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ORIGINAL ARTICLE

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Composition and content of glucosinolates in developing *Arabidopsis thaliana*

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Abstract The glucosinolate composition and content in various tissues of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia during development from seeds to bolting plants were determined in detail by high-performance liquid chromatography. Comparison of the glucosinolate profiles of leaves, roots and stems from mature plants with those of green siliques and mature seeds indicated that a majority of the seed glucosinolates were synthesized de novo in the silique. A comparison of the glucosinolate profile of mature seeds with that of cotyledons indicated that a major part of seed glucosinolates was retained in the cotyledons. Turnover of glucosinolates was studied by germination of seeds containing radiolabelled *p*-hydroxybenzylglucosinolate (*p*-OHBG). Approximately 70% of the content of [^{14}C]*p*-OHBG in the seeds was detected in seedlings at the cotyledon stage and [^{14}C]*p*-OHBG was barely detectable in young plants with rosettes of six to eight leaves. The turnover of *p*-OHBG was found to coincide with the expression of the glucosinolate-degrading enzyme myrosinase, which was detectable at very low levels in seedlings at the cotyledon stage, but which dramatically increased in leaves from plants at later developmental stages. This indicates that there is a continuous turnover of glucosinolates during development and not only upon tissue disruption.

Keywords *Arabidopsis* (glucosinolate) · *Brassica* (glucosinolate) · Development · Glucosinolate composition · Myrosinase

Abbreviations 4-bzb: 4-benzoyloxybutylglucosinolate · 3-bzp: 3-benzoyloxypropylglucosinolate · 1-meoind: 1-methoxyindol-3-ylmethylglucosinolate · 4-meoind: 4-methoxyindol-3-ylmethylglucosinolate · 4-msb: 4-methylsulphinylbutylglucosinolate · 3-OHp: 3-hydroxypropylglucosinolate · 4-OHb:4-hydroxybutylglucosinolate · *p*-OHBG: *p*-hydroxybenzylglucosinolate

Introduction

Glucosinolates are nitrogen- and sulfur-containing natural plant products found mainly within the order Capparales (Rodman et al. 1998), which includes the model plant *Arabidopsis thaliana* and agriculturally important crop plants such as oilseed rape and *Brassica* vegetables. Upon tissue disruption, glucosinolates are hydrolyzed by endogenous β-thioglucosidases (myrosinases), yielding isothiocyanates, nitriles or other products via unstable thiohydroximate-O-sulphonates (Halkier 1999; Rask et al. 2000). The glucosinolate-myrosinase system is believed to have multiple functions in plant-pest interactions, providing not only feeding and ovipositioning stimulants but also compounds acting as toxins and feeding deterrents to generalist insect herbivores. In addition, glucosinolates are used as flavouring compounds and as cancer-prevention compounds (Verhoeven et al. 1997; Mithen et al. 2000).

Glucosinolates are derived from relatively few amino acids and from chain-elongated derivatives of methionine and phenylalanine (Fahey et al. 2001). In *A. thaliana*

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ecotype Columbia 24 glucosinolates derived from tryptophan, homophenylalanine and chain-elongated derivatives of methionine have been identified in mature seeds and leaves of bolting plants (Hogge et al. 1988; Agerbirk et al. 2001). Recently, several genes with functions in the biosynthesis of the core structure of glucosinolates (Hull et al. 2000; Mikkelsen et al. 2000; Wittstock and Halkier 2000; Bak et al. 2001; Hansen et al. 2001a, b; Reintanz et al. 2001), as well as genes with functions in the enzymatic steps responsible for side-chain elongation (Graser et al. 2000; Kliebenstein et al. 2001), have been identified. Less is known about the enzymatic steps responsible for secondary modifications of the side chain. According to the proposed biosynthetic pathway of aliphatic glucosinolates, glucosinolates with ω-methylthioalkyl side chains are first formed (Graser et al. 2000; Kliebenstein et al. 2001). Subsequently, the 'parent' glucosinolates can be subject to various secondary modifications of the side chain, such as oxidations, alkenylations, hydroxylations and acylations (Mithen et al. 2000). The latter yields complex glucosinolates with, for example, benzoyloxyalkyl side chains. Acylations are not limited to the side chain, as glucosinolates with the glucose moiety esterified with, for example, sinapoyl or *iso*-feruloyl have been reported (Sørensen 1990).

In previous studies, the composition and content of glucosinolates have been found to vary widely both between different species and between different developmental stages and tissues within a given species (Porter et al. 1991; Fieldsend and Mildford 1994; Li et al. 1999; Koroleva et al. 2000). The present study provides a detailed analysis of the composition and content of glucosinolates in different tissues of *A. thaliana* (ecotype Columbia) during the development from seed to bolting plants. In parallel to changes in glucosinolate profiles, expression of myrosinase was investigated and correlated with the turnover of seed-derived endogenous glucosinolates, as well as the radiolabelled non-endogenous glucosinolate *p*-hydroxybenzylglucosinolate (*p*-OHBG), upon germination.

