et al. 2009). The positions were standardized to leaf number 3 (high) and 6 (low), starting from the top of the stem with the first leaf that in all directions exceeding a circular shape of 29 mm in diameter. Thus, one group of slugs was given the choice between leaves from the high position ($N = 50$) and the other group from the low position ($N = 50$). Each experiment arena was made up of one 100 mm-diameter Petri dish with a moist filter paper at the bottom and slugs were left to feed in these arenas for 24 h. The area eaten from each leaf disc was estimated with a 1 mm^2-mesh plastic screen.

Two-way analyses of variance (ANOVA) were used to test for the effect of line (Bt and Wt) and leaf position (high and low) on the amount of leaf area consumed by the slugs. In case of a significant interaction between the two factors,

subsequent contrasts were specified to examine the effect of each factor at each level of the other. The data were log [x + 1] transformed to meet the assumption of homogeneity and normality, and individuals that did not feed during the trials were excluded from the analyses ($n = 4$). All statistical analyses on bioassays were done with the statistical software SYSTAT 12.

The 10-day no-choice feeding trial was carried out in identical arenas as in the preference assay. Each slug was randomly assigned a diet consisting of leaf discs from either the Wt line ($n = 48$) or the Bt17 line ($n = 46$). All leaves in the 10-day no-choice feeding trial were derived from the "low" position on the aspens and the leaf discs were cut with a 10-mm Ø core borer. During the experiment, leaf discs were replaced with fresh discs once each day, and the consumption on the removed discs were noted. As we used five blocks of Wt and Bt plants, each block was presented to the slugs twice during the 10-day trial period. If a slug died, the day of death and the identity of the slug were noted. Slugs that refused to feed for three consecutive days were denoted as "starving" and eliminated from the experiment ($n = 47$). The filter papers in the arenas were re-moisturized twice a day, and halfway through the trials (at the end of day 5) the filter papers were replaced.

The survival and starving data from the 10-day trial were analyzed with an equality of two proportion test. The food intake data were log[x] transformed to meet the assumption of homogeneity and normality before being analyzed with a two-way ANOVA. In the ANOVA, we tested for the effect of the factors line (Bt and Wt) and the final condition of the slug (alive or dead) on food intake.

Results

Phytochemistry

The PERMANOVAs to explore phytochemical profiles revealed that both line and position had a significant effect ($P < 0.001$, in both cases). Phytochemical profiles were not affected by interactions between factors ($P = 0.345$), and the interaction term was thus omitted in the final analyses. Subsequent analyses of differences among groups showed that both of the Bt lines were significantly different from the Wt line ($P < 0.001$ in both cases) and that the two Bt lines also were different from each other ($P = 0.041$; Fig. 1). Subsequent SIMPER analyses showed that most substances decreased in concentration from the high position leaves to leaves at a low position (Table 1). The exception from this was salicortin that increased in low position leaves. The substances quercitrin, monocoumaroyl-kaempferol-glycoside and tremulacin contributed most to differences between Wt leaves and leaves from the Bt17 and Bt27 lines. Interestingly, these are the same substances that contributed the most also to the differences between high and low leaves (Table 1).

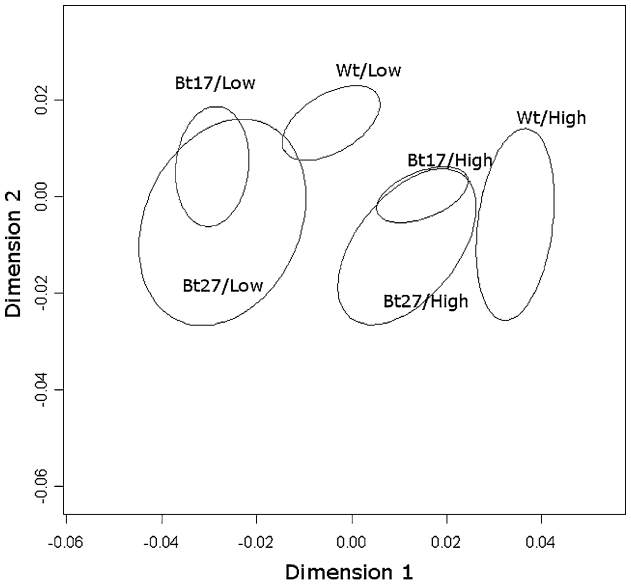

Fig. 1 MDS plot illustrating the significant differences in chemical profiles among lines (Wt, Bt17 and Bt27) and over leaf ontogeny (high and low). *Ellipsoids* show SE around mean positions of chemical profiles of leaves from the six different line and position combinations

Bioassay

The preference test revealed a significant line by leaf position interaction effect (Table 2). The subsequent analyses on differences between groups revealed that the consumption of the Bt line at the low position was significantly higher than that on both the Wt/low combination and the Bt/high combination ($P = 0.006$ and $P < 0.001$, respectively; Fig. 2).

