Comparative Investigations of the Glucosinolate–Myrosinase System in Arabidopsis Suspension Cells and Hypocotyls

Sophie Alvarez 1, Yan He 1 and Sixue Chen 1,2,*
1 Department of Botany, Genetics Institute, The Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, FL 32610, USA
2 College of Life Sciences, Heilongjiang University, Harbin 150080, PR China

Glucosinolates are secondary metabolites derived from amino acids. Upon hydrolysis by myrosinases, they produce a variety of biologically active compounds. In this study, the glucosinolate–myrosinase system was characterized in Arabidopsis suspension cells. A total of seven glucosinolates were identified and the myrosinase activity was determined. Plant suspension cells have been used as model systems in many areas of study. To investigate whether the glucosinolate–myrosinase system in suspension cells works similarly to that in planta, 10-day-old seedling hypocotyls were used for comparative studies. A total of 16 glucosinolates were identified in hypocotyls. The two types of samples were also treated with methyljasmonate (MeJA)—a signaling compound induced by herbivore attack and wounding to initiate plant defense processes. The glucosinolate levels and their responses to MeJA varied greatly with the age of the cells. Two-day-old cells were most responsive, with the levels of all seven glucosinolates induced by MeJA, while in 4-day-old cells only the levels of indole glucosinolates were increased. In hypocotyls, the levels of indole glucosinolates and aliphatic glucosinolates (especially 4-methylsulfinylbutyl- and 8-methylsulfinyloctylglucosinolates) were significantly increased by MeJA treatment. The transcript levels of several genes involved in glucosinolate biosynthesis were induced in both suspension cells and hypocotyls after MeJA treatment. Myrosinase levels and activities were also monitored. The molecular bases underlying the differences of glucosinolate metabolism in the suspension cells and hypocotyls were discussed.

Keywords: Arabidopsis — Glucosinolates — Hypocotyls — Methyljasmonate — Myrosinase — Suspension Cells.

Introduction

Glucosinolates are secondary metabolites, derived from amino acids, found in many plants of the order Capparales including Arabidopsis. Upon hydrolysis by thioglucosidases, also called myrosinases, a variety of biologically active compounds (e.g. isothiocyanates and derivatives) are produced. Because of their general toxicity and volatility, these degradation products play important roles in plant defense against pathogens and generalist herbivores, as well as serving as attractants to specialist insects (Rask et al. 2000, Barth and Jander 2006). In addition to lending distinctive flavors to cabbage and condiments, some of the degradation products, e.g. 4-methylsulfinylbutyl isothiocyanate found in certain Brassica vegetables, exhibit anticarcinogenic properties (Fahey et al. 1997).

The core glucosinolate biosynthetic pathway has been well studied in Arabidopsis using biochemical and genetics approaches (Chen and Andreasson 2001, Grubb and Abel 2006, Halkier and Gershenzon 2006). Five cytochrome P450 (CYP) gene products, CYP79A2, CYP79B2 and CYP79B3, CYP79F1 and CYP79F2, have been shown to catalyze the conversion of phenylalanine (Wittstock and Halkier 2000), tryptophan (Hull et al. 2000; Mikkelsen et al. 2000) or short-chain and long-chain elongated methionine substrates (Hansen et al. 2001, Chen et al. 2003) to the corresponding aldoximes, respectively. The aldoximes are further metabolized to form intact glucosinolates (Halkier and Gershenzon 2006). The activity of CYP79A2 in vivo is extremely low, making CYP79B and CYP79F the primary enzymes catalyzing the production of indole glucosinolates and aliphatic glucosinolates, respectively. These glucosinolates account for almost all the glucosinolates in Arabidopsis.

Plant glucosinolate metabolism is responsive to many environmental factors, plant hormones, abiotic and biotic stresses, and disease resistance pathway signaling molecules. Under pathogen or herbivore attack, plants often increase the production of glucosinolates (Mikkelsen et al. 2003, Mewis et al. 2005). Such an induction is at least partially mediated by jasmonic acid, salicylic acid and ethylene, the major hormones associated with defense responses.

