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Research article

Varied response of *Spodoptera littoralis* against *Arabidopsis thaliana* with metabolically engineered glucosinolate profiles

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ABSTRACT

Upon herbivory glucosinolates are known to be degraded into a cascade of secondary products that can be detrimental for certain herbivores. We performed herbivory bioassays using first and second instar generalist Lepidoptera larvae *Spodoptera littoralis* on *Arabidopsis thaliana* engineered to overexpress novel glucosinolates. A differential response in larval feeding patterns was observed on the plants engineered with novel glucosinolates. Larvae fed on plants overexpressing 4-hydroxybenzyl glucosinolate and isopropyl glucosinolate showed little response. Larvae fed on 35S:CYP79A2 plants engineered to overexpress benzyl glucosinolates, however, showed reduced larval and pupal weights. Upon herbivory a high expression of JA signalling gene LOX2 was observed on the 35S:CYP79A2 plants compared to the PR1a and VSP2 expression. To confirm the role of benzyl isothiocyanate (BITC), a degradation product of benzyl glucosinolate overexpressing plants, in the retarded larval growth we used Virus Induced Gene Silencing (VIGS) approach to silence LOX2 expression in the 35S:CYP79A2 plants. *S. littoralis* larvae fed on LOX2 silenced 35S:CYP79A2 plants exhibited a retarded larval growth thus indicating that BITC played a pivotal role in anti-herbivory and not only the JA signalling pathway.

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1. Introduction

Plants have developed various strategies to defend themselves against herbivorous enemies. One of the deterrent mechanisms studied widely in conjunction with plant-insect interactions is the glucosinolate–myrosinase system [1]. This system is known to be triggered by herbivores followed by the release of an array of downstream breakdown products, some of which are toxic for some herbivores [2]. There are a number of reports supporting the concept of increased plant defense due to higher accumulation of glucosinolates [3,4]. Certain herbivores are specialised in overcoming the toxic effects caused during the glucosinate–myrosinase interaction, which is primarily thought to be caused by wounding. These specialised insects may respond to glucosinolates other than those normally found in their host plants [5,6].

Cytochrome P450 monoxygenases (CYPs) play a crucial role during the biosynthesis of glucosinolates and cyanogenic glucosides. Three CYP79 genes have been overexpressed in *Arabidopsis thaliana*: CYP79A1 from *Sorghum bicolor* that converts tyrosine to 4-hydroxyphenylacetaldoxime [7], CYP79D2 from cassava (*Manihot esculenta*) that converts valine and isoleucine to aldoximes [8] and CYP79A2 from *A. thaliana* itself that catalyses the conversion of phenylalanine to phenylacetaldoxime [9]. The above transgenic *A. thaliana* lines overexpressing these CYP79s in the Col-0 background contain glucosinolates that are not present in high amounts in Col-0 wildtype plants [9–11].

In an earlier study [12] we investigated whether or not the presence of novel glucosinolates in *A. thaliana* had any effect on the oviposition preference and larval performance of the specialist *Plutella xylostella*. We observed that the specialist herbivore *P. xylostella* was not consistently affected by any of the lines. In the present study we attempt to understand the response of the generalist herbivore *Spodoptera littoralis* on the same transgenic lines engineered to contain 4-hydroxybenzyl glucosinolate, benzyl glucosinolate or 1-methyl propyl glucosinolate and isopropyl glucosinolate. On lines containing benzyl glucosinolate *S. littoralis* larvae weighed less than larvae fed on the wild type and other lines. We therefore also studied the insect's response to the degradation product of benzyl glucosinolate (benzyl isothiocyanate – BITC). In addition the defense signal activation from larval feeding was studied by monitoring marker genes VSP2, LOX2 and PR1a. As larval feeding caused a high level of LOX2 activation in the benzyl GS *A. thaliana* lines an experiment was done to ascertain if LOX2 expression was responsible for reduced larval weights.

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2. Materials and methods

2.1. Plants

A. thaliana of the following genotypes were used: wild-type Columbia-0 (Col-0), 35S:CYP79A1 (lines 1.8 and 6.3.1) producing 4-hydroxybenzylglucosinolate [10], 35S:CYP79A2 (lines 30.6 and 10.1) producing benzylglucosinolate [9], 35S:CYP79D2 (lines 28 and 5) producing isopropyl and 1-methylpropyl glucosinolate [11]. Seeds were surface sterilised for 20 min in 2% sodium hypochlorite followed by a brief rinse with 50% ethanol before planting in pots containing sterile soil. The plants were grown in controlled environment of a growth chamber using a 16/8 h photoperiod at 22/18 °C. In a previous study the mean values and standard errors for total and engineered glucosinolates as nmol/mg dry weight were as follows (see [12] for complete profiles): Col-0 (total glucosinolates, 11.57 (Standard Error (SE) = 3.15)), 35S:CYP79A1.18 (total glucosinolates, 5.79 (SE = 1.05); 4-hydroxybenzyl glucosinolate, 1.28 (SE = 0.28)), 35S:CYP79A1.6.3.1 (total glucosinolates, 2.92 (SE = 0.90); 4-hydroxybenzyl glucosinolate, 0.89 (SE = 0.38)), 35S:CYP79A2.30.6 (total glucosinolates, 12.93 (SE = 1.51); benzylglucosinolate, 4.85 (SE = 0.50)), 35S:CYP79A2.10.1 (total glucosinolates, 7.97 (SE = 1.31); benzylglucosinolate, 2.16 (SE = 0.40)), 35S:CYP79D2.28 (total glucosinolates, 8.03 (SE = 1.07), 1- Methylpropylglucosinolate, 0.65 (SE = 0.17) and isopropyl glucosinolate 0.24 (SE = 0.24)), CYP79D2.5 (total glucosinolates, 5.75 (SE = 1.03). Wild type Col-0 and CYP79D2.5 did not show accumulation of the 4-hydroxy benzyl, benzyl, 1-methylpropyl or isopropyl glucosinolates.

