Journal of Applied Botany and Food Quality 82, 131 - 135 (2009)

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Variability of aliphatic glucosinolates in *Arabidopsis thaliana* (L.) – Impact on glucosinolate profile and insect resistance

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(Received November 11, 2008)

Summary

The glucosinolate(GS)-myrosinase system of Brassicaceae, including the model plant *Arabidopsis thaliana* (L.), comprises a defence which is effective especially against generalist herbivores. Based on their side chain structure GS are grouped into aliphatic, aromatic, and indolyl GS. Indolyl GS are widely distributed among *A. thaliana* ecotypes and the Brassicaceae family, but the presence of aliphatic GS is variable and under strong genetic control. We investigated the effect of *AOP g*ene expression on the side chain modifications of GS and the impact on insect resistance. *AOP2* and *AOP3* genes from Mr-0 and Sap-0 ecotypes, respectively, were crossbred into the methylsulfinyl GS producing Gie-0. Successful crosses were heterozygote plants which produced allyl (*AOP2*) or 3-hydroxypropyl GS (*AOP3*). After self-pollination, the chemical profile of the F_3 generation of plants was screened to identify homozygote lines. Homozygote lines producing 3-hydroxypropyl GS were compared to methylsulfinyl GS, which were used to study the impact of GS structure on insect performance in first experiments. Our experiments revealed that methylsulfinyl GS containing ecotype lines were more resistant to the generalist caterpillar *Spodoptera exigua* (Hübner) and to the specialist caterpillar *Pieris brassicae* (L.) than the lines containing hydroxypropyl GS as main compounds.

Introduction

Plants live in a dangerous world and are constantly being attacked by various enemies, such as fungi, bacteria, and insects. They have developed effective strategies in order to overcome their enemies. The defense strategy of plants in families in the Brassicales order and of the model plant *Arabidopsis thaliana* (L.) comprises glucosinolates (GS) as their secondary plant metabolites. GS are stable, hydrophilic compounds localized in the plant's cell vacuole or in specialized cells, separated from their hydrolyzing enzymes, the myrosinases (β-thioglucoside glycohydrolases) (KOROLEVA et al., 2003). The principal biologically active compounds, such as isothiocyanates and nitriles, are released after tissue damage, when GS and myrosinase come into contact. The formation of the hydrolysis products depends on the chemical structure of the aglycone side chain, the presence of protein factors like the epithiospecifer protein (ESP), and other reaction conditions (LAMBRIX et al., 2001).

To date more than 120 GS varying in their aglycone side chain are described (FAHEY et al., 2000). Studies with *A. thaliana* and different Brassica species revealed that they are synthesized via different biochemical pathways (KLIEBENSTEIN et al., 2001a; RAYBOULDT and MOYES, 2001). In *A. thaliana*, three major classes of GS are distinguished: aliphatic GS, which derive principally from methionine precursors, aromatic GS from phenylalanine, and indolyl GS from tryptophan precursors. Indolyl GS are uniformly distributed in *A. thaliana* ecotypes and members of the Brassicaceae family (KLIEBENSTEIN et al., 2001a; FAHEY et al., 2001). In contrast, the presence of aliphatic GS is highly variable in the Brassicaceae and under strong genetic control. The variability of aliphatic GS is determined by polymorphism at only a few different loci. Studies on *AOP* genes and GS contents in ecotypes showed that they naturally produce either hydroxyalkyl (e.g 3-hydroxypropyl) or alkenyl (e.g. allyl) GS with production of either AOP2 or AOP3 (KLIEBENSTEIN et al., 2005). *AOP* genes are a result of gene duplication and encode for 2-oxoglutarate-dependent dioxygenases (KLIEBENSTEIN et al., 2001c). The gene product of *AOP2* causes the production of alkenyl GS of a methylsulfinyl precursor, whereas AOP3 leads to the formation of hydroxy-alkyl GS in presence of C_3 precursors are present. *A. thaliana* ecotypes that accumulate methylsulfinyl GS, like Columbia, possess a non-functional AOP2 and/or AOP3, or the expression of these genes is blocked.