The food intake of feeding slugs during the 10-day no-choice feeding trial was higher on Bt leaves than on Wt leaves, and slugs that survived the trials consumed more leaf area than slugs that did not, which is shown by the significant single factors line and condition, respectively (Fig. 3; Table 3). The proportion of slug individuals that did not eat for three consecutive days and thus denoted as "starving" (and excluded from the analyses) did not differ between lines (Wt = 58% and Bt = 41%, $P = 0.097$). Similarly, the proportion of the feeding individuals that survived the 10-day no-choice feeding trials was also not affected by the diet (Wt = 55% and Bt = 59%, $P = 0.770$).

Aspen growth

Analyses of stem biomass revealed that the line factor had a significant effect on stem biomass ($P < 0.001$) and

Table 1 Mean concentration (mg/g ± SD) of 25 chemical substances in leaves from high and low positions and from Wt, Bt27 and Bt17 populus plants

Substance	Position				Line						
	Mean concentration (mg/g ± SD)		High/low comparison		Mean concentration (mg/g ± SD)			Wt/Bt27 comparison		Wt/Bt17 comparison	
	High	Low	Contribution %	Direction	Wt	Bt27	Bt17	Contribution %	Direction	Contribution %	Direction
Quercitrin	10.82 ± 3.49	5.95 ± 2.42	7.75	(−)	10.90 ± 3.80	7.23 ± 3.79	7.01 ± 2.73	7.64	(−)	7.99	(−)
Monocoumaroyl-kaempferol-glycoside	1.50 ± 0.79	0.58 ± 0.36	7.38	(−)	1.05 ± 0.86	1.34 ± 0.82	0.74 ± 0.48	6.76	(+)	7.02	(−)
Tremulacin	146.32 ± 32.88	116.92 ± 22.29	6.79	(−)	142.75 ± 31.07	116.48 ± 30.23	135.62 ± 28.69	7.37	(−)	6.65	(−)
Kaempferol-3-rhamnoside	10.24 ± 1.62	6.21 ± 1.45	6.05	(−)	9.11 ± 2.38	7.58 ± 2.32	7.98 ± 2.77	4.50	(−)	5.38	(−)
Salicin	8.09 ± 3.88	6.04 ± 3.98	5.68	(−)	6.45 ± 3.73	8.56 ± 5.54	6.18 ± 1.52	6.26	(+)	4.61	(+)
Quercetin diglycoside 4	1.41 ± 0.65	0.79 ± 0.45	5.24	(−)	1.55 ± 0.65	0.90 ± 0.60	0.84 ± 0.40	5.78	(−)	6.12	(−)
Salicortin	126.99 ± 25.47	135.93 ± 20.81	5.00	(+)	129.24 ± 24.59	122.00 ± 26.58	143.13 ± 12.67	5.84	(−)	5.23	(+)
Tremulacin der 2	0.68 ± 0.57	0.41 ± 0.45	4.93	(−)	0.57 ± 0.62	0.64 ± 0.59	0.43 ± 0.34	5.04	(+)	4.77	(−)
Tremulacin der 1	0.68 ± 0.53	0.40 ± 0.42	4.91	(−)	0.56 ± 0.59	0.62 ± 0.53	0.44 ± 0.32	4.82	(+)	4.62	(−)