Materials and methods

Plant material, definition of stages and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia; cat. No. WT-02-17, Lehle seeds, Round Rock, Tucson, Ariz., USA) were sown in humid peat (Enhets K-jord, Weibulls, Sweden), supplemented with 1 g l^{-1} Bactimos (Wettable Products, Abbott Laboratories, Chicago, Ill., USA) in 12×15 cm polystyrene trays, allowing water uptake from the bottom. The seeds were sown at densities of ca. 400 and 4 seeds per 100 cm^2 when destined for plants of stages 2 or 3 and 4 or 5, respectively. The trays were incubated in the dark for 2 days at ca. 4 °C, transferred into light for 2 h, and incubated in darkness for an additional 2 days at room temperature, after which the seedlings were transferred to an controlled-environment *Arabidopsis* Chamber (AR-60L; Percival, Boone, Iowa, USA), where they were subjected to a photosynthetic flux of 100–120 μmol photons m^{-2} s^{-1}, 70% relative humidity, and a photoperiod of 12 h, 20 °C. The plants were watered at intervals of 4 days, and were not at any point during the life cycle subjected to aphid attack or any other visible pests.

The life cycle was divided into five developmental stages, which were defined as follows: mature seeds (stage 1), seedlings at the cotyledon stage (stage 2), young plants of stage 3 (rosette with 6–8 leaves and a diameter of 13–15 mm), plants of stage 4 (rosette with 16–18 leaves and a diameter of ca. 35 mm), bolting plants of stage 5 (rosette with a diameter of ca. 50 mm). Plants of stages 2–5 were divided into cotyledons/leaves and roots. In the case of stage-5 plants, stems and green siliques were also included, and the length of the siliques (10–15 mm) was additionally used to define this stage. When collecting root tissue, the roots were carefully rinsed under cold tap water, instantly cut, frozen in liquid nitrogen and stored at –80 °C. For determination of glucosinolate content per plant unit, i.e. a single seed or one pair of cotyledons or one intact rosette (stage 3), 100 mature seeds, 100 cotyledons and 30 whole rosettes (stage 3) were weighed (fresh weight) and subsequently lyophilized for more than 48 h and the dry weight (DW) obtained. One seed, one pair of cotyledons and one intact rosette (stage 3) were calculated to weigh on average 15, 64 and 693 μg (DW), respectively, thus allowing determination of the absolute glucosinolate content in one plant unit.

Mature seeds of *Brassica napus* L. cv. Polo were obtained from Danisco Seed, Holeby, Denmark.

Analysis of glucosinolates by HPLC and LC-MS

The content and composition of glucosinolates were determined by HPLC analysis of the desulphoglucosinolates as previously described (Petersen et al. 2001), with the exceptions that the sample amount was 20 mg DW instead of 100 mg DW, and the procedure for desulphoglucosinolate preparation was scaled down accordingly. Benzylglucosinolate (Merck) or *p*-hydroxybenzylglucosinolate (*p*-OHBG; Bioraf, Åkirkeby, Denmark) was added as an internal standard at the start of the extraction procedure and samples without an internal standard were included on a regular basis. The HPLC was done on a Shimadzu LC-10AT*vp* equipped with a Supelcosil LC-ABZ 59142 RP-amid C_{16} column (25 cm×4.6 mm, 5 mm) from Supelco (Holm & Halby, Denmark) and an SPD-M10AVP (Shimadzu) diode array detector, flow rate 1 ml min^{-1}. The desulphoglucosinolates were eluted by the following gradient: H_2O (2 min), a linear gradient of 0–60% methanol (48 min), a linear gradient of 60–100% methanol (3 min), and 100% methanol (3 min). Analyses of the different plant tissues were done in triplicate, and identification and quantification of individual glucosinolates were done as previously described (Petersen et al. 2001). In addition, desulphoglucosinolate preparations, analyzed by HPLC as described above, were subjected to LC-MS analysis on a regular basis. LC-MS was done on an HP1100 LC coupled to a Bruker Esquire-LC ion-trap mass spectrometer (Bruker Daltonik, Germany). The reversed-phase LC conditions were as follows: A C_{18} column (Chrompack Inertsil 3 ODS-3 S15×3 COL CP 29126) was used. The mobile phases were A: water doped with sodium acetate (50 μM), B: methanol. The flow rate was 0.25 ml min^{-1} and the gradient program was: 0–2 min: isocratic 100% A; 2–40 min: linear gradient 0–60% B; 40–45 min: linear gradient 60–100% B; and 45–50 min: isocratic 100% B. The mass spectrometer was run in positive-ion mode. A 15-μl aliquot of each glucosinolate preparation was injected. The TICs (Total Ion Currents), RICs (Reconstructed Ion Chromatograms) and UV traces were used to locate peaks, and the $[M+Na]^+$ adduct ions in conjunction with diode array UV spectra were used for identifications.

Analysis of turnover of [14C]*p*-OHBG introduced into *A. thaliana* seeds

For introduction of the traceable non-endogenous glucosinolate [^{14}C]*p*-OHBG into the seeds of *A. thaliana*, three 40-μl aliquots of [^{14}C]*p*-OHBG (35 Bq μl^{-1}, 1.295×10^{10} Bq mol^{-1}), dissolved in 100 mM Tris-HCl (pH 7.0), were applied at 5-day intervals to an abraded rosette leaf on 28 intact *A. thaliana* plants at the early

bolting stage (growth conditions as described above). During the development of the inflorescence, fully mature siliques with the characteristic yellow color starting at the tip were harvested, left to dry, and the mature seeds obtained. The applied [^{14}C]*p*-OHBG was purified and quantified as described by Chen and Halkier (2000).