There is a lack of studies that investigate the importance of aliphatic GS variability in regard to plant defense against herbivorous insects. Preliminary examinations indicated that the variability of the *AOP* genes in *A. thaliana* ecotypes influences the host plant's resistance. Further investigations shall reveal the influence of *AOP g*ene expression on the side chain modifications of GS and the impact on insect resistance in more detail. The first approach was to crossbreed *AOP2* and *AOP3* genes into a methylsulfinyl GS producing C_3 ecotype to obtain allyl and 3-hydroxypropyl GS containing plants in the filial generation. To evaluate the host-plant resistance of homozygote lines with a chemically different phenotype, we tested the feeding performance of two lepidopteron pest species on these lines. We used the specialist *Pieris brassicae* (L.) and the generalist *Spodoptera exigua* (Hübner) for the bioassays. In the present study, the first results for methylsulfinyl GS producing lines versus hydroxypropyl GS containing lines are presented.

Material and methods

Crossbreeding of *AOP2* and *AOP3* into a methylsulfinyl GS containing ecotype

The 3-methylsulfinylpropyl GS producing ecotype Gie-0 (N1193) was selected for the experiments, because preliminary bioassays revealed that this ecotype is highly resistant to insects. Its crossing partners were the hydroxypropyl GS accumulating ecotype Sap-0 (N1506) and Mr-0 (N1373), whereby Sap-0 was most suitable to insects in pilot studies. Female-sterile crossing lines of Gie-0 were produced by making the stamen unripe. Then the pistil was spread with pollen of Sap-0. To guarantee that at least one cross contained the desired GS profile (hydroxypropyl or allyl GS) in the F_1, 10 parallel crossings were carried out each time. The seeds of successful crosses were used to obtain at least five plants. Subsequently the GS profiles of filial generations (self-pollination) were examined. Plants containing 3-hydroxypropyl, allyl, and 3-methylsulfinyl GS were used for seed collection. Once can only tell which plant is homozygous for *AOP3, AOP2* or *AOP0* (non-functional AOP2/3) in the F_3 generation, because of the constant GS profile. Only homozygote lines which are stable for *AOP* and *ESP* were used for insect bioassays. Plants used for the bioassays were cultivated in a climate chamber at 21 ± 1 °C, 60 ± 5% relative humidity, at 200 µmol $m^{-1}s^{-1}$ light intensity and a 10 : 14 (L : D) photoperiod.

Glucosinolate analysis

For GS analysis, 4 to 5 leaves of 2-week old plants were frozen in liquid nitrogen, freeze-dried, and grinned with a pestle directly in the tube. GS were extracted as described in detail in MEWIS et al. (2005). 4-hydroxybenzyl GS (sinalbin, purified from *Sinapsis alba* seeds as potassium salt) was used as internal standard for quantification of GS in extracts. GS in extracts were desulfated with 75 µl aryl sulfatase solution (H-1 from *Helix pomatia*, Sigma-Aldrich) on DEAE Sephadex A-25 mini columns. 40 µl of desulpho GS extracts were run on a Dionex P680A HPLC system equipped with a narrow bore column (Acclaim™ 120, 250 - 2.1, 5 µm, RP18, Dionex). For analysis of GS a 43 min gradient program was used consisting of the following eluents: A) Millipure Water and B) 40% acetonitrile (HPLC grade). The run was composed of 0.5% B (1 min), 0.5 - 20% B (7 min), 20% B (2 min), 20 - 50% B (9 min), 50% B (3 min), 50 - 99% B (6 min), a 5 min hold at 99% B, 99 - 0.5% B (3 min), and a 7 min final hold at 0.5% B. GS were monitored at 229 nm. For GS identification, internal standards, retention time, and UV spectra were used.