Quercetin diglycoside 3	1.11 ± 0.38	0.57 ± 0.23	4.76	(−)	1.12 ± 0.43	0.72 ± 0.38	0.67 ± 0.28	4.59	(−)	5.04	(−)
HCH-salicortin	1.21 ± 0.72	1.03 ± 1.00	4.12	(−)	0.98 ± 0.71	1.34 ± 1.11	1.03 ± 0.72	4.70	(+)	4.10	(+)
Disalicortin	2.51 ± 1.47	2.19 ± 1.61	4.07	(−)	2.09 ± 1.45	2.74 ± 1.86	2.22 ± 1.23	5.04	(+)	4.52	(+)
Quercetin diglycoside 2	0.38 ± 0.16	0.20 ± 0.10	3.72	(−)	0.39 ± 0.16	0.26 ± 0.17	0.23 ± 0.10	3.75	(−)	3.94	(−)
Kaempferol-diglycoside 2	0.32 ± 0.07	0.15 ± 0.05	3.67	(−)	0.28 ± 0.11	0.22 ± 0.10	0.21 ± 0.09	2.77	(−)	3.17	(−)
Apigenin diglycoside der 2	0.67 ± 0.15	0.37 ± 0.10	3.52	(−)	0.61 ± 0.19	0.47 ± 0.18	0.49 ± 0.19	2.89	(−)	3.14	(−)
p-OH-cinnamic acid der 1	3.42 ± 0.60	2.52 ± 0.57	3.17	(−)	3.35 ± 0.66	2.70 ± 0.70	2.87 ± 0.73	3.04	(−)	3.18	(−)
Chlorogenic acid der	6.50 ± 0.57	4.97 ± 0.56	2.92	(−)	6.19 ± 0.93	5.60 ± 0.93	5.42 ± 0.87	2.25	(−)	2.69	(−)
Kaempferol-diglycoside 1	0.97 ± 0.13	0.66 ± 0.15	2.66	(−)	0.90 ± 0.17	0.76 ± 0.21	0.79 ± 0.23	2.15	(−)	2.45	(−)
Soluble condensed tannins	3.50 ± 1.43	3.24 ± 1.06	2.60	(−)	4.11 ± 1.60	3.13 ± 1.16	2.89 ± 0.28	3.77	(−)	3.76	(−)
Quercetin diglycoside 1	0.17 ± 0.05	0.11 ± 0.05	2.55	(−)	0.17 ± 0.06	0.13 ± 0.07	0.11 ± 0.04	2.37	(−)	2.82	(−)
SaOH-diglucoside	2.54 ± 0.44	2.02 ± 0.25	2.24	(−)	2.07 ± 0.30	2.41 ± 0.45	2.35 ± 0.50	1.99	(+)	2.24	(+)
Myricitrin	0.60 ± 0.14	0.48 ± 0.15	2.12	(−)	0.64 ± 0.13	0.47 ± 0.16	0.51 ± 0.12	2.61	(−)	2.16	(−)
Apigenin diglycoside der 1	0.76 ± 0.15	0.67 ± 0.15	1.60	(−)	0.71 ± 0.14	0.67 ± 0.14	0.76 ± 0.18	1.57	(−)	1.78	(+)
p-OH-cinnamic acid der 2	0.32 ± 0.08	0.27 ± 0.06	1.46	(−)	0.30 ± 0.07	0.29 ± 0.08	0.29 ± 0.06	1.54	(−)	1.58	(−)
Chlorogenic acid	0.28 ± 0.03	0.23 ± 0.03	1.08	(−)	0.27 ± 0.04	0.26 ± 0.04	0.24 ± 0.03	0.95	(−)	1.04	(−)

Comparisons reflect the contribution (%) of that substance to the differences between groups in SIMPER analyses and directions illustrate if the change is positive (+) or negative (−)

Table 2 ANOVA table showing the effect of the factors line and leaf position on the consumption of leaf discs by slugs in preference tests

Factor	*df*	SS	*F*	*P*
Line (L)	1	0.985	1.883	0.172
Leaf position (P)	1	7.756	14.832	<0.001
L × P	1	3.372	6.448	0.012
Error	188	98.315		