Turnover of [^{14}C]*p*-OHBG was studied by germinating seeds containing [^{14}C]*p*-OHBG (obtained as described above) in petri dishes containing 1× MS medium (Murashige and Skoog 1962), 2% sucrose and 0.8% agar, pH 5.6 (growth conditions as described above). Seeds (100 mg total weight) were grown to the cotyledon stage (stage 2) or to stage 3. Subsequently, all plant material was harvested and the total content of glucosinolates was obtained (as desulphoglucosinolates), as previously described (Petersen et al. 2001), and analyzed by TLC, LC-MS and HPLC. The MS-agar media, in which the stage-2 and -3 plants were grown, respectively, were collected, melted, and 1 ml of each was subjected to liquid scintillation counting. As a positive control, 100 mg of [^{14}C]*p*-OHBG-containing seeds were ground in 70% methanol and added to identical amounts of agar used for the growth of stage-2 or -3 plants. All experiments were done in triplicate.

Thin-layer chromatography

TLC was performed on Silica Gel 60 F_{254} sheets (Merck) using isopropanol/ethyl acetate/distilled H_2O (7:1:2, by vol.) as eluent. Radiolabelled bands were visualized and quantified by a STORM 840 phosphoimager (Molecular Dynamics, Sunnyvale, Calif., USA).

Western blot analysis and measurement of myrosinase activity

Total protein from ca. 0.2 g plant material (fresh weight) was extracted in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. SDS-PAGE and immunoblotting was done as described earlier (Petersen et al. 2001). A 10-μg sample of total protein was applied to each lane. Myrosinase activity was monitored semi-quantitatively as described earlier (Chen and Halkier 1999). Briefly, crude plant extract was passed through a mini Sephadex G25 column and 2 μg total protein was incubated at 30 °C in a 10-μl reaction mixture containing 50 mM citrate buffer (pH 4.5), 0.3 mM ascorbate and 37 Bq [^{14}C]*p*-OHBG (ca. 5 nmol). Hydrolysis of [^{14}C]*p*-OHBG was analyzed by TLC.

Results

Determination of glucosinolate content

We used HPLC analysis to determine the composition and content of glucosinolates in major tissues of *A. thaliana* ecotype Columbia during five stages of development from seed to bolting stage (see *Materials and methods*). All preparations of glucosinolates were analyzed as desulphoglucosinolates by HPLC. In addition, selected preparations from all tissues of the five developmental stages were analyzed by LC-MS. The ion-trace chromatograms and mass spectra were used to verify the identity of the assigned peaks in the HPLC chromatograms. With the exception of 2-phenylethylglucosinolate (Phee), which shifted place with indole-3-ylmethylglucosinolate, the order of the eluted desulphoglucosinolates was identical in the two systems (data not shown).

Composition and content of glucosinolates in vegetative tissues

Methylsulphinylalkyl glucosinolates constituted the major methionine-derived glucosinolates, 4-methylsulphinylbutylglucosinolate (4-msb) being the dominant aliphatic glucosinolate in stems, roots and leaves during stages 2–5 (Tables 1, 2, 3). In leaves of stage-5 plants, 4-msb accounted for 45% of the total glucosinolate content and 8-methylsulphinyloctyl- (8-mso) and

Table 1 Glucosinolate contents of cotyledons and rosette leaves of *Arabidopsis thaliana*, ecotype Columbia, at various stages of development. Glucosinolate content was determined in seedlings (stage 2), plants with 6–8 rosette leaves (stage 3), with 18–20 rosette leaves (stage 4) and bolting plants (stage 5). Mean values ± SD ($n = 3$). *n.d.* Not detected