Abbreviations: 4MOI3M, 4-methoxy-indol-3-ylmethylglucosinolate; 4MSOB, 4-methylsulfinylbutylglucosinolate; 4OH13M, 4-hydroxy-indol-3-ylmethylglucosinolate; 5MSOP, 5-methylsulfinylpentylglucosinolate; 5MTP, 5-methylthiopentylglucosinolate; 8MSOO, 8-methylsulfinyloctylglucosinolate; 8MTO, 8-methylthiooctylglucosinolate; CYP, cytochrome P450; I3M, indol-3-ylmethylglucosinolate; MeJA, methyljasmonate; MS, mass spectrometry; NMOI3M, N-methoxy-indol-3-ylmethylglucosinolate; RT-PCR, reverse transcription-PCR; TLC, thin-layer chromatography.

*Corresponding author: E-mail, schen@ufl.edu; Fax, +1-352-273-8284.
Glucosinolates in suspension cells and hypocotyls

(Rask et al. 2000, Meiw et al. 2005). Methyljasmonate (MeJA) treatment, which simulates the response to insect attack and necrotrophic pathogens, leads to significant increases in indole glucosinolate levels, while having little effect on aliphatic glucosinolates (Bodnaryk 1994, Doughty et al. 1995, Bartlet et al. 1999, Mikkelsen et al. 2003). The regulation seems to occur at the transcriptional level, since the expression of CYP79B2 and CYP79B3 is significantly up-regulated, but the expression of CYP79F1 and CYP79F2 is barely affected by MeJA (Mikkelsen et al. 2003).

Plant suspension cells have been widely used in many areas of research, including gene expression (Axelos et al. 1992), plant regeneration (Chawla 2000), nutrient deprivation (LeFèbvre et al. 1990), hormone action (Brault et al. 2004), stress physiology (Krause and Durner 2004, Duval et al. 2005), secondary metabolism (Zook 1998, Hirai et al. 2007) and membrane transport (Sterling et al. 1990). The use of suspension cells has a number of advantages. For example, the cells are homogenous, rapidly growing, and easy to maintain and propagate to theoretically unlimited quantities. The biochemical machinery in suspension cells can be harnessed to produce useful chemicals (Yeoman and Yeoman 1996). Experiments using suspension cells are also facilitated by the ease of controlling the environment, culture conditions and treatment. The obvious disadvantage is that the cells are dedifferentiated, so the biochemical and physiological processes might not reflect those that occur in planta. Most of the work on the glucosinolates and myrosinases has been performed using different plant tissues and organs (Chen and Andreasson 2001, Grubb and Abel 2006, Halkier and Gershenzon 2006). Plant suspension cells are in theory totipotent and have the potential to synthesize any of the compounds normally associated with intact tissues. However, only a few studies have used suspension cells to investigate the glucosinolate–myrosinase system. Indole glucosinolates were identified in suspension cells derived from Brassica napus and B. oleracea, but in suspension cells of another crucifer species, Hirschfeldia incana, no glucosinolates could be found (Belkhiri and Lockwood 1994). Suspension cells derived from hairy roots of Tropaeolum majus were found to produce an aromatic glucosinolate benzylglucosinolate and myrosinase (Wielanek and Urbane 1999). Recently, a Myb28 transcriptional factor that specifically regulates aliphatic glucosinolate biosynthetic genes was transformed into Arabidopsis suspension cells. Ectopic expression of Myb28 led to the production of different glucosinolates. However, no glucosinolate could be found in non-transformed and empty vector-transformed control cells (Hirai et al. 2007). In this report, we describe an extensive biochemical analysis of the glucosinolate–myrosinase system in wild-type Arabidopsis suspension cells as well as in 10-day-old hypocotyls, the source tissue used to generate the suspension cells. In addition, we conducted MeJA treatment of the two types of samples and compared the responses of the glucosinolate–myrosinase system. The goal of these experiments is to characterize the glucosinolate–myrosinase system in suspension cells and determine whether it works similarly to that in planta.