2.2. Insects

S. littoralis eggs were obtained from the Department of Plant Biology, Faculty of Landscape planning, Horticulture and Agricultural Sciences, SLU, Alnarp and incubated at 22 °C until they hatched.

2.3. Herbivore bioassay

2.3.1. A. thaliana lines, larval and pupal weights

For the first instar larvae, newly hatched larvae were maintained on an artificial diet for 24 h and on the second day groups of 4 larvae were placed on three-week-old wild type and transgenic lines and allowed to feed for 10 days. For the second instar larvae, newly hatched larvae were maintained on an artificial diet for 5 days and then four larvae were placed on an experimental plant and allowed to feed for 10 days. During the experimental period those plants completely consumed by the larvae were replaced with new plants of the respective lines. After 10 days the larvae were removed from the plants and weighed together. Two experiments were done. In the first there were 6 replicates per plant line and in the second there were 10 replicates per plant line. Average larval weight per replicate was used in the statistical analysis. To obtain pupal weights second instar larvae were placed on each of the seven genotypes until pupation; 5 larvae were fed on each plant genotype. The 5 pupae from each genotype were weighed together and an average weight per pupae was calculated. The pupal experiment was also repeated twice, with 6 replicates the first time and 10 the second. All experiments used independently grown sets of plants. An Analysis of Variance (ANOVA) was performed in order to see if the larval or pupal weights differed depending on what plant line they fed upon. Weights were not transformed as they were normally distributed. A preliminary ANOVA considering the two experiments together showed significant interactions between plant lines and experiments (1st instar: $F = 10.02$; df = 6,98; $P < 0.0001$; 2nd instar: $F = 3.36$; df = 6,98; $P = 0.005$; pupae: $F = 3.28$; df = 6,98; $P = 0.006$). Therefore the two experiments were analysed separately. A Student Newman Keuls test was used to separate means. SAS version 9.1 for Windows (SAS Institute, USA) was used for statistics.

2.3.2. A. thaliana lines, leaf area consumed

In a no-choice experiment second instar larvae were gently placed on a plant with a brush and allowed to feed for 12 h. The damaged leaves were photographed using the NIKON SMZ1500 microscope and the percentage leaf area consumed by the larvae was calculated by using the NIS-Elements D 3.00 Imaging software (Nikon). The leaf size was similar for all lines tested. The experiment was replicated in 10 sets. Each replicate contained all 6 transgenic lines and the wild type Col-0. The bioassay plants were maintained in a controlled growth chamber using a 16/8 h photoperiod at 22/18 °C. An Analysis of Variance (ANOVA) was performed in order to see if the percentage of leaf area eaten differed depending on what plant line was fed upon. Leaf area was normally distributed and no transformation was used. A Student Newman Keuls test was used to separate means. SAS version 9.1 for Windows (SAS Institute, USA) was used for statistics.

2.3.3. Benzyl isothiocyanate, larval weights

A bioassay was performed to study if the reduced larval weight after feeding on CYP79A2 lines was due to accumulation of BITC, the major glucosinolate degradation product in these lines. We assessed the role of BITC by performing in vitro herbivore bioassays to simulate the effect of accumulating glucosinolate degradation product in the wild type Col-0. The rosettes of three-week-old Col-0 plants grown in soil were placed in petri dishes (8.5 cm × 1.5 cm) and sprayed with 10 μM, 25 μM, 50 μM and 100 μM benzyl isothiocyanate (BITC, Sigma–Aldrich, USA) solutions. Different BITC stock solutions were diluted in 2% dimethyl sulfoxide (DMSO). A separate set of plants were sprayed with 2% DMSO and water as controls. The petridishes were sealed with cellophane tape to prevent evaporation. Five larvae (first instar) were placed on each plant and allowed to feed for 10 days. To maintain BITC levels during the duration of the feeding, every 48 h the larvae were taken out and fresh plants were sprayed with BITC and the larvae were placed back on the plants. During the experimental period those plants completely consumed by the larvae were replaced with new plants. At the end of the experiment all larvae were weighed individually. This experiment was replicated five times. The weight of larvae raised on the different treatments of BITC, DMSO, and H_2O were subjected to an Analysis of Variance (ANOVA). The actual weights were normally distributed and no transformation of data was used. Two factors were considered: the treatments and the different replicates. The effect of these two factors as well as their interaction was studied. SAS version 9.1 for Windows (SAS Institute, USA) was used for statistics.