Glucosinolate	Glucosinolate content (μmol g^{-1})			
	Cotyledons		Rosette leaves	
	Stage 2	Stage 3	Stage 4	Stage 5
3-Methylsulphinylpropyl-	0.04 ± 0.01	0.94 ± 0.09	0.75 ± 0.22	0.99 ± 0.22
3-Hydroxypropyl-	0.57 ± 0.13	0.40 ± 0.12	n.d.	n.d.
3-Benzoyloxypropyl-	0.77 ± 0.39	n.d.	n.d.	n.d.
4-Methylthiobutyl-	2.73 ± 0.46	2.02 ± 0.23	3.83 ± 0.75	1.04 ± 0.32
4-Methylsulphinylbutyl-	2.33 ± 0.91	6.54 ± 0.99	5.68 ± 0.66	8.88 ± 2.19
4-Hydroxybutyl-	1.57 ± 0.56	0.30 ± 0.08	n.d.	n.d.
4-Benzoyloxybutyl-	0.66 ± 0.23	n.d.	n.d.	n.d.
5-Methylthiopentyl-	0.20 ± 0.05	n.d.	0.07 ± 0.05	0.14 ± 0.04
5-Methylsulphinylpentyl-	0.30 ± 0.06	0.22 ± 0.03	0.64 ± 0.32	0.46 ± 0.17
6-Methylthiohexyl-	n.d.	n.d.	n.d.	n.d.
6-Methylsulphinylhexyl-	0.06 ± 0.04	0.03 ± 0.02	n.d.	0.04 ± 0.02
7-Methylthioheptyl-	0.21 ± 0.07	0.14 ± 0.11	0.05 ± 0.05	0.27 ± 0.11
7-Methylsulphinylheptyl-	0.43 ± 0.12	0.13 ± 0.02	0.04 ± 0.01	0.17 ± 0.05
8-Methylthiooctyl-	0.19 ± 0.03	0.21 ± 0.02	0.10 ± 0.09	0.05 ± 0.04
8-Methylsulphinyloctyl-	0.94 ± 0.26	0.82 ± 0.11	0.53 ± 0.21	1.17 ± 0.63
Indol-3-ylmethyl-	0.83 ± 0.51	2.95 ± 0.38	2.85 ± 1.01	3.31 ± 1.25
4-Hydroxy-indol-3-ylmethyl-	n.d.	0.01 ± 0.02	0.01 ± 0.02	n.d.
4-Methoxy-indol-3-ylmethyl-	n.d.	0.41 ± 0.03	1.47 ± 0.26	2.06 ± 0.38
1-Methoxy-indol-3-ylmethyl-	n.d.	0.57 ± 0.05	0.61 ± 0.62	0.21 ± 0.07
2-Phenylethyl-	0.01 ± 0.02	n.d.	0.02 ± 0.03	n.d.
Total	11.99 ± 2.44	15.86 ± 1.75	16.67 ± 2.25	19.40 ± 2.12

Table 2 Glucosinolate contents of roots of seedlings and plants of *A. thaliana* at various developmental stages (stages 2–5). Mean value ± SD ($n = 3$). *n.d.* Not detected

Glucosinolate	Root glucosinolate content (μmol g^{-1})			
	Stage 2	Stage 3	Stage 4	Stage 5
3-Methylsulphinylpropyl-	0.05 ± 0.02	0.08 ± 0.01	0.60 ± 0.23	1.06 ± 0.18
3-Hydroxypropyl-	n.d.	n.d.	n.d.	n.d.
3-Benzoyloxypropyl-	n.d.	n.d.	n.d.	n.d.
4-Methylthiobutyl-	0.09 ± 0.03	0.07 ± 0.02	0.03 ± 0.03	0.16 ± 0.06
4-Methylsulphinylbutyl-	0.20 ± 0.02	0.68 ± 0.16	3.20 ± 1.84	9.94 ± 1.32
4-Hydroxybutyl-	n.d.	n.d.	n.d.	n.d.
4-Benzoyloxybutyl-	n.d.	n.d.	n.d.	n.d.
5-Methylthiopentyl-	n.d.	n.d.	n.d.	0.07 ± 0.02
5-Methylsulphinylpentyl-	0.13 ± 0.04	0.03 ± 0.06	0.19 ± 0.03	0.38 ± 0.03
6-Methylthiohexyl-	n.d.	n.d.	0.07 ± 0.12	n.d.
6-Methylsulphinylhexyl-	0.05 ± 0.01	n.d.	0.19 ± 0.21	n.d.
7-Methylthioheptyl-	0.29 ± 0.09	0.03 ± 0.03	0.10 ± 0.12	0.12 ± 0.10
7-Methylsulphinylheptyl-	0.24 ± 0.05	0.02 ± 0.01	0.02 ± 0.01	0.16 ± 0.06
8-Methylthiooctyl-	0.98 ± 0.39	0.23 ± 0.01	0.02 ± 0.03	0.12 ± 0.16
8-Methylsulphinyloctyl-	1.29 ± 0.21	0.17 ± 0.02	0.16 ± 0.05	0.25 ± 0.07
Indol-3-ylmethyl-	1.43 ± 0.37	0.27 ± 0.06	0.43 ± 0.13	0.22 ± 0.07
4-Hydroxy-indol-3-ylmethyl-	n.d.	n.d.	n.d.	n.d.
4-Methoxy-indol-3-ylmethyl-	0.40 ± 0.08	0.17 ± 0.02	0.25 ± 0.05	0.82 ± 0.31
1-Methoxy-indol-3-ylmethyl-	1.04 ± 0.14	8.11 ± 1.16	9.52 ± 2.35	9.29 ± 2.69
2-Phenylethyl-	n.d.	n.d.	n.d.	n.d.
Total	6.19 ± 1.07	9.97 ± 1.08	14.97 ± 1.58	22.52 ± 1.72

Table 3 Glucosinolate contents in stems, siliques (stage 5) and mature seeds (stage 1) of *Arabidopsis thaliana*. Mean values ± SD ($n = 3$). *n.d.* Not detected