**Results**

**Arabidopsis suspension cells produce glucosinolates**

Glucosinolates in Arabidopsis suspension cells were converted to desulfoglucosinolates using established procedures (Petersen et al. 2000, Petersen et al. 2002, Chen et al. 2003) and determined using HPLC online with a quadrupole ion trap mass spectrometer. The HPLC chromatogram of desulfoglucosinolate samples prepared from suspension cells showed approximately 17 major peaks (Fig. 1). In order to identify glucosinolate peaks, the chromatogram was compared with those of desulfoglucosinolates prepared from Arabidopsis seeds, leaves and roots. The glucosinolate identities and quantities have been well characterized in these tissues (Petersen et al. 2000, Reintanz et al. 2001, Petersen et al. 2002, Chen et al. 2003). Based on retention time, UV spectra and relative peak areas, seven glucosinolates could be tentatively identified in the suspension cells (Fig. 1). To confirm the identity of each glucosinolate in the suspension cells, tandem mass spectrometry (MS/MS) data acquisition directed by enhanced MS scan and neutral loss scan (m/z 162.2 corresponding to the glucose moiety) was conducted. The MS/MS fragmentation pattern of each individual peak was searched against an internal metabolite database constructed from the MS/MS spectra of glucosinolates in Arabidopsis seeds, leaves and roots using Analyst 1.4.1 software (Applied Biosystems Inc., Foster City, CA, USA). Based on precursor mass and MS/MS fragmentation, the seven glucosinolates were unambiguously identified in the suspensions cells (Fig. 2).

The other 10 peaks that we observed on the HPLC chromatogram were not glucosinolates. For some of the peaks, we obtained molecular mass and quality MS/MS spectra (Supplementary Fig. S1). Since these compounds bind to Sephadex DEAE resin and can be eluted using sulfatase treatment, they are likely to contain sulfate groups. Further structural analysis using nuclear magnetic resonance technology is under way.

**Glucosinolate profiles in suspension cells and hypocotyls are different**

To investigate whether the glucosinolate profile in suspension cells (derived from the hypocotyls) is similar to that in organized plant tissue, glucosinolates from a subculture time series of suspension cells and hypocotyls were analyzed by HPLC-MS. For simplicity, the number of
days after subculture was used to indicate the age of the cells. All subcultures contained the seven glucosinolates described in the previous section, although the concentrations of individual glucosinolates as well as total glucosinolates varied in the course of cell division and development, with day 3 cells containing the highest amounts of glucosinolates (Fig. 3A). In hypocotyls, 16 different glucosinolates were identified (Table 1). Compared with suspension cells, hypocotyls did not have 5-methylthiopentylglucosinolate (5MTP), but contained 10 additional aliphatic glucosinolates. For the six common glucosinolates, the levels of 4-methylsulfinylbutylglucosinolate (4MSOB) in hypocotyls were >4 times higher than those in suspension cells. In contrast, the levels of 8-methylsulfinyloctylglucosinolate (8MSOO) were >4 times less than those in suspension cells. The levels of different indole glucosinolates in hypocotyls differed from those in suspension cells. Suspension cells contained about three times more indol-3-ylmethylglucosinolate (I3M) and 4-methoxy-indol-3-ylmethylglucosinolate (4MOI3M), as well as >15 times more 4-hydroxy-indol-3-ylmethylglucosinolate (4OHI3M). Hypocotyls contained about four times more N-methoxy-indol-3-ylmethylglucosinolate (NMOI3M) (Table 1). Overall, the total content of glucosinolates in suspension cells was approximately twice as much as that in hypocotyls.

Glucosinolate changes in response to MeJA are dependent on the age of the suspensions cells

Since the suspension cells were subcultured into fresh medium every 7 d, the effect of the age of the cells on glucosinolate levels in response to MeJA treatment was studied. MeJA was added to the culture medium to a final concentration of 50 μM from day 1 to day 5 after subculture, and the cells were subsequently harvested 24 h after the treatment was initiated. As can be seen from Fig. 3A, MeJA treatment caused increased levels of total glucosinolates and the response was dependent on the age of the cells. The highest increase in glucosinolate levels was observed at day 2 after subculture, followed by day 3. MeJA-treated cells contained >4 times as much glucosinolates as the control cells. At day 4, the glucosinolate accumulation in treated cells was only about twice as high as in control cells. When these changes were examined at the level of individual glucosinolates, it became apparent that not all glucosinolates responded to MeJA similarly (Fig. 3B). MeJA induced all the glucosinolates detected at day 2. At day 4, no significant changes in aliphatic glucosinolate levels due to MeJA were observed, but there was a marked induction of all the indole glucosinolates. However, the individual glucosinolate levels were generally lower at day 4 than at day 2 (Fig. 3B).

To investigate the MeJA effect on glucosinolate levels in hypocotyls, 10-day-old seedlings were treated with 50 μM MeJA for 24 h before hypocotyls were harvested. Glucosinolates in both control and treated hypocotyls were extracted, separated and quantified. Not all the glucosinolates were induced by MeJA treatment. Among the short-chain aliphatic glucosinolates, only 4MSOB and 5-methylsulfinylpentylglucosinolate (5MSOP) were significantly induced. The three long-chain glucosinolates also accumulated in response to MeJA. Among the indole glucosinolates, only two glucosinolates (I3M and NMOI3M) showed induction by MeJA (Fig. 4). Overall,
the extent of indole glucosinolate changes was much smaller than that observed in suspension cells.