2.4. Real-time PCR analysis

To investigate the defense signal activation during larval feeding in the glucosinolate overexpressing lines, we analysed the expression of the JA responsive wound inducible marker gene VSP2, LOX2 and SA responsive gene PR1a. Second instar *S. littoralis* larvae were placed on the first leaf of each of the 35S:CYP79 lines and Col-0 wild type and allowed to feed. After 12 h the larvae were removed and the damaged (local) leaves and the undamaged (systemic) leaves were snap frozen in liquid nitrogen and used for quantitative RT-PCR. Plants without any larvae feeding were used as a control. As a template for quantitative RT-PCR (qRT-PCR), cDNA synthesised from total RNA was used. Total RNA was extracted using Plant RNeasy Mini Kit (QIAGEN, Germany) and followed by DNase I (Ambion, UK)

Table 1
Mean weights (mg) and standard errors (in parentheses) of *Spodoptera littoralis* after feeding on different lines of *A. thaliana*. In experiment I, 24 larvae per line were used. In experiment II, 40 larvae per line were used. Means followed by different letters in the same column are statistically different from each other (Student Newman Keuls test).

Arabidopsis lines	Larvae fed from 1st instar for 10 days		Larvae fed from 2nd instar for 10 days		Pupae fed from 2nd instar until pupation	
Experiment	I	II	I	II	I	II
Col-0	50.2 (3.40) ab	59.5 (0.99) b	72.0 (2.36) a	81.1 (1.14) a	86.3 (3.63) a	92.4 (2.41) ab
CYP79A.1.18	49.6 (3.82) ab	33.0 (0.56) d	63.8 (2.45) ab	71.2 (1.05) b	85.3 (2.81) a	95.5 (0.67) a
CYP79A1.6.3.1.1	42.4 (3.25) bc	38.7 (0.82) c	65.1 (1.92) ab	65.00 (1.02) c	84.0 (1.59) a	90.0 (1.16) bc
CYP79A2.30.6	34.0 (1.87) c	31.4 (0.43) d	58.7 (1.78) bc	61.9 (1.36) d	65.5 (1.88) b	69.7 (0.86) d
CYPA2.10.1	35.1 (3.40) c	34.5 (1.45) d	52.2 (2.48) c	60.3 (0.89) d	64.2 (1.49) b	73.2 (1.39) d
CYP79D2.28	54.8 (4.27) ab	68.7 (1.48) a	67.3 (3.98) ab	80.7 (0.54) a	83.3 (3.63) a	89.5 (1.15) bc
CYP79D2.5	61.0 (3.26) a	68.2 (2.98) a	74.0 (2.34) a	79.3 (1.16) a	91.3 (2.03) a	86.6 (1.09) c

treatment to remove any genomic DNA contamination. The first strand cDNA was synthesised from 500 ng of each DNase-treated total RNA in 25 μl reaction volume using SuperScript III (Invitrogen, USA) and then diluted up to 50 μl with 3 mM TE buffer, pH 7.5. Primer Express 2.0 software (PE Applied Biosystems, USA) was used to design the gene specific forward and reverse primers to amplify PR1a (At2g14610; forward primer 5′-tgatcctcgtgggaattatgt-3′, reverse primer 5′-tgcatgatcacatcattacttcat-3′), LOX2 (At3g45140; forward primer 5′-cttacccgcggatctcatc-3′, reverse primer 5′-actccatgttctgcggtctt-3′), VSP2 (At5g24770; forward primer 5′-gttagg gaccggagcatcaa-3′ and reverse primer 5′-aacggtcactgagtatgatgggt-3′), tubulin transcript (At5g62700; forward primer 5′-cgatgttgttcg taaggaagc-3′ and reverse primer 5′-tcctcccaatgagtgacaaa-3′) and UBQ5 (At3g62270; forward primer 5′-cgatggatctggaaaggttc-3′ and reverse primer 5′-agctccacaggttgcgttag-3′) by qRT-PCR. ABI Prism 7000 Sequence Detection System and Software (PE Applied Biosystem, USA) was used. qRT-PCR reactions (20 μl) included SYBR Green PCR master mix (Applied Biosystem, USA) supplemented with 5 μM primers and 1 μl cDNA as a template. As a negative control, reaction mixtures without cDNA were used. PCR reactions were performed using the following parameters: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C in 96-well optical reaction plates (Applied Biosystem). The identities of the amplicons and the specificity of the reactions were verified by melting curve analysis. The data were analysed by comparative C_T method [13] with PCR efficiency correction. PCR efficiency was determined based on the slope of standard curves. The gene expression level obtained by qRT-PCR was normalised using the tubulin and UBQ5 genes and subsequently the fold differences in the transcript levels and mean standard error were calculated using the Q-GENE software [14]. Three different RNA isolations and cDNA synthesis products were used for quantification and each cDNA was measured in triplicate.