Glucosinolate	Glucosinolate content (μmol g^{-1})		
	Stem (stage 5)	Siliques (stage 5)	Seeds (stage 1)
3-Methysulphinylpropyl-	0.58 ± 0.03	0.82 ± 0.04	0.09 ± 0.01
3-Hydroxypropyl-	n.d.	0.63 ± 0.07	2.29 ± 0.16
3-Benzoyloxypropyl-	n.d.	0.61 ± 0.11	4.88 ± 0.24
4-Methylthiobutyl-	n.d.	4.07 ± 0.95	31.84 ± 2.03
4-Methylsulphinylbutyl-	3.90 ± 0.36	9.24 ± 0.52	3.77 ± 0.21
4-Hydroxybutyl-	0.01 ± 0.02	2.06 ± 0.21	6.73 ± 0.39
4-Benzoyloxybutyl-	n.d.	1.63 ± 0.45	7.51 ± 0.40
5-Methylthiopentyl-	n.d.	0.25 ± 0.02	3.02 ± 0.18
5-Methylsulphinylpentyl-	0.22 ± 0.02	0.59 ± 0.04	0.71 ± 0.04
6-Methylthiohexyl-	n.d.	n.d.	0.60 ± 0.45
6-Methylsulphinylhexyl-	n.d.	0.18 ± 0.03	0.30 ± 0.02
7-Methylthioheptyl-	n.d.	0.28 ± 0.24	4.21 ± 0.33
7-Methylsulphinylheptyl-	0.05 ± 0.01	0.97 ± 0.15	2.07 ± 0.45
8-Methylthiooctyl-	0.02	0.41 ± 0.11	6.47 ± 0.48
8-Methylsulphinyloctyl-	0.14 ± 0.03	3.22 ± 0.47	8.95 ± 0.48
Indol-3-ylmethyl-	0.50 ± 0.12	1.31 ± 0.12	2.56 ± 0.11
4-Hydroxy-indol-3-ylmethyl-	0.11 ± 0.05	0.23 ± 0.06	0.03 ± 0.06
4-Methoxy-indol-3-ylmethyl-	0.06 ± 0.01	0.01 ± 0.02	0.26 ± 0.36
1-Methoxy-indol-3-ylmethyl-	0.11 ± 0.07	n.d.	n.d.
2-Phenylethyl-	0.02 ± 0.02	0.08 ± 0.03	0.28 ± 0.01
Total	5.72 ± 0.68	26.61 ± 3.19	86.57 ± 3.66

3-methylsulphinylpropyl- (3-msp) glucosinolates accumulated as the second and third most abundant aliphatic glucosinolates, respectively (Table 1). 4-Methoxy-indol-3-ylmethylglucosinolate (4-meoind) accumulated in leaves and accounted for ca. 40% and 10% of the content of indole and total glucosinolates, respectively, in leaves of stage-5 plants (Table 2). In the roots of stage-5 plants, 1-meoind accounted for ca. 90% and 40% of the content of indole and total glucosinolates, respectively (Table 2, Fig. 1).

Composition and content of glucosinolates in floral tissues

In green siliques and mature seeds, four groups of glucosinolates accumulated in substantial amounts:

i. the silique-, seed- and cotyledon-specific hydroxylated and benzoyl acylated methylthioalkyl-derived glucosinolates, i.e. 3-hydroxypropyl- (3-OHp), 4-hydroxybutyl- (4-OHb), 3-benzoyloxypropyl- (3-bzp), and 4-benzoyloxybutylglucosinolate (4-bzb);

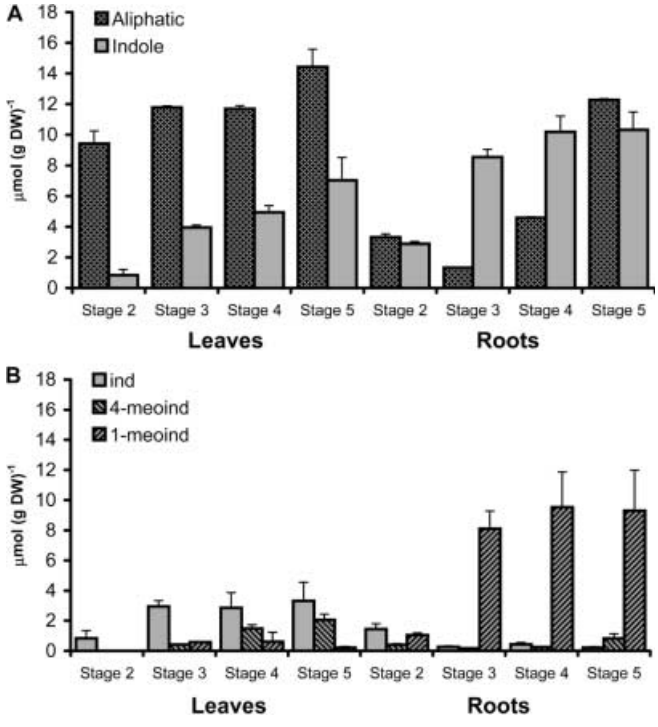

Fig. 1 **a** Total content of aliphatic and indole glucosinolates in *Arabidopsis thaliana*, ecotype Columbia, plants at stages 2–5. **b** Composition of indole glucosinolates in leaves and roots. Mean values ± SD ($n = 3$)

ii. the 'parent' methylthioalkyl glucosinolates, i.e. 4-methylthiobutyl-, 5-methylthiopentyl-, 6-methylthiohexyl-, 7-methylthioheptyl-, and 8-methylthiooctylglucosinolate;
iii. 8-methylsulphinyloctylglucosinolate;
iv. the 'parent' indole glucosinolate indol-3-ylmethylglucosinolate