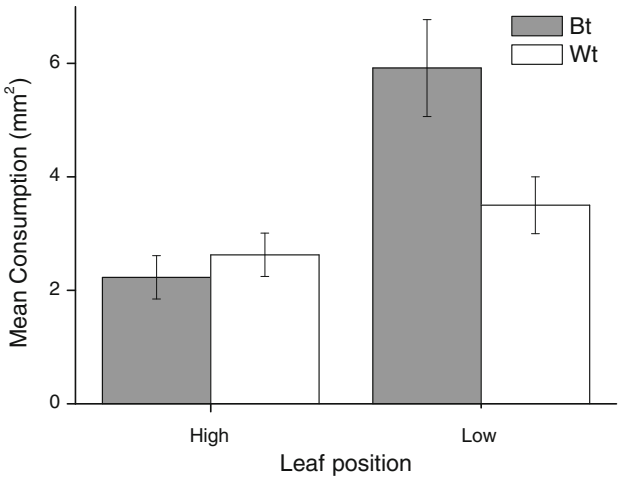

Fig. 2 Leaf consumption ($mm^2 \pm 1$ SE) by slugs when presented with both Bt and Wt leaf discs from either the high or low leaf positions of Bt and Wt plants. Contrasts between groups revealed that the consumption of the Bt line at the low position was significantly higher than that on both the Wt/low combination and the Bt/high combination ($P < 0.006$ and $P < 0.001$, respectively)

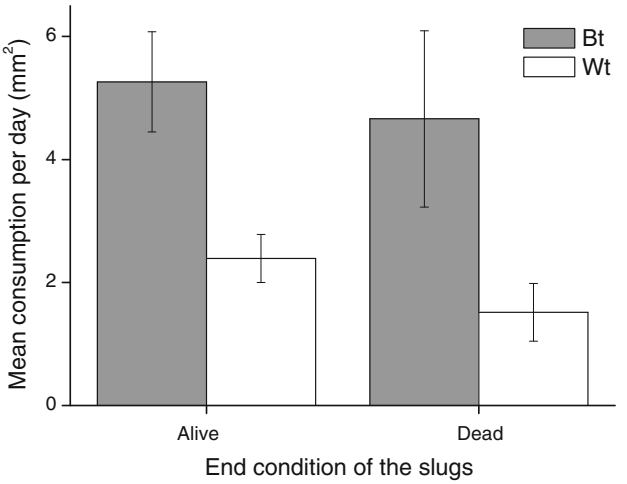

Fig. 3 The mean daily leaf consumption ($mm^2 \pm$ SE) by slugs ending up as either alive or dead, when presented with leaf discs from both Bt and Wt plants in a 10-day no-choice feeding trail. ANOVA analyses showed that both line (Bt and Wt) and condition (alive and dead) affected consumption

Table 3 ANOVA table showing the effect of the factors line and condition on the leaf consumption by slugs in a 10-day no-choice feeding trial

Factor	*df*	SS	*F*	*P*
Line (L)	1	9.869	16.149	<0.001
Condition (C)	1	2.928	4.791	0.034
L × C	1	0.310	0.507	0.480
Error	43	26.278		

subsequent comparisons showed that Wt was different from both of the Bt lines ($P < 0.001$), but that the two Bt lines were not different from each other ($P = 0.33$). While growing under our greenhouse conditions for 10 weeks, the unmodified Wt line produced more stem biomass than both Bt17 and the Bt27 line (52.3 g ± 8.4 SD, 21.6 g ± 7.0 SD and 28.9 ± 16.5 SD, respectively).

Discussion

Effects on plant phytochemicals

We showed that two independent modifications for resistance in aspens caused significant changes in the phytochemical profiles of leaf tissue, and likewise that the two insect-resistant lines also differed from each other. The contributors to these differences include several phytochemical compounds of importance for plant–herbivore interactions. For example, salicortin and soluble condensed tannins have previously shown resistance properties toward insect herbivores (Donaldson and Lindroth 2007; Orians et al. 1997) and slugs (Albrectsen et al. 2004; Fritz et al. 2001), suggesting that changes in their concentrations may alter plant–herbivore interactions. A majority of the substances, including condensed tannins but with the exception of salicortin, were lower in the Bt17 line compared to Wt. This indicates that compounds other than salicortin are likely to explain the higher consumption of Bt leaves by slugs. It is also possible that the combined effect of several phytochemicals as illustrated by the differences in chemical profiles also influenced the response in the slugs. Alteration of phytochemical traits have been reported before, both in genetically modified crops (Saxena and Stotzky 2001) and in modified trees (Axelsson et al. 2010; Hjältén et al. 2007).