2.5. Virus induced gene silencing

To confirm if LOX2 expression was responsible for the reduced larval weight in the 35S:CYP79A2 lines, we used a loss of function approach with the tobacco rattle virus (TRV) mediated VIGS optimised for *A. thaliana*. Two Agrobacterium expression vectors (pTRV1 and pTRV2) were used. For controls, plants were co-infiltrated with pTRV1 and pTRV2 carrying a partial sequence of PDS or an empty pTRV2 vector for any effect caused due to VIGS. pTRV1 (pYL192) and pTRV2 (pYL156) vectors described in [15] were used in this study. pTRV2-AtPDS encoding Phytoene desaturase was used for the VIGS control experiment to visualise bleaching in the infiltrated plants. The silencing of PDS produces a typical white color that is the result of photo- bleaching. To generate pTRV2-lox2, the *A. thaliana* LOX2 cDNA fragment was PCR amplified from *A. thaliana* cDNA using primers 5′-TGCTCGCCAGACACTTGCCG -3′ and 5′-TGGCGTGCACGAGCGTTGAT -3′. The resulting PCR product was cloned into EcoRI cut pTRV2. The constructs were then electroporated into competent cells of *Agrobacterium tumefaciens* strain GV3101 by a Gene Pulser (Bio-Rad, USA).

2.5.1. Plant growth and agroinfiltration

Wild-type *A. thaliana* ecotype Col-0 and 35S:CYP79A2 lines were grown in pots at 23 °C in a growth chamber under a 16/8 h

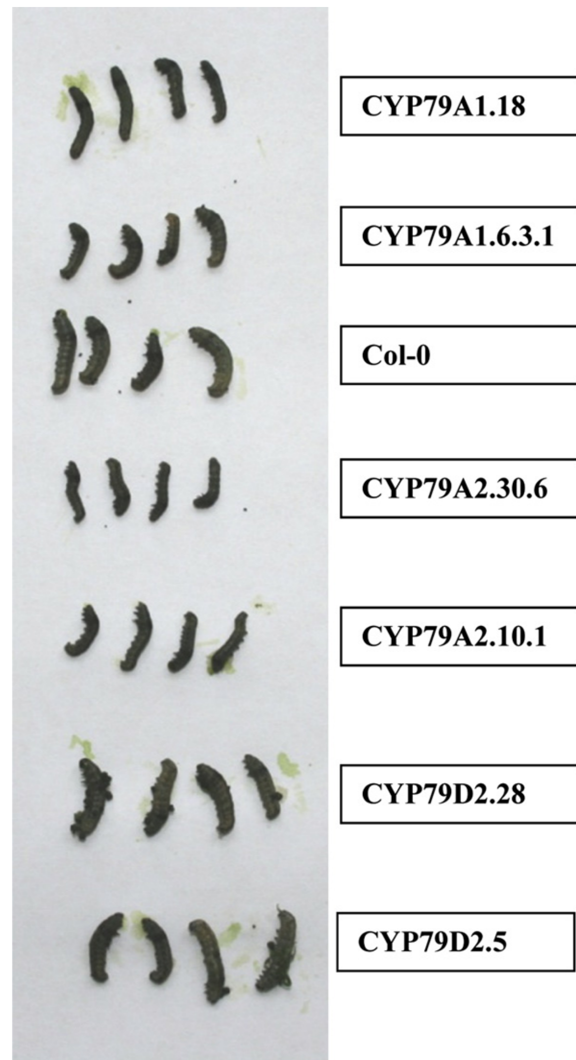

Fig. 1. *Spodoptera littoralis* larval size 10 days after feeding on wild type (Col-0) and transgenic plants. The larvae are representative of 44 larvae used in each experiment. Similar results were obtained in all replicates.

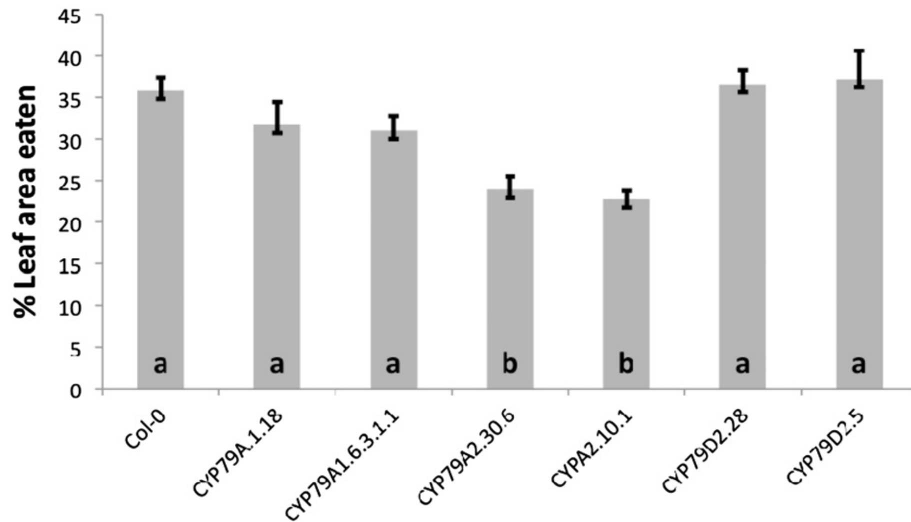

Fig. 2. Percentage of leaves consumed after 12 h of feeding by *Spodoptera littoralis* on different lines of Arabidopsis. Ten larvae per line were used. Bars are the means of replicates and standard error bars are shown. Bars with different letters at the bottom of the bars are significantly different (SNK test).