The transcription of glucosinolate biosynthetic genes is responsive to MeJA treatment

To identify the molecular basis underlying the differential glucosinolate changes in response to MeJA treatment in suspension cells and hypocotyls, the relative transcript levels of the genes involved in the biosynthesis of aliphatic glucosinolates, CYP79F1 and CYP79F2, and of indole glucosinolates, CYP79B2 and CYP79B3, were determined using reverse transcription–PCR (RT–PCR). Since day 2 cells and day 4 cells displayed different responses to MeJA treatment (Fig. 3), RNA was extracted from these samples for gene transcription analysis together with 10-day-old hypocotyls with and without MeJA treatment. The PDF1.2 gene and actin 1 gene were included as positive controls for MeJA treatment and equal loading, respectively (Mikkelsen et al. 2003). Except for CYP79F1, all gene transcripts were detected in suspension cells (Fig. 5A). After MeJA treatment, PDF1.2, CYP79B2 and CYP79B3 were induced in both 2-day-old and 4-day-old cells, but CYP79F2 transcripts increased only in the 2-day-old cells. In hypocotyls, CYP79F1, CYP79F2, CYP79B2 and

**Fig. 2** LC-MS/MS spectra of glucosinolates in suspension cells. The fragment ions were annotated with the corresponding molecular structure. (A) 4MSOB; (B) 5MTP; (C) 8MSOO; (D) I3M; (E) 4OHI3M; (F) 4MOI3M; (G) NMOI3M; (F) Benzylglucosinolate (internal standard). Refer to the footnotes to Table 1 for glucosinolate abbreviations.
CYP79B3 showed strong induction by MeJA. However, the induction of the PDF1.2 gene in hypocotyls was not as pronounced as in suspension cells (Fig. 5B). 

Myrosinase activities and contents in suspension cells and hypocotyls are different

Myrosinase activities were analyzed in day 2 cells, day 4 cells and hypocotyls that were subjected to MeJA treatment. Fig. 6A shows the results from thin-layer chromatography (TLC) of glucosinolate degradation products produced by incubating myrosinase extracts from the hypocotyls and suspension cells with \( p \)-hydroxybenzyl-glucosinolate (\( p \)-OHBG) substrate. Myrosinase activity in hypocotyls (as indicated by \( p \)-OHBG degradation) was detectable after 30 min of incubation, while it took >2h to observe \( p \)-OHBG degradation by myrosinases from suspension cells at day 2 and day 4 after subculture. Based on \( p \)-OHBG depletion, the myrosinase activity in hypocotyls was much higher than that in suspension cells. In response to MeJA, the myrosinase activity in hypocotyls decreased. Meanwhile the activity increased slightly in 2-day-old suspension cells, but strongly increased in 4-day-old cells (Fig. 6A, B).

The Arabidopsis genome contains at least six myrosinase genes. Thioglucoside glucohydrolase 1 (TGG1) and TGG2 were shown to be expressed in shoots (Barth and Jander 2006, Ueda et al. 2006), while TGG4 and TGG5 were expressed in roots (Zimmermann et al. 2004, Toufighi et al. 2005). To determine which myrosinases were present and whether protein expression accounted for the differences in activity, Western blotting was conducted using TGG1- and TGG2-specific antibodies. TGG1 protein was much more highly expressed in hypocotyls than in suspension cells, where only a weak signal was detected (Fig. 6C). TGG1 protein levels were not induced by MeJA in hypocotyls or suspension cells. TGG2 signal could not be detected in either hypocotyls or suspension cells (data not shown).