The reason why unintended changes in phytochemistry occurred in the Bt trees is not clear. Under our greenhouse conditions, we observed reduced growth of the Bt lines, which might have influenced our results through secondary effects on phytochemistry and further the preference of the slugs. Such a pronounced growth reduction as seen here

was not detected in another study with these same lines (Hjältén et al. unpublished). This suggests that the growth of these lines may be conditionally determined and that further studies are needed to validate the generality of our results. Nevertheless, growth may affect phytochemistry of poplar trees (Kleiner et al. 1998) and plant vigor may indeed influence resistance properties (Albrectsen et al. 2004; Price 1991). It has been shown that slugs may prefer slow growing over vigorous willows, a response potentially explained by the higher concentration of condensed tannins in the vigorously growing plants (Albrectsen et al. 2004). However, the reason why these combined effects of reduced growth and changes in phytochemistry occurred is still not explained. Pleiotropic effects of genetic modification may arise if the locations of the transgenic insertion are not fully controlled (Novak and Haslberger 2000) or if the inserted genes influence the metabolic expressions of the receiving plant (Conner and Jacobs 1999). Such effects could potentially work directly on genes responsible for the resistance phenotypes or influence resistance through secondary effects caused by growth–phytochemistry associations. The random insertion explanation is supported by the fact that two independent modifications seemed to have caused divergent changes in phytochemical profiles. However, the two Bt lines used here also expresses different concentrations of Bt toxins, which make them different also in this respect. Thus, we cannot with certainty state the precise mechanism causing the observed effects.

Non-target herbivore effects

The bioassays show that genetic modification for resistance in trees can influence the preference of a non-target herbivore. When presented with leaves from the low position, the non-target slugs preferred to feed on leaves from the Bt trees: a result also supported by the 10-day no-choice feeding trial. In contrast, using leaves from the high position, no such difference was observed suggesting that leaf ontogeny is an interacting factor in these relationships.

Our results add to those from previous studies on trees showing that genetic modification of sucrose phosphate synthase (Hjältén et al. 2007) and lignin (Brodeur-Campbell et al. 2006; Tiimonen et al. 2005) may affect insect preference. Together, such findings give support to the importance of studying non-target organisms in risk assessments of GM plants, as also suggested previously (Andow 2006). Increased attraction of a non-target herbivore toward insect-resistant transgenic trees is of principal importance, as it indicates that genetic modifications for insect resistance can affect plant resistance, so that GM plants are preferred over Wt. Commercially, attraction of non-target herbivores may intuitively be seen as counterproductive. Accumulation of secondary pest species has previously been mentioned as a potential problem with genetic modifications for resistance (Balestrazzi et al. 2006), and genetic modifications of trees have also been shown to change trees' susceptibility toward pathogens (Blomberg 2007). However, similar differences in resistance can also be seen among clones of the same species as well as among hybrids (Bingaman and Hart 1992; Ramirez et al. 2004). For example, Bingaman and Hart (1992) showed that feeding and oviposition preferences of cottonwood leaf beetles (*Chrysomela scripta*) differed among six *Populus* clones in both multiple-choice and no-choice tests. It is important to emphasize that all of the lines used here derived from the same isogenic clone and should, with the exception of the changes introduced by the genetic modification, therefore be considered genetically identical. Further, our results may be of relevance also to resistance evolution processes, such as those recently reported in *Helicoverpa armigera*, the primary target of Bt cotton in China (Liu et al. 2010). Genissel et al. (2003a) detected Bt-resistant alleles in a field population of the leaf beetle *Chrysomela tremulae* indicating a potential for resistance evolution and suggesting that such processes may need considerations also in relation to insect-resistant Bt trees. Resistance evolution depends among other factors on the fitness costs linked with the resistant alleles (Wenes et al. 2006), and changes in the innate resistance of the host plant could thus be of importance in such processes.