photoperiod with 60% humidity. Two to three leaf seedlings were used for VIGS. For the VIGS assay, pTRV1 and pTRV2 were introduced into *A. tumefaciens* strain GV3101. The Agrobacterium cultures were grown at 28 °C in LB medium containing 50 mg/L gentamycin and 50 mg/L kanamycin antibiotics, 10 mM MES and 20 mM acetosyringone. The following day *A. tumefaciens* cells were harvested and resuspended in infiltration media (10 mM MgCl2, 10 mM MES, and 200 mM acetosyringone), adjusted to an OD_{600} of 1.5, and left at room temperature for 4 h. Agroinfiltration was performed with a needleless 1-mL syringe into two leaves of two to three leaf stage plants, infiltrating the entire leaf.

Newly hatched larvae were maintained on an artificial diet for 24 h and on the second day groups of four 1st instar larvae were placed on three-week-old Col-0 and 35S:CYP79A2 lines that were either antisenselox2 or VIGS vector control plants and allowed to feed for 10 days. During the experimental period those plants completely consumed by the larvae were replaced with new plants. After 10 days the larvae were removed from the plants and the larval weight was recorded. The experiment was repeated 5 times, average larval weight per replicate was used in the statistical analysis. An Analysis of Variance (ANOVA) was performed in order to see if the weights differed depending on what plant line and treatment they fed upon. Weights were normally distributed and not transformed for the analysis. Student Newman Keuls test was used to separate means. SAS version 9.1 for Windows (SAS Institute, USA) was used for statistics.

3. Results

3.1. Herbivore bioassay with intact plants

In general weights were higher in the second experiment, but the standard error was lower. There was a significant difference between larval weight when starting feeding in the 1st or 2nd instar depending on what plant lines larvae fed upon (experiment I: 1st instar: $F = 8.78$; df $= 6{,}35$; $P < 0.0001$; 2nd instar: $F = 8.59$; df $= 6{,}35$; $P < 0.0001$ and experiment II: 1st instar: $F = 132.28$; df $= 6{,}63$; $P < 0.0001$; 2nd instar: $F = 75.08$; df $= 6{,}63$; $P < 0.0001$). In the analysis of the larval weight of the larvae that started feeding as 1st instar, those that fed on the 35S:CYP79D2 lines were heavier than those on all other lines in both experiments (Table 1). Larvae fed on 35S:CYP79A2.30.6, 35S:CYP79A2.10.1, and 35S:CYP79A1.6.3.1 weighed significantly less than those fed on the wild type Col-0 in the first experiment, the same was true in the second experiment

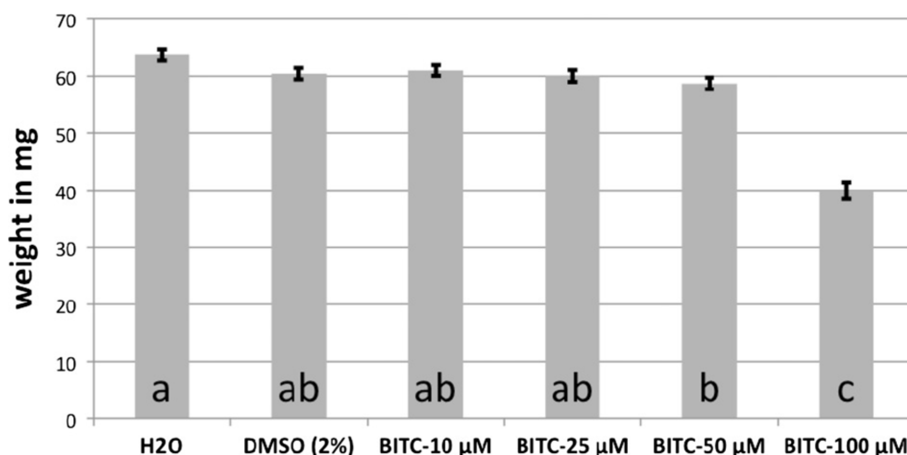

Fig. 3. Final weights of larvae raised for 10 days on leaves treated with different amounts of benzyl isothiocyanate (BITC). 50 larvae per treatment were used. Bars are the mean weight of all replicates and standard error bars are shown. Bars with different letters at the bottom of the bars are significantly different (SNK test).

where 35S:CYP79A1.1.8 larvae also weighed significantly less than those fed on the wild type Col-0 (Table 1, Fig. 1). For larvae that began the experiment as 2nd instars, the larvae on the two 35S:CYP79A2 lines were significantly lighter than all larvae on other plant lines in both experiments (Table 1). In the second experiment larvae feeding on the 35S:CYP79D2 lines and the wild type weighed significantly more than those on all the other lines (Table 1). Pupal weights differed significantly depending on what lines they fed upon (experiment I: $F = 17.28$; df = 6,35; $P < 0.0001$ and experiment II: $F = 53.49$; df = 6,63; $P < 0.0001$). The smallest pupae came from the two 35S:CYP79A2 lines and these differed significantly from all other lines in both experiments (Table 1).