(Fig. 2a–c; Tables 1, 3). These glucosinolates accounted for ca. 45% and over 90% of the total glucosinolate content in the green siliques and mature seeds, respectively. In contrast, these glucosinolates accounted for only 25%, 3% and 9% of the total glucosinolate content in leaves, roots and stems of stage 5, respectively. Comparison of the composition and content of glucosinolates in vegetative parts with those of siliques and mature seeds suggests that a least a substantial part of the seed glucosinolates is synthesized de novo in the silique.

Phenylalanine-derived glucosinolates

Only one phenylalanine-derived glucosinolate, namely 2-phenylethylglucosinolate (Phee), was identified. Phee was found in minute amounts in mature seeds, siliques, cotyledons and leaves of plants at stages 3–5. Phee was not detected in root or stem tissue.

Comparison of glucosinolate profiles of seeds, seedlings and young plants

The composition of glucosinolates in cotyledons correlated with the composition of glucosinolates in mature seeds, which again correlated with the composition of glucosinolates in the siliques (see above). The major glucosinolates, which accounted for over 90% of the total glucosinolate content in mature seeds accounted for ca. 75% of the total glucosinolate content in the cotyledons (Tables 1, 3). As an example, the hydroxylated (3-OHp, 4-OHb) and acylated (3-bzp, 4-bzb) glucosinolates accumulated during development of the silique, reached the highest concentration [µmol (g DW)$^{-1}$] in the seeds, and were found again in the expanding cotyledons (Fig. 2a–c; Tables 1, 3). The composition of similar glucosinolates in mature seeds (stage 1) and cotyledons (stage 2) indicated that seed glucosinolates were retained in the cotyledons. We determined the content of each glucosinolate in a single plant unit, defined as one seed (stage 1), one pair of cotyledons (stage 2) and one intact rosette (stage 3). When normalized to the glucosinolate content in one seed, net synthesis (fold increase > 1) was detected in cotyledons (stage 2) and in the rosette of stage-3 plants (Fig. 2d).

Turnover of [14C]*p*-OHBG during the early stages of development

Radiolabelled *p*-OHBG was introduced into seeds by application of [^{14}C]*p*-OHBG to the rosette leaves of flowering *A. thaliana* plants. The presence of intact [^{14}C]*p*-OHBG in the seeds was confirmed by LC-MS giving a peak at the expected retention time and a $[M + Na]^+$ ion at m/z 368 (the desulpho form) (Fig. 3a). In a parallel study, we have provided evidence that transport from leaves to seeds of *p*-OHBG, endogenously synthesized in the leaves of transgenic CYP79A1 *A. thaliana* plants (Bak et al. 1999), mimics the transport of endogenous glucosinolates (Chen et al. 2001). Pools of seeds (100 mg) containing [^{14}C]*p*-OHBG were germinated to the cotyledon stage (stage 2) or early rosette stage (stage 3) and the glucosinolate content (obtained as desulphoglucosinolates) of the total biomass was determined. The seed-specific endogenous glucosinolates 3-bzp and 4-bzb were not detectable in plants of stage 3 (data not shown). In agreement with this finding, TLC analysis showed that 70% of the content of [^{14}C]*p*-OHBG in the seeds was present in seedlings (stage 2) and barely detectable in plants of stage 3 (Fig. 3b). The absence of radioactivity in the growth medium (MS-agar) upon harvest of the stage-2 and -3 plants is consistent with the reduced amount of [^{14}C]*p*-OHBG being due to turnover, rather than diffusion into the medium (data not shown).