Ontogenetic effects

Our preference tests indicate that unintended effects on herbivores may not be static throughout the plant and can be influenced by plant ontogeny, e.g., slugs preferred Bt over Wt leaves only when presented with mature leaves. Genetically controlled ontogenetic changes in resistance can differ dramatically within poplar trees (Holeski et al. 2009), and our result suggested that such ontogenetic patterns were affected in the GM lines. A similar phenomenon was noted by Kleiner et al. (2003), who showed that condensed tannin concentrations in control poplar plants increased with leaf plastochron index as expected, but that concentrations in Bt poplar plants did not. That plant material of different age or in different ontogenetic stages can differ greatly to suit individual herbivores (Albrectsen et al. 2004; Bingaman and Hart 1992; Fritz et al. 2001; Hjältén et al. 2007) and even whole communities (Kearsley and Whitham 1989; Waltz and Whitham 1997) is well documented, and similar patterns can be seen in the compounds conferring resistance (Albrectsen et al. 2004; Boege and Marquis 2005; Donaldson 2006; Fritz et al. 2001; Osier et al. 2000; Rehill et al. 2006). Such patterns of resistance may be particularly important in long-lived

plants that have to endure many seasons of herbivory during their life cycle and, in the context of their commercial use, several seasons before harvest.

Acknowledgments This study was supported by FORMAS (grant to J. Hjältén). We thank Marie Nygren for propagation of the plants and the personnel at the facilities at SLU Umeå for nursing the plants in the greenhouse.

References

Albrectsen BR, Gardfjell H, Orians CM, Murray B, Fritz RS (2004) Slugs, willow seedlings and nutrient fertilization: intrinsic vigor inversely affects palatability. Oikos 105(2):268–278

Alvarez-Alfageme F, Ortego F, Castanera P (2009) Bt maize fed-prey mediated effect on fitness and digestive physiology of the ground predator *Poecilus cupreus* L. (Coleoptera: Carabidae). J Insect Physiol 55(2):143–149

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Aust Ecol 26(1):32–46

Andow DA (2006) Assessing environmental risks of transgenic plants. Ecol Lett 9(2):196–214

Axelsson EP, Hjältén J, LeRoy CJ, Julkunen-Tiitto R, Wennström A, Pilate G (2010) Can leaf litter from genetically modified trees affect aquatic ecosystems? Ecosystems 13(7):1049–1059

Balestrazzi A, Allegro G, Confalonieri M (2006) Genetically modified trees expressing genes for insect pest resistance. Tree Transgenesis: Recent Developments, part C. Springer, Heidelberg, pp 253–273

Beyer WN, Saari DM (1977) Effect of tree species on distribution of slugs. J Anim Ecol 46(3):697–702

Bingaman BR, Hart ER (1992) Feeding and oviposition preferences of adult cottonwood leaf beetles (Coleoptera, Chrysomelidae) among *Populus* clones and leaf age classes. Environ Entomol 21(3):508–517

Blomberg P (2007) Genetically engineered aspen (*Populus tremula x tremuloides*) alters its interaction with the non-target pathogen *Venturia tremulae*. Umeå University, Umeå

Boege K, Marquis RJ (2005) Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends Ecol Evol 20(8):441–448

Bradshaw AH, Strauss SH (2001) Plotting a course for GM forestry [2]. Nat Biotechnol 19(12):1103–1104

Brodeur-Campbell SE, Vucetich JA, Richter DL, Waite TA, Rosemier JN, Tsai CJ (2006) Insect herbivory on low-lignin transgenic aspen. Environ Entomol 35(6):1696–1701

Carson WP, Root RB (2000) Herbivory and plant species coexistence: community regulation by an outbreaking phytophagous insect. Ecol Monogr 70(1):73–99

Clarke KR, Gorley RN (2001) Primer v5: user manual/tutorial. Primer-E Ltd, Plymouth

Conner AJ, Jacobs JME (1999) Genetic engineering of crops as potential source of genetic hazard in the human diet. Mutat Res-Genet Toxicol Environ Mutagen 443(1–2):223–234

de Vaufleury A, Kramarz PE, Binet P, Cortet J, Caul S, Andersen MN, Plumey E, Coeurdassier M, Krogh PH (2007) Exposure and effects assessments of Bt-maize on non-target organisms (gastropods, microarthropods, mycorrhizal fungi) in microcosms. Pedobiologia 51(3):185–194

Dickson LL, Whitham TG (1996) Genetically based plant resistance traits affect arthropods, fungi, and birds. Oecologia 106(3): 400–406

Donaldson JR (2006) Age-related shifts in leaf chemistry of clonal aspen (*Populus tremuloides*). J Chem Ecol 32(7):1415–1429

Donaldson JR, Lindroth RL (2007) Genetics, environment, and their interaction determine efficacy of chemical defense in trembling aspen. Ecology 88(3):729–739