Insect bioassay

Pieris brassicae (L.) larvae were obtained from an established rearing in the urban horticultural department at Humboldt University. Eggs were originally ordered from Insect Service GmbH (Berlin). For the rearing, the adult laid its eggs on kohlrabi (*Brassica oleraceae var. gongylodes*) and were reared until the 2nd instar on these plants. Then they were transferred to savoy cabbage (*Brassica oleraceae var. sabauda*) and maintained there until pupation. *Spodoptera exigua* (Hübner) eggs and larvae were kindly given by Bayer Crop Science (Monheim, Germany). Rearing is described in ROHR et al. (2006). For the bioassays, 10 lines of methylsulfinyl (MSOP) GS producing plants and 10 lines of hydroxypropyl (OHP) GS producing plants were used. The test insects were 2nd instars of *S. exigua* and *P. brassicae*. The experiments were conducted in plastic cages covered with fine mesh gauze containing one larva per plant. The initial and final (after 72 h) larval weights were determined. In addition, the plant damage was estimated, according to STOTZ et al. (2000).

Analysis of glucosinolate hydrolysis product

Leaf samples (200 mg) of ecotypes were ground by using a pestle with 1.0 ml of MilliQ water in 4 ml glass vials. Phenyl cyanide (50 µl; diluted 1 : 5000 in 1% MeOH/H_2O) was added as internal standard. The tubes were quickly sealed with a septum cap and left standing for 10 min at room temperature. After the addition of 2 ml of dichloromethane through the septum, the tube was vortexed for 10 s and centrifuged at 5000 g. The dichloromethane layer was then removed, dried, and filtered by passing through a short column of anhydrous sodium sulfate to remove water residues. The aqueous layer of the first extraction was re-extracted with 2 ml dichloromethane. Extracts were combined and concentrated under nitrogen to 200 µl and analyzed by GC-MS using an Agilent 6890 series gas chromatograph (Agilent Technologies, Waldbronn, Germany) with a DB5 column (J & W Scientific, 30 m, 0.25 mm I.D., and 0.25 µm film). A 1 µl sample was split-less injected at 200 °C. A temperature program of 35 °C for 3 min, a 10 °C/min ramp to 230 °C and a cool down with a total analysis time of 37 min was used, and helium was used as carrier gas. For product identification by using standards and MS libraries, the column was coupled to an Agilent 5973N quadrupole mass detector.

RT-PCR analysis

Total RNA of Mr-0, Gie-0, Sap-0, and Col-0 (reference ecotype) was isolated from frozen rosettes with Trizol® reagent (Invitrogen, Karlsruhe) following the standard protocol and including the high salt precipitation step. RNA was converted to cDNA by reverse transcription according to the Promega (Madison, WI) protocol. A primer (0.5 µg), oligodT12-18 (Invitrogen, Carlsbad, CA) was added to 2 µg of total RNA with a total of 8 µl volume and was heated to 65 °C for 5 min. cDNA was synthesized by adding 0.5 mM dNTPs, 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) and the buffer supplied for this enzyme in a total volume of 20 µl. The mixture was incubated at 37 °C for 1 h. The volume was adjusted to 50 µl followed by heating for 10 min at 70 °C.

The PCR reaction was optimized and 2 µl of RT reaction was used as a template for the tests. The following reaction condition were used for the PCR in a total volume of 20 µl, 1 X PCR buffer (Promega), 0.2 mM dNTP's, 2.1 mM $MgCl_2$, 0.5 µmol of the forward and reverse primer, 1 unit of *Taq* DNA polymerase (Promega). The following PCR program was performed using a Biometra gradient cycler: 2 min 96 °C, 30 cycles of 15 s at 94 °C, 30 s 54 °C, and 20 s at 72 °C, followed by a 5 min final at 72 °C. *Actin8* (AC8, At1g49240) was used as reference gene and was designed to be intron spanning for possible detection of DNA contamination. The *AC8* forward (f) primer was 5'-ATGAAGATTAAGGTCGTGGCAC and the reverse (r), 5'-GTTTTTATCCGAGTTTGAAGAGGC. Following primers were designed to amplify AOP genes:

AOP2 (At4g03060)	f: 5'CACGTGTCCAAAACCGGAC
	r: 5'ATTGTCGAAACTCGGAATCAAG
AOP3 (At4g03050)	f: 5'GAAAGAAGACGAGATACGCAG
	r: 5'CTTGAAACCACGTCCAAACA

The intensities of bands were visualized by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide on a Kodak Image Station by using Kodak MI™ software. All primers successfully amplified a band of correct size. PCR products were cloned into the T-overhang vector pCR2.1 with the TOPO TA cloning kit (Invitrogen). PCR products were fully sequenced to confirm their fidelity.