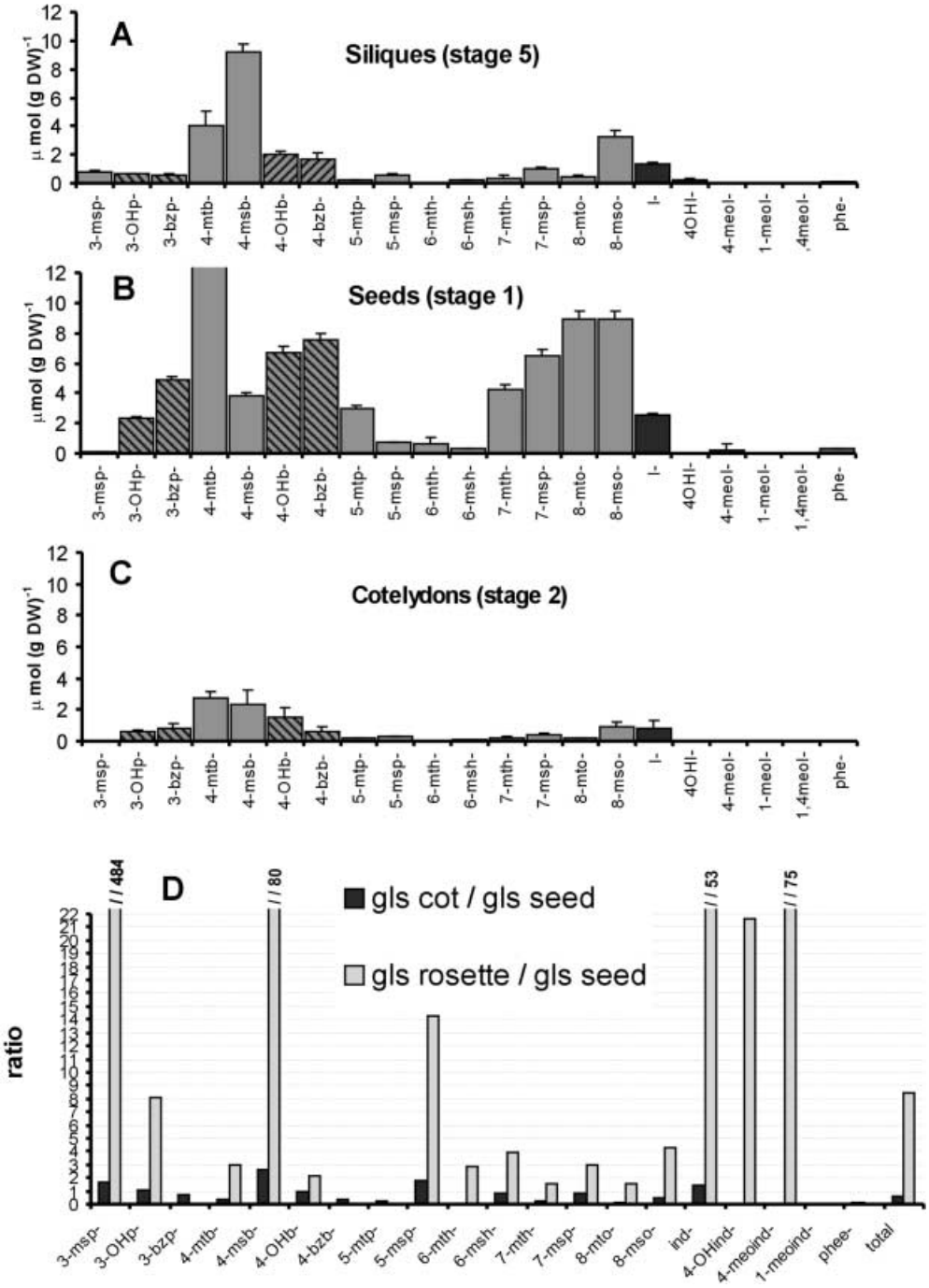

Fig. 2a–d Analysis of glucosinolates and net synthesis of glucosinolates during the early developmental stages of *A. thaliana*. Glucosinolate contents of siliques (**a**), seeds (**b**) and cotyledons (**c**). *Hatched bars* represent glucosinolates (3-OHp, 3-bzp, 4-OHb, 4-bzb) that accumulate to high amounts in seeds. Mean values ± SD ($n = 3$.). **d** The ratio of individual glucosinolates between one pair of cotyledons and one seed (*gls cot*/*gls seed*) and between one rosette (stage 3) and one seed (*gls rosette*/*gls seed*). Abscissa values > 1 indicates net synthesis, i.e. accumulation of the particular glucosinolate

Western blot analysis and measurements of myrosinase activity

When the monoclonal anti-myrosinase antibody 3D7 (Lenman et al. 1990) was used as primary antibody in Western blot analysis, a large increase in myrosinase protein was observed in leaves from *A. thaliana* plants of later developmental stages (stages 3–5; Fig. 4a). In contrast, myrosinase protein could not be detected in mature seeds and was barely detectable in seedlings at the cotyledon stage. Using [^{14}C]*p*-OHBG as substrate, 'potential' myrosinase activity, measured at an ascorbate concentration of 0.3 mM, was found to follow a similar pattern. In mature seeds, a moderate activity (Fig. 5), which was dependent on the presence of ascorbate and which could be irreversibly abolished by boiling, was detected. This suggests that myrosinase protein was present in amounts below the detection limit of the Western blot analysis.

Discussion

The present study provides a detailed analysis of the composition and content of glucosinolates in *A. thaliana*