Ellison AM, Bank MS, Clinton BD, Colburn EA, Elliott K, Ford CR, Foster DR, Kloeppel BD, Knoepp JD, Lovett GM, Mohan J, Orwig DA, Rodenhouse NL, Sobczak WV, Stinson KA, Stone JK, Swan CM, Thompson J, Von Holle B, Webster JR (2005) Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Frontiers Ecol Environ 3(9): 479–486

Fritz RS, Hochwender CG, Lewkiewicz DA, Bothwell S, Orians CM (2001) Seedling herbivory by slugs in a willow hybrid system: developmental changes in damage, chemical defense, and plant performance. Oecologia 129(1):87–97

Genissel A, Augustin S, Courtin C, Pilate G, Lorme P, Bourguet D (2003a) Initial frequency of alleles conferring resistance to *Bacillus thuringiensis* poplar in a field population of *Chrysomela tremulae*. Proc Roy Soc Lond Series B-Biol Sci 270(1517): 791–797

Genissel A, Leple JC, Millet N, Augustin S, Jouanin L, Pilate G (2003b) High tolerance against *Chrysomela tremulae* of transgenic poplar plants expressing a synthetic *cry*3Aa gene from *Bacillus thuringiensis* ssp. *tenebrionis*. Mol Breeding 11(2): 103–110

Griffiths J, Phillips DS, Compton SG, Wright C, Incoll LD (1998) Responses of slug numbers and slug damage to crops in a silvoarable agroforestry landscape. J Appl Ecol 35(2):252–260

Hjältén J, Lindau A, Wennström A, Blomberg P, Witzell J, Hurry V, Ericson L (2007) Unintentional changes of defence traits in GM trees can influence plant–herbivore interactions. Basic Appl Ecol 8(5):434–443

Holeski LM, Kearsley MJC, Whitham TG (2009) Separating ontogenetic and environmental determination of resistance to herbivory in cottonwood. Ecology 90(11):2969–2973

Jennings TJ, Barkham JP (1975) Food of slugs in mixed and deciduous woodland. Oikos 26(2):211–221

Julkunen-Tiitto R, Sorsa S (2001) Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. J Chem Ecol 27(4):779–789

Kearsley MJC, Whitham TG (1989) Developmental-changes in resistance to herbivory—implications for individuals and populations. Ecology 70(2):422–434

Kerney MP, Cameron RAD (1979) A field guide to the land snails of Britain and north-west Europe. Collins, Britain

Kleiner KW, Raffa KF, Ellis DD, McCown BH (1998) Effect of nitrogen availability on the growth and phytochemistry of hybrid poplar and the efficacy of the *Bacillus thuringiensis* cry1A(a) d-endotoxin on gypsy moth. Can J For Res-Revue Canadienne De Recherche Forestiere 28(7):1055–1067

Kleiner KW, Ellis DD, McCown BH, Raffa KF (2003) Leaf ontogeny influences leaf phenolics and the efficacy of genetically expressed *Bacillus thuringiensis* cry1a(a) d-endotoxin in hybrid poplar against gypsy moth. J Chem Ecol 29(11):2585–2602

Kolehmainen JC, Julkunen-Tiitto R, Roininen H, Tahvanainen J (1995) Phenolic glucosides as feeding cues for willow-feeding leaf beetles. Entomologia experimentalis et applicata 74(3):235–243

Lankau RA (2007) Specialist and generalist herbivores exert opposing selection on a chemical defense. The New phytol 175(1): 176–184

Liu FY, Xu ZP, Zhu YC, Huang FN, Wang YH, Li HL, Li H, Gao CF, Zhou WJ, Shen JL (2010) Evidence of field-evolved resistance to Cry1Ac-expressing Bt cotton in *Helicoverpa armigera* (*Lepidoptera: Noctuidae*) in northern China. Pest Manag Sci 66(2): 155–161

Maron JL (1998) Insect herbivory above- and belowground: individual and joint effects on plant fitness. Ecology 79(4):1281–1293

Mauricio R, Rausher MD (1997) Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. Evolution 51(5): 1435–1444

Mueller RC, Wade BD, Gehring CA, Whitham TG (2005) Chronic herbivory negatively impacts cone and seed production, seed quality and seedling growth of susceptible pinyon pines. Oecologia 143(4):558–565