Results

Characteristics of parent ecotypes

In order to investigate the influence of *AOP g*enes on side chain modifications of GS and their impact on insect resistance, ecotypes with different C_3 main GS were crossed to obtain ecotype lines containing methylsulfinyl, hydroxypropyl or allyl GS. The parent ecotypes had the following characteristics. Gie-0 is accumulating mainly 3-methylsulfinylpropyl GS and does not show *AOP3* expression (Fig. 1). Low expression of *AOP2* was detected, indicating a non-functional enzyme like in Col-0. *AOP2* in the allyl GS containing ecotype Mr-0 was greatly expressed, whereas expression of AOP3 was not detected. The predominantly 3-hydroxypropyl GS accumulating ecotypes Sap 0 was the only ecotype with *AOP3* expression. The GC-MS analysis revealed that Gie-0 produces nitriles as hydrolysis products, whereas the dominating hydrolysis product of Mr-0 and Sap-0 were isothiocyanates.

GS profil of Gie-0 x Sap-0 crosses

Although we were able to successfully cross the methylsulfinyl GS producing ecotype Gie-0 with Sap-0 and Mr-0 to achieve the desired GS profiles (either 3-hydroxypropyl or allyl GS, in the F_1 generation) detailed data will be presented only for Gie-0 x Sap-0. The seeds of successful crosses were used, and GS profiles of filial generations after self-pollination were examined. 3-hydroxypropyl, allyl, and 3-methylsulfinylpropyl GS containing plants were used for seed

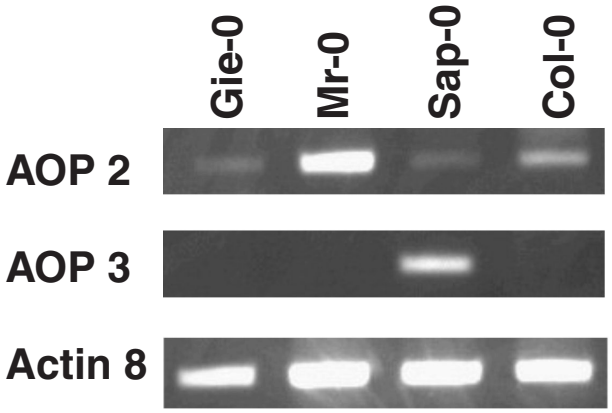

Fig. 1: Expression of AOP genes in *A. thaliana* ecotypes compared to the reference gene Actin 8.

collection. In Fig. 2 (A, B) HPLC chromatogram examples for ecotype lines producing 3-hydroxypropyl as well as 3-methylsulfinylpropyl GS used for screening, are presented. To obtain homozygous plant lines for *AOP3*, GS profiles of five plants per line were analyzed by HPLC in the F_3 generation. For methylsulfinyl GS producing plants (genotype *AOP0*, nonfunctional AOP2/AOP3), only one plant was analyzed each time. According to their stable GS profiles, 10 homozygote lines containing aliphatic 3-methylsulfinyl (3MSOP) GS, as well as 10 lines producing aliphatic 3-hydroxypropyl (3OHP) GS as main compounds, were identified and selected for the bioassays with insects (Tab. 1). The main indolyl GS of lines were 3-indolylmethyl GS followed by 4-methoxy-3-indolylmethyl GS and 1-methoxy-3-indolylmethyl GS. The total GS content in OHP producing lines was up to twofold higher than in MSOP producing lines (Tab. 1). Plants obtained from seeds of these lines were used for the bioassays with different insect herbivores.

Tab. 1: Aliphatic, indolyl, and total glucosinolate content in leaves of *A. thaliana* ecotype lines producing 3-hydroxypropyl GS (OHP) or 3-methylsulfinylpropyl GS (MSOP).