Table 1 Glucosinolate content (nmol g FW\(^{-1}\)) in 3 d suspension cells and hypocotyls

<table>
<thead>
<tr>
<th>Glucosinolate</th>
<th>3OHPr</th>
<th>3MSOP</th>
<th>3BOP</th>
<th>4OHB</th>
<th>4MSOB</th>
<th>4BOB</th>
<th>5MTP</th>
<th>5MSOP</th>
<th>6MSOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>n.d.(^a)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>17.7±1.3</td>
<td>n.d.</td>
<td>78.8±7.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>8.0±0.4</td>
<td>3.0±0.2</td>
<td>4.9±1.1</td>
<td>48.2±1.4</td>
<td>70.3±11.5</td>
<td>8.3±1.8</td>
<td>n.d.</td>
<td>6.3±0.9</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>6BOH</td>
<td>7MTH</td>
<td>8MTO</td>
<td>8MSOO</td>
<td>13M</td>
<td>40HI3M</td>
<td>4MOI3M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>35.7±5.1</td>
<td>870.3±59.3</td>
<td>88.8±13.4</td>
<td>276.5±30.5</td>
<td>38.7±17.2</td>
<td></td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>4.6±0.4</td>
<td>3.5±0.4</td>
<td>4.4±0.5</td>
<td>8.3±0.3</td>
<td>275.4±24.2</td>
<td>5.9±0.8</td>
<td>89.4±10.4</td>
<td>159.2±16.6</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)3OHPr, 3-hydroxypropyl; 3MSOP, 3-methylsulfinylpropyl; 3BOP, 3-benzoyloxypropyl; 4OHB, 4-hydroxybutyl; 4MSOB, 4-methylsulfinylbutyl; 4BOB, 4-benzoyloxybutyl; 5MTP, 5-methylthiopentyl; 5MSOP, 5-methylsulfinylpentyl; 6MSOH, 6-methylsulfinylhexyl; 6BOH, 6-benzoyloxyhexyl; 7MTH, 7-methylthioheptyl; 8MTO, 8-methylothiooctyl; 8MSOO, 8-methylsulfinyloctyl; 13M, indol-3-ylmethyl; 40HI3M, 4-hydroxy-indol-3-ylmethyl; 4MOI3M, 4-methoxy-indol-3-ylmethyl; NMOI3M, N-methoxy-indol-3-ylmethylglucosinolate.

\(^b\)n.d., not detected. ±, represents standard error.
shown), although it was readily detectable in leaf samples of Arabidopsis plants (Supplementary Fig. S2). Although TGG1 and TGG2 share 70% identity, the antibodies do not cross-react because they were generated using unique epitopes (Ueda et al. 2006).

Discussion

Many plant secondary metabolites have been reported to be produced by plant suspension cell cultures (Yeoman and Yeoman 1996, Zook 1998, Chawla 2000). However, only a few studies have used suspension cells to investigate the glucosinolate–myrosinase system (Belkhiri and Lockwood 1994, Wielanek and Urbanek 1999, Hirai et al. 2007). In these limited studies, comparison of the glucosinolate–myrosinase system between source tissues and suspension cell cultures generated from these same tissues has been rare. The lack of such information makes it impossible to judge whether the biochemical and physiological processes taking place in cell cultures reflect those in planta. In this work, we demonstrate that the Arabidopsis suspension cells derived from 10-day-old hypocotyls produce glucosinolates (before and after MeJA treatment). The glucosinolate profiles differ from those in hypocotyls in an age-dependent manner. These differences may be due to multiple layers of regulation, including lower myrosinase activities in suspension cells vs. hypocotyls.
Arabidopsis suspension cells generally produce fewer glucosinolates at higher levels vs. intact hypocotyls

Compared with the glucosinolate profile in the 10-day-old hypocotyls from which the suspension cells were derived, suspension cells produced only seven glucosinolates (Figs. 1, 2). Except for the glucosinolates 4MSOB and NMOI3M, all other measured glucosinolates were produced at much higher levels in the cell cultures (Table 1). In total, the suspension cells produced twice as much total glucosinolate as the hypocotyls. The high concentration of glucosinolates in suspension cells is not due to the presence of 2,4-D in the medium because depletion of 2,4-D actually increased the levels of indole glucosinolates and an aliphatic glucosinolate 8MSOO (Supplementary Fig. S3). This is in strong contradiction to the general belief that undifferentiated plant cell cultures usually produce low quantities of secondary metabolites because growth without differentiation is thought to be incompatible with the expression of plant secondary metabolic pathways (Granicher et al. 1992, Maldonado-Mendoza et al. 1993). One recent report investigated glucosinolate levels in Arabidopsis suspension cells (Hirai et al. 2007). No glucosinolates were detected in Arabidopsis T87 cells unless they were transformed with a Myb28 gene, which regulates aliphatic glucosinolate biosynthesis. It seems that the T87 cells cannot express Myb28, but have all the biochemical machinery for glucosinolate biosynthesis. Apparently, the cells used in our study were different from T87 cells, which were prepared from whole seedlings of A. thaliana (Axelos et al. 1992). The differences in glucosinolate levels might be explained by the origin of the suspension cells, the degree of dedifferentiation, the culture conditions and the stage of cells. Indeed, as shown in our study, glucosinolate profiles and the response to MeJA vary with the age of the cells after subculture (Fig. 3). In the previous study using T87 cells, information regarding the number of cell generations and the stage of cultures is not available (Hirai et al. 2007). In addition to glucosinolates, we also detected several potentially sulfonated compounds in suspension cells (Fig. 1). These compounds were not detected in hypocotyl samples. This indicates that besides glucosinolates, the biosynthesis of other chemicals in suspensions cells may also differ from that in their corresponding plant tissues.