Leaf area consumed by the second instar larvae after feeding for 12 h was similar in the wild type and the D2 and A1 transgenic lines, but both A2 lines showed significantly less feeding damage than the other lines ($F = 7.89$; df = 6,63; $P < 0.0001$). The leaf consumption observed in the 35S:CYP79A2 lines was only about two-thirds of that observed on the wild type (Fig. 2).

3.2. Effect of BITC treatment on herbivory

Different treatments had different effects on larval weights ($F = 65.44$; df = 6,120; $p < 0.0001$). Weights were different between replicates ($F = 3.85$; df = 6,120; $p = 0.006$), but there was not a significant interaction between treatments and replicates ($F = 0.73$; df = 20,120; $p = 0.79$). We therefore performed a Student-Newman Kuels test (SNK) to ascertain the differences among treatments. Larvae treated with 100 μM of BITC weighed significantly less than larvae that ate plants just treated with water and 2% DMSO (Fig. 3).

3.3. Marker gene expression analysis

At the constitutive level the expression of LOX2, VSP2 and PR1a did not seem to vary significantly among the transgenic lines compared with the wild type (Fig. 4a–c). Upon larval feeding the local leaves had a 2–3-fold increase of the LOX2 transcripts in both 35S:CYP79A2 lines compared to the 35S:CYP79A1, 35S:CYP79D2 and the wild type plants. A consistent systemic gene expression of the LOX2 was found among all the overexpressing lines and the wild type (Fig. 4a). A similar pattern was observed with the JA responsive marker gene VSP2 with the highest transcript accumulation in both the 35S:CYP79A2 lines upon herbivory (Fig. 4b). The SA responsive marker PR1a showed a different pattern of expression among the transgenic lines with the highest observed in the 35S:CYP79A1 and 35S:CYP79D2 lines (Fig. 4c). Compared to the LOX2 and VSP2, a two-fold lower basal expression of PR1a was observed in the wild type and the transgenic plants. A 4–5 fold increased expression was observed in the 35S:CYP79A1 lines compared to the wild type Col-0 in the local leaves, whereas the systemic expression was found to be similar in both the plants.

3.4. VIGS bioassay

S. littoralis larvae fed on pTRV-lox2 plants showed significantly increased weight when compared to the larvae fed on pTRV-empty vector plants (Col-0: $F = 46.08$; df = 1,8; $P < 0.0001$; 35S:CYP 79A2.10.1: $F = 14.95$; df = 1,8; $P < 0.0001$; 35S:CYP79A2.30.6: $F = 23.55$; df = 1,8; $P < 0.0001$; Fig. 5). Larvae fed on Col-0 plants were always heavier than those fed on 35S:CYP79A2 lines (pTRV-lox2: $F = 61.91$; df = 2,12; $P < 0.0001$; pTRV- empty vector plants: $F = 26.00$; df = 2,12; $P < 0.0001$). VSP2 gene expression upon herbivory was lower in the lox2 silenced 35S:CYP79A2 plants compared non-silenced plants indicating that JA signalling was compromised in the pTRV-lox2 plants (Supplement Fig. 1). This indicated that reduced larval weight is partially independent of LOX2 expression in the benzylglucosinolate overexpressing 35S:CYP79A2 lines and that benzylglucosinolate accumulation in these plants is directly linked to reduced larval feeding.