Line	Glucosinolate content [µmol/g dry weight]		
	Aliphatic	Indolyl	Total
OHP1	55.87	5.15	61.02
OHP2	72.54	10.42	82.97
OHP3	40.52	6.32	46.84
OHP4	60.73	9.39	70.12
OHP5	52.23	7.53	59.77
OHP6	61.56	9.46	71.03
OHP7	57.04	7.90	64.95
OHP8	42.20	6.32	48.53
OHP9	27.28	5.54	32.83
OHP10	35.06	7.18	42.25
MSOP1	12.94	4.72	17.67
MSOP2	9.45	3.72	13.17
MSOP3	8.10	5.40	13.51
MSOP4	19.07	6.06	25.14
MSOP5	43.57	11.53	55.10
MSOP6	26.80	5.65	32.45
MSOP7	7.52	4.30	11.82
MSOP8	12.04	5.70	17.74
MSOP9	23.64	4.94	28.59
MSOP10	26.58	5.55	32.13

Host plant suitability of ecotype lines for *Spodoptera exigua* and *Pieris brassicae* larvae

Insect performance data, measured as percentage weight gain of larvae within three days, on MSOP or OHP producing lines are presented in Fig. 3. The percentage larval weight gain on chemically

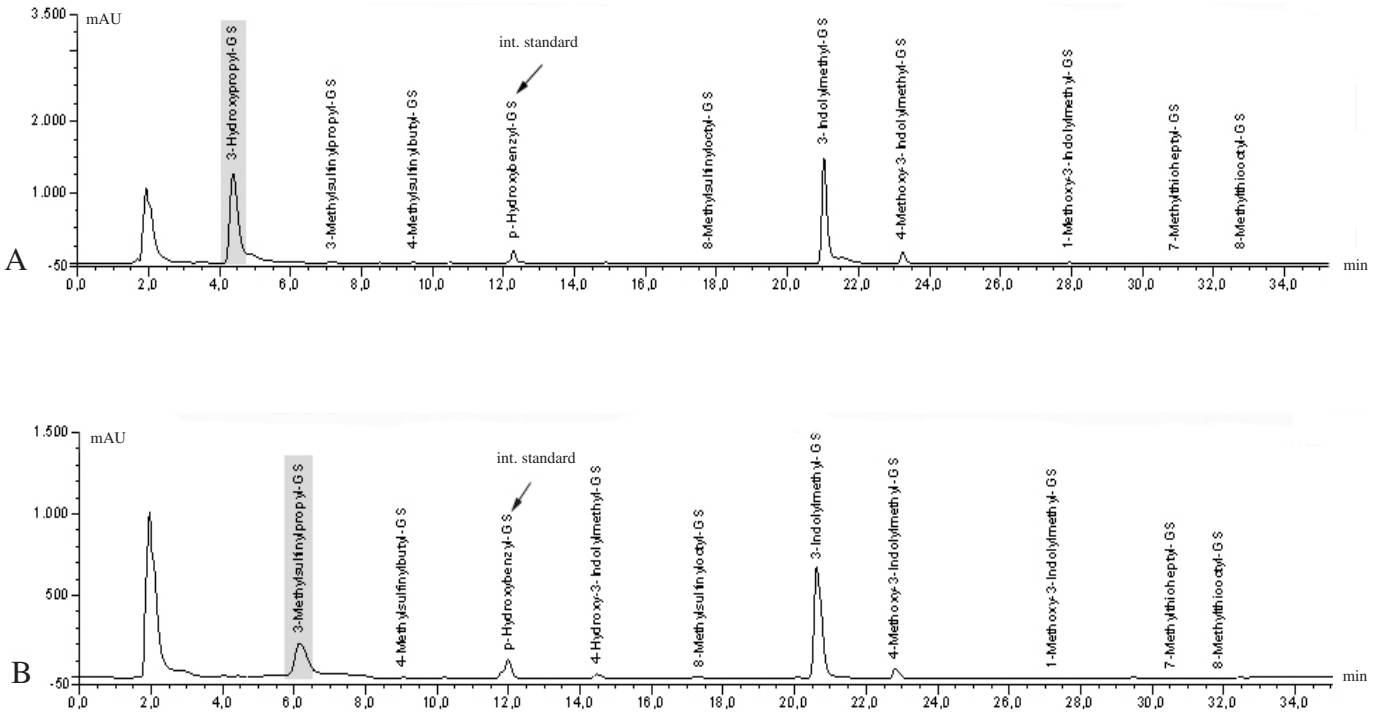

Fig. 2: HPLC chromatogram examples of lines producing 3-hydroxypropyl GS (A) or 3-methylsulfinylpropyl GS.

different ecotype crosses was significant different for both lepidopteron species tested (Fig. 3). When the larval weight increase on the MSOP and OHP phenotypes is compared, the generalist *S. exigua* and the specialist *P. brassicae* performed better on 3-hydroxypropyl GS producing lines than on lines containing 3-methylsulfinylpropyl GS.