Glucosinolate biosynthesis is under different layers of control

Our investigations of glucosinolate production in Arabidopsis suspension cells demonstrated the biosynthetic capabilities of the cells and the differences of such capabilities from the source tissue hypocotyls in the absence and presence of MeJA. As described below, multiple layers of control may account for the differences. (i) The age after subculture affected glucosinolate production and responses to MeJA (Fig. 3). Cells at day 2 and day 3 of subculture produced high levels of glucosinolates and became more MeJA responsive. This correlated with the rapid biomass increase of the cell cultures (data not shown). Although the molecular basis underlying this correlation is not clear, this phenomenon seems to occur in other systems as well. For example, high production of benzylglucosinolate in T. majus hairy root cultures took place in parallel with the rapid growth phase (Wielanek and Urbanek 1999). (ii) MeJA signaling regulates glucosinolate production in suspension cells and hypocotyls (Figs. 3, 4). Both aliphatic and indole glucosinolates were dramatically induced by MeJA in cells at day 2. This pattern was very similar to the response of glucosinolate production in hypocotyls, though the extent of indole glucosinolate induction was much smaller in hypocotyls. Previous studies showed that MeJA treatment of B. napus and B. juncea selectively induced only indole glucosinolate synthesis, with aliphatic and aromatic glucosinolates being unaffected (Bodnaryk 1994, Doughty et al. 1995, Bartlet et al. 1999). In Arabidopsis, treatment with MeJA strongly induced indole glucosinolate biosynthesis (Brader et al. 2001, Mikkelsen et al. 2003, Mewis et al. 2005). While total aliphatic glucosinolate changes were not significant, induction of 5MSOP, 8-methylthiooctylglucosinolate (8MTO) and 8MSOO levels was marked (Mikkelsen et al. 2003, Mewis et al. 2005). In this study, we observed significant induction of total aliphatic glucosinolates as well as several individual aliphatic glucosinolates in hypocotyls and in day 2 suspension cells (Figs. 3, 4). This is consistent with a dependence on MeJA signaling for the accumulation of aliphatic and indole glucosinolates. (iii) Glucosinolate biosynthesis is partly under transcriptional control. In both suspension cells and hypocotyls under control conditions, we could detect the expression of several glucosinolate biosynthetic genes (Fig. 5). The induction of CYP79 gene transcripts in hypocotyls in response to MeJA explains the changes of corresponding glucosinolates. For example, the strong induction of CYP79F1 paralleled the significant accumulation of 4MSOB and 8MSOO (Fig. 4). In suspension cells, the strong increase in indole glucosinolate levels in response to MeJA correlated very well with the strong induction of the CYP79B2 and CYP79B3 genes. Similarly, CYP79F2 was highly induced by MeJA in day 2 cells, and the concentration of 8MSOO increased in response to the treatment. In contrast, in day 4 cells CYP79F2 expression was not induced by MeJA, and the concentration of aliphatic glucosinolates did not show changes (Fig. 5). One of the most striking features of suspension cells was that no CYP79F1 gene expression was detected by RT-PCR, but the day 2 cells accumulated significant amounts of short-chain aliphatic glucosinolates, 4MSOB and 5MTP upon MeJA treatment. We have excluded potential technical problems as the explanation for the lack of the detection of CYP79F1 (Supplementary Fig. S4).
It may be possible that the CYP79F1 enzyme was present and active, while the CYP79F1 transcript was not stable. (iv) S-oxygenating activity specific for 5MTP is lacking in suspension cells. Except for seeds, 5MTP levels are often low or below detection in vegetative tissues during Arabidopsis development (Reintanz et al. 2001, Petersen et al. 2002). The presence of high levels of 5MTP in suspension cells (Fig. 3B) suggests that the S-oxygenating activity converting 5MTP to 5MSOP could be lacking in the suspension cells. In Arabidopsis T87 cells overexpressing Myb28, 5MTP was also detected (Hirai et al. 2007). In summary, the various layers of control of glucosinolate biosynthesis in suspension cells and hypocotyls appear different.

