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 gene 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 crossed 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 F3 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 glycosylhydrodrolases) (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 epithiospecifier 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 Moyses, 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 C3 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 gene 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 C3 ecotype to obtain allyl and 3-hydroxypropyl GS containing plants in the filial generation. To evaluate the host-plant resistance of homozgygote 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 ecotypes 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 F1, 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 F1 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-2 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 grinded 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 Sinapis 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) Millipore 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 oleracea var. gongylodes) and were reared until the 2nd instar on these plants. Then they were transferred to savoy cabbage (Brassica oleracea 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/H2O) 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), oligodiT12-18 (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 MgCl2, 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 cyclers: 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, A11g49240) 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’-ATGAAAGATTAAGGCTGCGAATCAAG and the reverse (r), 5’-GTTTATTCGCCATGTTGAAAGGC. Following primers were designed to amplify AOP genes:

- AOP2 (At4g03060) f: 5’CACGTGTCAAAACCCGGAC r: 5’ATTTGTCAAGACCTCGGAATCAAG
- AOP3 (At4g03050) f: 5’GAAAGAAGACAGGATACGCAAG r: 5’CTTGAACACGTCGTTCAACAA

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 MF™ software. All primers successfully amplified a band of correct size. PCR products were cloned into 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 genes on side chain modifications of GS and their impact on insect resistance, ecotypes with different C3 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 isothiocyanates. AOP2 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 F1 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

132 F. Rohr, Ch. Ulrichs, I. Mewis
Glucosinolates and insect resistance

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 F3 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).

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

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

**Fig. 2:** HPLC chromatogram examples of lines producing 3-hydroxypropyl GS (A) or 3-methylsulfinylpropyl GS.
different ecotype crosses was significant different for both lepidopteran 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-methylsulfanylpropyl 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 Lambrrix et al. (2001) showed a feeding preference of the generalist Trichoplusia ni (Hübner) for F2 lines of ecotype crosses Da1(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**


Raybould, A.F., Moves, C.L., 2001: The ecological genetics of aliphatic...


Address of the authors:
Franziska Rohr, Institute for Horticulture Science, Urban Horticulture, Humboldt University Berlin, Lentzeallee 55-57, 14195 Berlin-Dahlem, Germany.