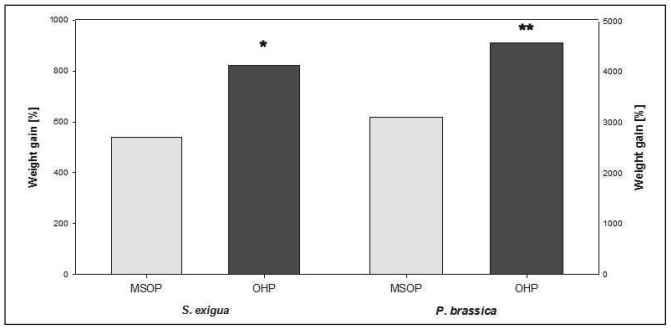

Fig. 3: Average percentage weight gain of second instars of caterpillars on ecotype lines (ANOVA, ** p <0.05. * p <0.1).

Discussion

The GS-myrosinase defence system has been an object of extensive study in the past several years. However, many aspects are far from being clear, such as the function of variability of aliphatic GS in *A. thaliana* and other Brassicaceae. In our study we examined the impact of *AOP* gene expression on the side chain modification of aliphatic GS and the impact on host-plant resistance.

Our study supports the finding of KLIEBENSTEIN et al. (2001b), which state that the two genes, *AOP2* and *AOP3*, which encode for 2-oxoglutarate-dependent dioxygenases, are responsible for side chain hydroxylation of aliphatic GS. *AOP2* from Mr-0 crossed into the 3-methylsulfinylpropyl GS producing ecotype Gie-0 resulted in an alkenyl-producing phenotype. AOP3 introduced into Gie-0 leads to the conversion of methylsulfinylalkyl to the hydroxyalkyl form, whereby *AOP3* expression was detected only for Sap-0. The methylsulfinyl GS accumulating ecotype Gie-0 is caused by the absence of AOP3 and a non-functional *AOP2*.

Ecotypes vary not only in their main GS, but also other factors like plant morphology and other secondary metabolites than GS can influence host-plant resistance (LARKIN et al., 1996; HARREWIJN et al., 2001). Therefore, we created ecotype lines which posses a chemically distinct GS profile with a preferably similar genetic background to run feeding studies with insects of different specialization. Weight gain of lepidopteron larvae, one generalist: *S. exigua* and one specialist: *P. brassicae*, was significantly higher on 3-hydroxypropyl GS (OHP) containing ecotype lines than on 3-methylsulfinyl GS (MSOP) producing ecotype lines. The better performance of OHP lines corresponds to a higher mean GS content compared to GS contents in MSOP lines. This suggests a strong effect of side chain hydroxylation on insect performance. Our results are consistent with our previous study with common ecotypes (ROHR et al., 2006).

It is known that GS and different types of hydrolysis-product produced influence the plant resistance especially against generalist insects (KLIEBENSTEIN et al., 2002b; KROYMANN et al., 2003). But different compounds within a chemical group, like the GS, can have effects on specialized herbivorous insects as well (BARTLETT et al., 1994). The reason for OHP lines being more suitable for generalist and specialist lepidopteron species than MSOP lines might be due to the short chemical structure of 3-hydroxypropyl GS and/or different reactivity of this compound compared to 3-methylsulfinylpropyl GS. Further investigations regarding the reaction of this compound within the insect gut could confirm this assumption. The preference of generalist and specialist insect species for short chain aliphatic GS like 3-hydroxypropyl GS has been reported also in other studies (GIAMOUSTRIS and MITHEN, 1995). Also LAMBRIX et al. (2001) showed a feeding preference of the generalist *Trichoplusia ni* (Hübner) for F_2 lines of ecotype crosses Da(1)-12 x Ei-2, containing 3-hydroxypropyl GS. Furthermore, they determined that *T. ni* preferred feeding on lines producing nitriles as hydrolysis products. In our study with ecotype lines, we did not find any dependence of hydrolysis products, isothiocyanates or nitriles, on host plant suitability (data not shown).