Low myrosinase activity may facilitate glucosinolate accumulation

Considering that myrosinases are mainly responsible for glucosinolate hydrolysis and turnover (Chen and Andreasson 2001, Petersen et al. 2002), it was of great interest to study the myrosinase activity and content in Arabidopsis suspension cells and hypocotyls. Using a very sensitive myrosinase activity assay (Chen and Halkier 1999), we could just barely detect glucosinolate degradation using enzyme extracts from suspension cells, while dramatic glucosinolate degradation could be observed with hypocotyl extracts in a short period of time. It is clear that suspension cells derived from hypocotyls contain low levels of myrosinase (Fig. 6). These results correlated with the abundance of glucosinolates in suspension cells. In intact Arabidopsis plants, myrosinase was localized to guard cells and phloem cells (Husebye et al. 2002), and was identified in vacuoles isolated from leaves (Carter et al. 2004). In suspension cells, there are no previous studies showing the levels of myrosinases, although myrosinase-associated proteins as well as glycosyl hydrolases have been identified in the vacuoles isolated from suspension cells (Shimaoka et al. 2004)

Under MeJA treatment, myrosinase activities in suspension cells increased, although no significant changes at the myrosinase protein level could be detected by Western blotting. Previous studies using other plant materials showed that MeJA stimulated glucosinolate production and myrosinase gene expression or activity (Taipalensuu et al. 1997, Wielanek and Urbanek 1999, Schenk et al. 2000). Our observation with suspension cells is consistent with these findings. However, in hypocotyls, the MeJA effect on myrosinase activity and content was the opposite (Fig. 6). The differences in glucosinolate metabolism between suspension cells and hypocotyls are significant. These findings highlight the fact that care needs to be taken when applying findings obtained using suspension cell systems to plant tissues or intact plants.

Materials and Methods

Chemicals and plant materials

Unless specified otherwise, all chemicals and reagents were obtained from Sigma-Aldrich, Fluka, Merck or Fisher Scientific. Seeds from A. thaliana ecotype Col-0 were obtained from the Arabidopsis Biological Resource Center. The seeds were sterilized using 50% bleach for 10 min, and thereafter by washing 4-5 times with sterilized water by vortexing for 1 min. Seeds were germinated on a half-strength Murashige-Skoog agar medium containing 1% sucrose and transferred to a growth chamber under a photosynthetic flux of 140 μmol photons m⁻² s⁻¹ with a photoperiod of 16 h at 24°C and 18°C at night for 10 d. Suspensions cells were obtained from callus generated from hypocotyls of the 10-day-old seedlings as previously described (Fein and Laugher 1989). They were subcultured for 12 generations (3 months) in these conditions before being used for the experiment. The established suspension cells were maintained in 50 ml of Murashige-Skoog liquid medium containing 3% (w/v) sucrose and 0.5 mg l⁻¹ 2,4-D, pH 5.7 in 250 ml flasks on a rotary shaker at 120 r.p.m., for 24°C in the dark. The cells were subcultured into fresh medium every 7 d.

MeJA treatment

When the Arabidopsis seedlings were 9 d old, MeJA was applied to the surface of the agar medium at a final concentration of 50 μM and incubated for 24 h. Hypocotyls were dissected, weighed and immediately frozen in liquid nitrogen. At each time point, the tissues were harvested and pooled. Three to four pools were generated as replicates. For suspension cells, MeJA was added directly to the medium to a final concentration of 50 μM. For sampling, the suspension cells were filtered using fine Miracloth, washed, blotted dry briefly on filter paper, weighed and frozen in liquid nitrogen. Three replicates from three independently generated cultures were harvested at each time point. For the control samples, all steps were the same except that MeJA was replaced with 0.004% (v/v) ethanol in water.