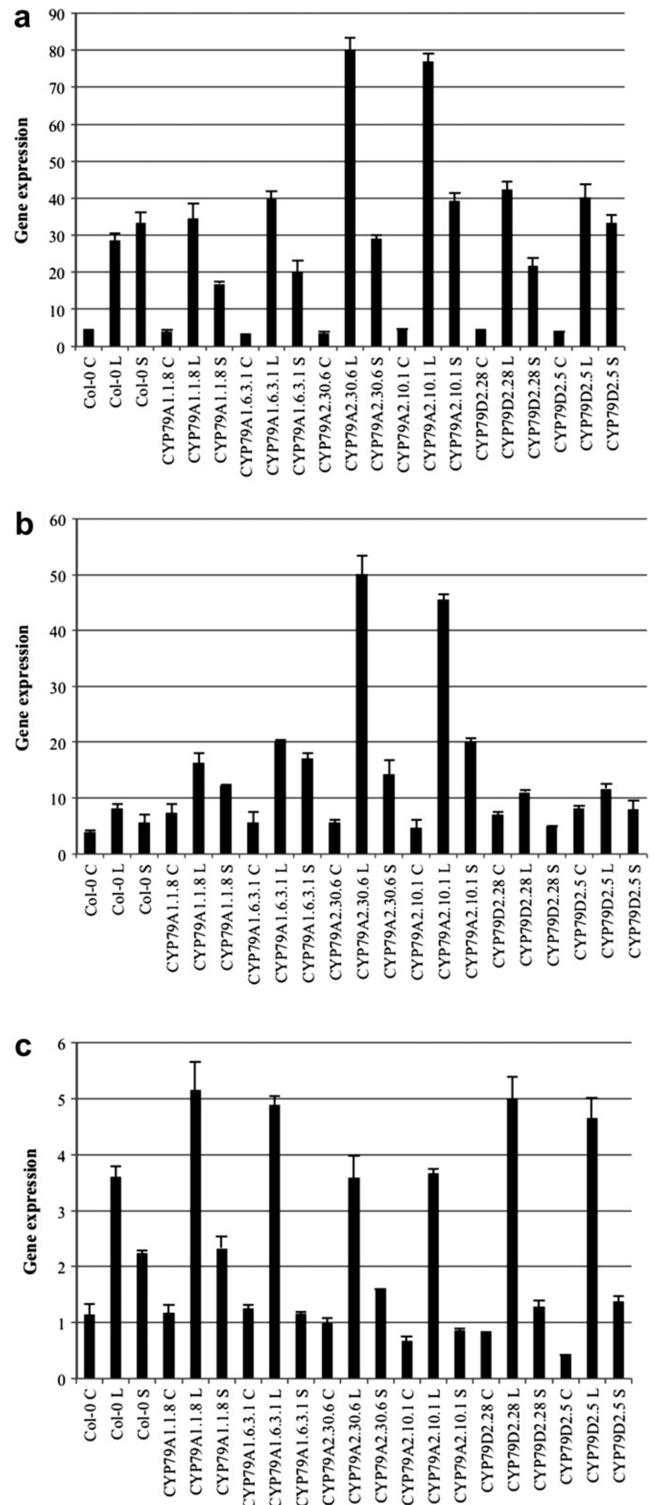

Fig. 4. Quantitative RT-PCR for (a) LOX2, (b) VSP2 and (c) PR1a gene expression after feeding by *Spodoptera littoralis* larvae. Plants without insect feeding were used as controls (C), plants fed on by second instar larvae are designated by (L) and the undamaged systemic leaf from the plant on which the larvae had been feeding are designated by (S). The data were normalised to Tubulin expression. Values correspond to the average of three replicates ± standard error.

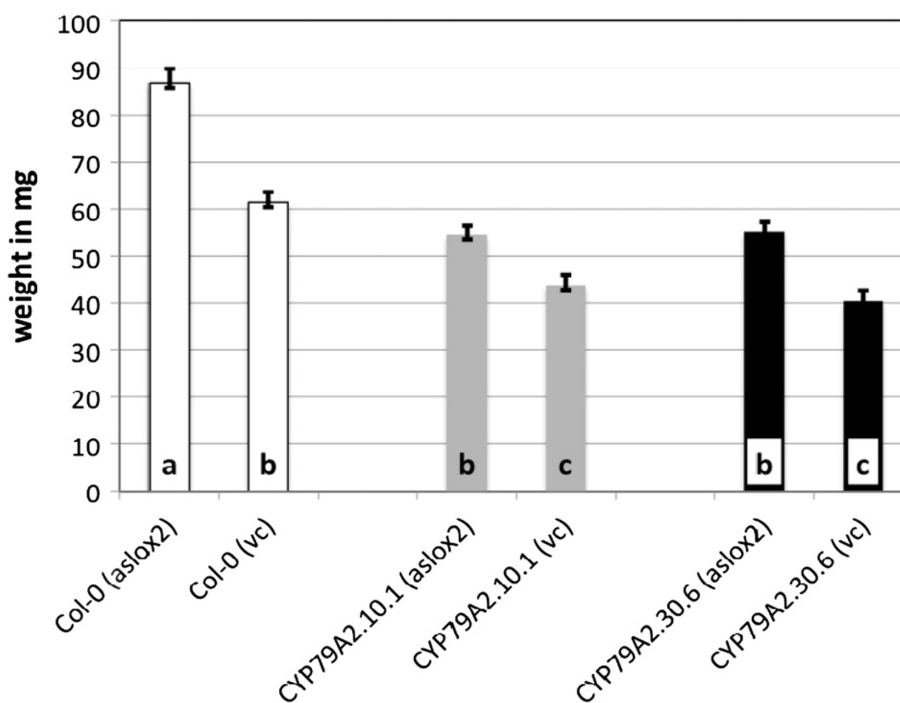

Fig. 5. Larval mass of *Spodoptera littoralis* after feeding on VIGS silenced Col-0 (aslox2), vector control Col-0 (vc), VIGS silenced CYP79A2.10.1 (aslox2), vector control CYP79A2.10.1 (vc), VIGS silenced CYP79A2.30.6 (aslox2), vector control CYP79A2.30.6 (vc). 20 larvae were used per treatment. Bars are the mean weight of all replicates and standard error bars are shown. The different plant types are grouped by color. Bars of the same color with different letters at the bottom of the bars are significantly different (SNK test).

4. Discussion

S. littoralis larvae feeding on 35S:CYP79A2 lines generally weighed less than those on other lines, the same was true for the weight of pupae that developed on 35S:CYP79A2 lines. In addition 35S:CYP79A2 lines received less feeding damage than other plant lines. JA and wound inducible marker LOX2 was found to be induced to a higher level upon larval feeding in the 35S:CYP79A2 lines compared to the other glucosinolate overexpressing lines. Because the two 35S:CYP79A2 lines were the most detrimental to larval growth and most resistant to larval feeding, benzylglucosinolate appears to have a negative effect on these generalist larvae. The conclusion is supported by the negative effect on larval weights when spraying benzyl isothiocyanate on *A. thaliana* leaves.