Although our study indicates that GS side chain influences plant resistance against insect, further studies are needed to reveal the biochemical basics, which lead to the distinct effects of chemically different compounds to insects.

Acknowledgement

We thank Dr. Peter Meisner and Gerd Trautmann (Bayer Crop Science, Mohnheim, Germany) for sending the start population of *S. exigua*. The authors further thank Dr. Michael Reichelt and Dr. Jonathan Gershenzon (Max-Planck Institute for Chemical Ecology Jena, Germany) for their collaborative help in analyzing the glucosinolate hydrolysis products. The project was funded by DFG (Deutsche Forschungsgesellschaft, GZ ME 2095/4-1).

References

BARTLET, E., KIDDLE, G., WILLIAMS, I., WALLSGROVE, R.M., 1999: Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist. Entomol. Exp. Appl. 91, 163-167.

FAHEY, J.W., ZALCMANN, A.T., TALALAY, P., 2001: The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56, 5-51.

HARREWIJN, P., OOSTEN, VAN A.M., PIRON, P.G.M., 2001: Natural terpenoids as messengers: a multidisciplinary study of their production, biological functions, and practical applications. Dordrecht, Boston: Kluwer Academic 2001, 440.

KLIEBENSTEIN, D.J., GERSHENZON, J., MITCHELL-OLDS, T., 2001a: Comparative quantitative trait loci mapping of aliphatic, indolic and benzylic glucosinolate production in *Arabidopsis thaliana* leaves and seeds. Genetics 159, 359-370.

KLIEBENSTEIN, D.J., LAMBRIX, V.M., REICHELT, M., GERSHENZON, J., MITCHELL-OLDS, T., 2001b: Gene duplication in the diversification of secondary metabolism: Tandem 2-Oxogluterate-dependent dioxygenases control glucosinolate biosynthesis in *Arabidopsis*. Plant Cell 13, 681-693.

KOROLEVA, O.A., DAVIES, A., DEEKEN, R., THORPE, M.R., TOMOS, A.D., HEDRICH, R., 2000: Different myrosinase and ideoblast distribution in *Arabidopsis* and *Brassica napus*. Plant Physiol. 127, 1750-1763.

LAMBRIX, V., REICHELT, M., MITCHELL-OLDS, T., KLIEBENSTEIN, D.J., GERSHENZON, J., 2001: The *Arabidopsis* epithiospecifer protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. Plant Cell 13, 2793-2807.

LARKIN, J.C., YOUNG, N., PRIGGE, M., MARKS, M.D., 1996: The control of trichome spacing and number in *Arabidopsis*. Development 122, 997-1105.

MEWIS, I., APPEL, H.M., HOM, A., RAINA, R., SCHULTZ, J.C., 2005: Major signaling pathways modulate *Arabidopsis thaliana* (L.) glucosinolate accumulation and response to both phloem feeding and chewing insects. Plant Physiol. 138, 1149-1162.

RAYBOULD, A.F., MOYES, C.L., 2001: The ecological genetics of aliphatic

glucosinolates. Heredity 87, 383-391.

ROHR, F., ULRICHS, C., MUCHA-PELZER, T., MEWIS, I., 2006: Variability of aliphatic glucosinolates in Arabidopsis and their influence on insect resistanc. Comm. Appl. Biol. Sci, Ghent University, 71/ 2b, 507-515.

STOTZ, H.U., PITTENDRIGH, B.R., KROYMANN, J., WENIGER, K., FRITSCHE, J., BAUKE, A., MITCHELL-OLDS, T., 2000: Induced plant defense responses against chewing insects ethylene signalling reduces resistance of *Arabidopsis* against egyptian cotton worm but not diamondback moth. Plant Physiol. 124, 1007-1017.

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