Novak WK, Haslberger AG (2000) Substantial equivalence of antinutrients and inherent plant toxins in genetically modified novel foods. Food Chem Toxicol 38(6):473–483

Orians CM, Huang CH, Wild A, Dorfman KA, Zee P, Dao MTT, Fritz RS (1997) Willow hybridization differentially affects preference and performance of herbivorous beetles. Entomologia Experimentalis Et Applicata 83(3):285–294

Osier TL, Hwang SY, Lindroth RL (2000) Within- and between-year variation in early season phytochemistry of quaking aspen (*Populus tremuloides* Michx.) clones. Biochem Syst Ecol 28(3): 197–208

Price PW (1991) The plant vigor hypothesis and herbivore attack. Oikos 62(2):244–251

Raffa KF (1989) Genetic-engineering of trees to enhance resistance to insects—evaluating the risk of biotype evolution and secondary pest outbreak. Bioscience 39(8):524–534

Ramirez CC, Zamudio F, Verdugo JV, Nunez ME (2004) Differential susceptibility of poplar hybrids to the aphid *Chaitophorus leticomelas* (*Homoptera: Aphididae*). J Econ Entomol 97(6): 1965–1971

Rank NE, Köpf A, Julkunen-Tiitto R, Tahvanainen J (1998) Host preference and larval performance of the salicylate-using leaf beetle *Phratora vitellinae*. Ecology 79(2):618–631

Rehill BJ, Whitham TG, Martinsen GD, Schweitzer JA, Bailey JK, Lindroth RL (2006) Developmental trajectories in cottonwood phytochemistry. J Chem Ecol 32(10):2269–2285

Ricroch A, Bergé JB, Kuntz M (2010) Is the German suspension of MON810 maize cultivation scientifically justified? Transgenic Res 19(1):1–12

Rose R, Dively GP (2007) Effects of insecticide-treated and lepidopteran-active Bt transgenic sweet corn on the abundance and diversity of arthropods. Environ Entomol 36(5):1254–1268

Rosi-Marshall EJ, Tank JL, Royer TV, Whiles MR, Evans-White M, Chambers C, Griffiths NA, Pokelsek J, Stephen ML (2007) Toxins in transgenic crop byproducts may affect headwater stream ecosystems. Proc Nat Acad Sci USA 104(41): 16204–16208

Saxena D, Stotzky G (2001) BT corn has a higher lignin content than non-BT corn. Am J Bot 88(9):1704–1706

Schweitzer JA, Bailey JK, Hart SC, Wimp GM, Chapman SK, Whitham TG (2005) The interaction of plant genotype and herbivory decelerate leaf litter decomposition and alter nutrient dynamics. Oikos 110(1):133–145

Sisterson MS, Biggs RW, Manhardt NM, Carrière Y, Dennehy TJ, Tabashnik BE (2007) Effects of transgenic Bt cotton on insecticide use and abundance of two generalist predators. Entomologia Experimentalis et Applicata 124(3):305–311

Tiimonen H, Aronen T, Laakso T, Saranpaa P, Chiang V, Ylioja T, Roininen H, Haggman H (2005) Does lignin modification affect feeding preference or growth performance of insect herbivores in transgenic silver birch (*Betula pendula* Roth)? Planta 222(4): 699–708

van Frankenhuyzen KU, Beardmore T (2004) Current status and environmental impact of transgenic forest trees. Can J For Res 34(6):1163–1180

Waltz AM, Whitham TG (1997) Plant development affects arthropod communities: opposing impacts of species removal. Ecology 78(7):2133–2144

Waterman PG, Mole S (1994) Analysis of phenolic plant metabolites. Blackwell Scientific Publications, Oxford

Wenes AL, Bourguet D, Andow DA, Courtin C, Carre G, Lorme P, Sanchez L, Augustin S (2006) Frequency and fitness cost of resistance to *Bacillus thuringiensis* in *Chrysomela tremulae* (*Coleoptera: Chrysomelidae*). Heredity 97(2):127–134

Whitham TG (2006) A framework for community and ecosystem genetics: From genes to ecosystems. Nature reviews. Genetics 7(7):510–523

Zurbrugg C, Nentwig W (2009) Ingestion and excretion of two transgenic Bt corn varieties by slugs. Transgenic Res 18(2): 215–225