Glucosinolate extraction, HPLC and MS analysis

Glucosinolates from Arabidopsis suspension cells and different tissues were extracted and analyzed as desulfoglucosinolates as previously described (Petersen et al. 2000, Petersen et al. 2002, Chen et al. 2003). The individual glucosinolates were separated using HPLC (Promincence, Shimadzu, GA, USA) and identified by MS/MS system. Enhanced MS scan- and/or neutral loss scan-(m/z 162.2) directed MS/MS functions were used. Quantification was based on integrative peak areas and normalized against a known amount of an internal standard, deisoslated benzylglucosinolate, which was added at the start of the glucosinolate extraction procedure. The relative response factors of different glucosinolates were also taken into consideration (Petersen et al. 2000). Data presented are means ±SE of at least three replicates per sample.

Gene expression analysis by RT–PCR

Total RNA was extracted from Arabidopsis cells and 10-day-old hypocotyls using an RNaseasy Mini Kit following the instruc-
The PCRs were performed in a total volume of 50 μl of PCR buffer (Invitrogen) containing 200 μM dNTPs, 1.5 mM MgCl₂, 50 pmol of forward and reverse primers and 1 U of Platinum Taq DNA polymerase (Invitrogen). The PCR program was as follows: 2 min at 94°C, 23–50 cycles (transcript dependent as specified below) of 30 s at 94°C, 30 s at 53–57°C (transcript dependent as specified below) and 60 s at 72°C. The following primers (all listed from 5’ to 3’) and conditions were used: CYP79F1 forward, TTTTTAGACACCATCCTTGTTTCCCTTC; CYP79F1 reverse, AAAGCTCAATG-CTAGAAT (40 cycles, 53°C); CYP79F2 forward, AAAGCTCAATGCGTCGAAT; CYP79F2 reverse GCGTCGAAACACATCACAGAG (25 cycles, 53°C); CYP79B2 forward, AACCCCACTAATGAGC; CYP79B2 reverse, TCATAAAATATATAGGCCGTCG (25 cycles, 53°C); CYP79B3 forward, AAACACCCTATTAGGAACT; CYP79B3 reverse, TCCTCGCGTACGTCAC-GG (26 cycles, 54°C); PDF1.2 forward, TATGCTCAAG-CTTCTTTCT; PDF1.2 reverse AACACACAGTATTAGCACCC (29 cycles, 53°C); actin1 forward, TGGAACTGGAATGTAGAAGGCTG; and actin1 reverse, TCTCCAGGAIGTGACCAAT-ACCG (42 cycles, 57°C). Most primer sets were designed to be intron spanning, and with these primers no PCR products from genomic DNA were detected. All primers successfully amplified a band of the correct size as expected. An aliquot of each PCR (10 μl) was visualized by ethidium bromide staining and quantified on a Gel Doc Transilluminator (ImageQuant 300, GE Healthcare, Milwaukee, WI, USA). Actin-specific primers were used to ensure the correct size as expected. An aliquot of each PCR (10 μl) was analyzed by gel electrophoresis on 1% (w/v) agarose gels. Bands were visualized by ethidium bromide staining and quantified on a Gel Doc Transilluminator (ImageQuant 300, GE Healthcare, Milwaukee, WI, USA). Actin-specific primers were used to ensure that equal amounts of RNA were used, and reverse transcriptase reactions were equally efficient for all the samples. PDF1.2-specific primers were used as positive controls for MeJA-dependent induction. RNA extraction and RT-PCR were performed at least twice, and similar patterns of expression were obtained.

Myrosinase activity measurement and Western analysis

Frozen material was homogenized in extraction buffer composed of 50 mM Tris–HCl (pH 7.0), 0.15 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 20,000×g, 4°C for 20 min. The supernatant was used for the myrosinase activity assay and Western blotting. Myrosinase activity was analyzed as previously described (Chen and Halkier 1999) with minor modifications. Briefly, equal amounts of total protein extracts were incubated at 37°C in a 5 μl reaction mixture containing 50 mM Tris–HCl buffer (pH 7.0), 0.3 mM ascorbate and 5 mM [14C]-p-0HBB (Chen and Halkier 2000). Hydrolysis of the glucosinolate was analyzed by TLC (Chen and Halkier 1999, Petersen et al. 2002). Western analysis was carried out as described before (Chen and Halkier 1999) using a polyclonal anti-myrosinase TGG1 antibody at a 5,000-fold dilution. The antibody was generated in rabbit using a synthetic peptide (Ueda et al. 2006). Proteins were determined using a BioRad protein assay kit (Cat# 500-0002) with bovine serum albumin as a standard.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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References

Glucosinolates in suspension cells and hypocotyls


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