In this study, overexpression of glucosinolates in Arabidopsis appears to influence *S. littoralis* larval feeding behaviour. In an earlier study, the specialist herbivore *Plutella xylostella* larvae in similar no-choice bioassays did not show a significant effect of feeding on any of the transgenic lines or the wild type [12]. *P. xylostella* was not affected by feeding on benzylglucosinolate overexpressing 35S:CYP79A2 lines [12], but this does not mean that it is tolerant to BITC. *P. xylostella* possesses sulfatase activity that modifies glucosinolates and prevents the formation of toxic hydrolysis products such as BITC [16]. In another study [17], *P. xylostella* was not affected when feeding on glucosinolate-overexpressing *A. thaliana* plants but *S. littoralis* did show a negative effect upon feeding. Specialist herbivores are believed to have evolved to overcome the defense/toxic substances of their natural hosts [25]. *S. littoralis* being a generalist does not contain the necessary machinery to digest the toxic products generated by the glucosinolate-myrosinase complex. Similarly, transgenic *A. thaliana* with higher total glucosinolate levels did not have any effect on specialists *Phyllotreta nemorum* and *P. cruciferae* [5]. A negative effect was found on the generalist herbivore *Trichoplusia ni* on *A. thaliana* with varying glucosinolate composition [18]. An increase in *Spodoptera exigua* larval weight gain was observed when fed on *cyp79B2 cyp79B3 myb28 myb29* quadruple mutants deficient in indole and aliphatic glucosinolates compared to the wild type plants [14]. In contrast, specialist herbivore *P. xylostella* did not show any significant preference in feeding and pupation on the plants devoid of indole and aliphatic glucosinolates.

In our study we also found a selective feeding pattern among the glucosinolate overexpressing *A. thaliana* plants in comparison with the wild type. We also observed that sub-lethal concentrations of BITC on the wild type Col-0 plants had a significant affect on the feeding that subsequently lead to reduced larval weight. This demonstrated that 35:CYP79A2 plants produced sufficient amounts of benzyl glucosinolates to repress larval feeding. It is indeed tempting to speculate that the overexpression of the 35:CYP79A2 in *A. thaliana* is responsible for herbivore resistance. In our previous study [12], the expression of benzylglucosinolate was the highest in dry weight and percentage of the total glucosinolate among the novel glucosinolates so we cannot say if the other novel glucosinolates would have had an effect or not if their concentrations had been higher.

Marker gene analysis corresponding to different plant defense pathways indicated a possible cross-talk between SA and JA pathways upon *S. littoralis* feeding on the glucosinoate overexpressing *A. thaliana* lines. Compared to the expression of VSP2 and PR1a, LOX2 had a pronounced fold increase in expression upon herbivory in the 35S:CYP79A2 plants. The lower larval weights on the 35S:CYP79A2 lines provides evidence concerning the prominent role of isothiocyanates as a deterrent to insect feeding. Previous results have shown herbivores prefer plants with simple nitriles over those with isothiocyanates [19,20]. JA signalling and wound inducible marker LOX2 gene expression was elevated upon larval feeding in the CYP79A2 lines compared to the other glucosinolate overexpressing lines. Several genes including LOX2 involved in the JA biosynthesis have been reported to be induced upon feeding by *Pieris rapae* [21]. This could be due to the tissue damage caused by

the larvae which in turn released some aromatic products triggering wound inducible gene and subsequent JA mediated defense responses. *Manduca sexta* larvae when fed with LOX3 silenced *Nicotiana attenuata* plants showed an increase in body mass compared to the wild type [22]. In another study [23] it was shown that *A. thaliana coi1* mutant impaired in JA signalling were more susceptible to *Spodoptera exigua* compared to the wild type plants, in contrast *npr1* mutants impaired in SA signalling were more resistant compared to the wild type plants. In our study we found that VIGS mediated pTRV2-lox2 35S:CYP79A2 plants were found to be more appetising than the VIGS empty vector inoculated 35S:CYP79A2 plants. Intriguingly the larvae that fed on pTRV2-lox2 Col-0 plants gained more body mass than the pTRV2-lox2 35S:CYP79A2 lines. This demonstrated that LOX2 mediated JA defense response is not solely responsible for reduced larval weight in the 35S:CYP79A2 lines. It appears that the benzylglucosinolate hydrolysis products also have an effect on larval growth. Recently the TRV based VIGS approach has been used to demonstrate that PDS silenced *A. thaliana* and Brassica plants that show a bleached phenotype were less preferred for oviposition by *Pieris rapae* [24]. They also reported that the neonate *P. rapae* larval growth was significantly lower on the PDS silenced plants than on the wild type.

This study demonstrates that plants engineered to express higher levels of benzyl glucosinolate can inhibit herbivory by a generalist Spodoptera. Unlike specialists, which are better equipped to digest the glucosinolates, generalists find it hard to face disparate defensive mechanisms of plants. We show that reduced herbivory by *S. littoralis* on 35S:CYP79A2 lines could be due to the direct toxicity of the breakdown products of benzylglucosinolates and not only the JA signalling pathway. Further studies using signalling mutants and metabolic profiling of the over expressing *A. thaliana* lines would provide supporting evidence for the role of the specific glucosinolates against herbivory.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.plaphy.2011.07.014.

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