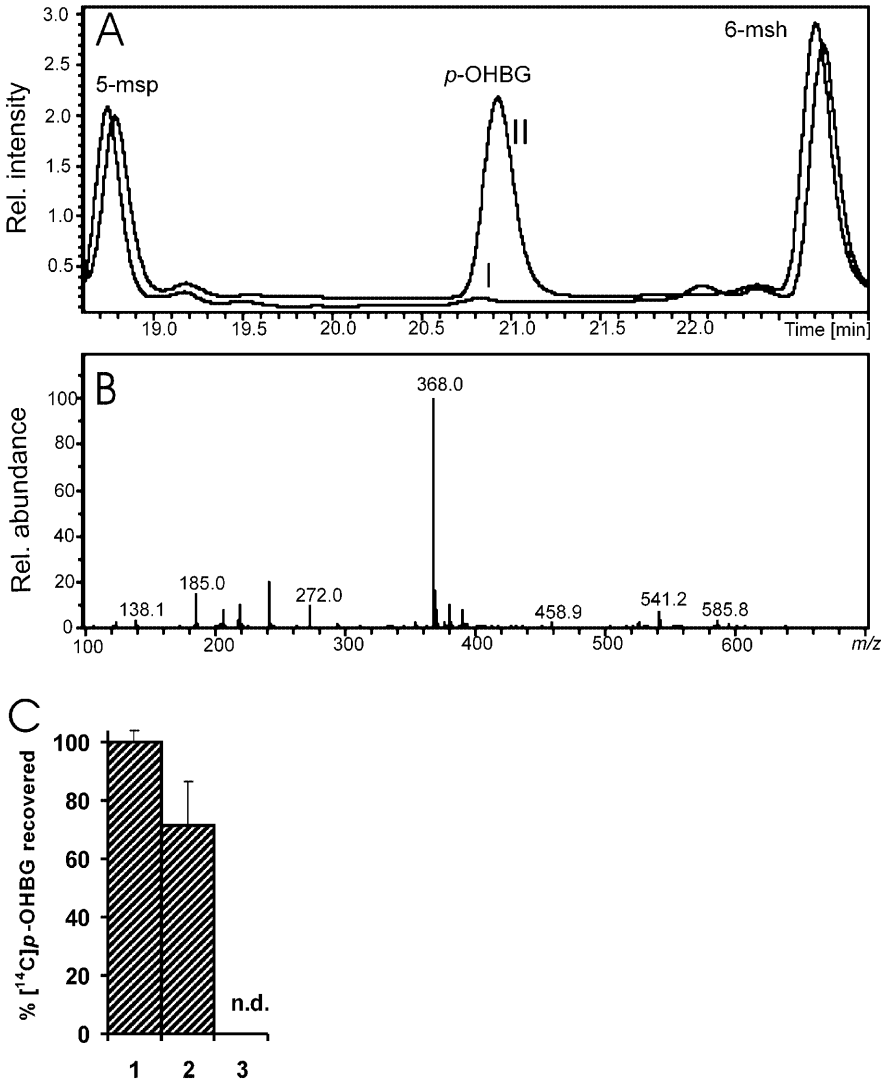

Fig. 3a–c Turnover of [^{14}C]*p*-OHBG in seeds of *A. thaliana* upon germination. **a** UV-trace (229 nm) from LC-MS analysis of wild-type seeds (*I*) and seeds containing exogenously introduced radiolabelled *p*-OHBG (*II*). **b** Mass spectrum of the peak at 20.9 min showing the expected $[M+Na]^+$ ion at *m/z* 368 corresponding to [^{14}C]*p*-OHBG (desulpho form). **c** TLC analysis of [^{14}C]*p*-OHBG in seeds (stage 1) (100%), seedlings of the cotyledon stage (stage 2) and young plants (stage 3). Glucosinolate content, including [^{14}C]*p*-OHBG, was determined as desulphoglucosinolates. Mean values ± SD ($n=3$). *n.d.* Not detected

during development from seeds to bolting plants. In parallel to changes in glucosinolate profiles, expression of myrosinase was monitored and correlated with the turnover of endogenous and exogenous glucosinolates in mature seeds upon germination.

The indole glucosinolates 4-meoind and 1-meoind accumulated in leaves and roots of plants of stages 3–5 accounting for ca. 40% and 85%, respectively, of the total content of indole glucosinolates at stage 5 (Tables 2, 3; Fig. 1). In these tissues, hydroxylated indole glucosinolates were either undetectable (roots) or barely detectable (leaves). In stems and siliques, however, 4-hydroxyindol-3-ylmethylglucosinolate was as prominent as, or more prominent than, the methoxylated derivative (Table 3). Variations in the amount of the hydroxylated and methoxylated forms could indicate that hydroxylation and methylation are not tightly coupled.

The vegetative tissues and stem tissue contained predominantly glucosinolates with secondary modifications (e.g. methylsulphinylalkyl- and methoxylated indolyl- glucosinolates), while siliques and mature seeds contained predominantly high amounts of 'parent' glucosinolates (e.g. methylthioalkylglucosinolates and indol-3-yl-methylglucosinolate; Tables 1, 2, 3). This difference indicates that a major part of the glucosinolates in the seeds is synthesized in the siliques. It has been shown that both intact glucosinolates and desulphoglucosinolates possess the physico-chemical properties allowing phloem-mediated transport (Brudenell et al. 1999). Furthermore, the presence of intact glucosinolates in the phloem has recently been demonstrated, indicating intact glucosinolates as the transport form (Chen et al. 2001). In developing seedlings of *B. napus*, however, the recovery of radiolabelled 2-hydroxy-3-butenylglucosinolate and 3-butenylglucosinolate, upon administration of radiolabelled *β*-D-glucopyranosyl-4-pentenethiohydroxamic acid, was found to be appreciably higher than the recovery of the intact administered radiolabelled glucosinolates (Rossiter and James 1990; Rossiter et al. 1990; Thangstad et al. 2001), suggesting that glucosinolates might be transported as desulphoglucosinolates.

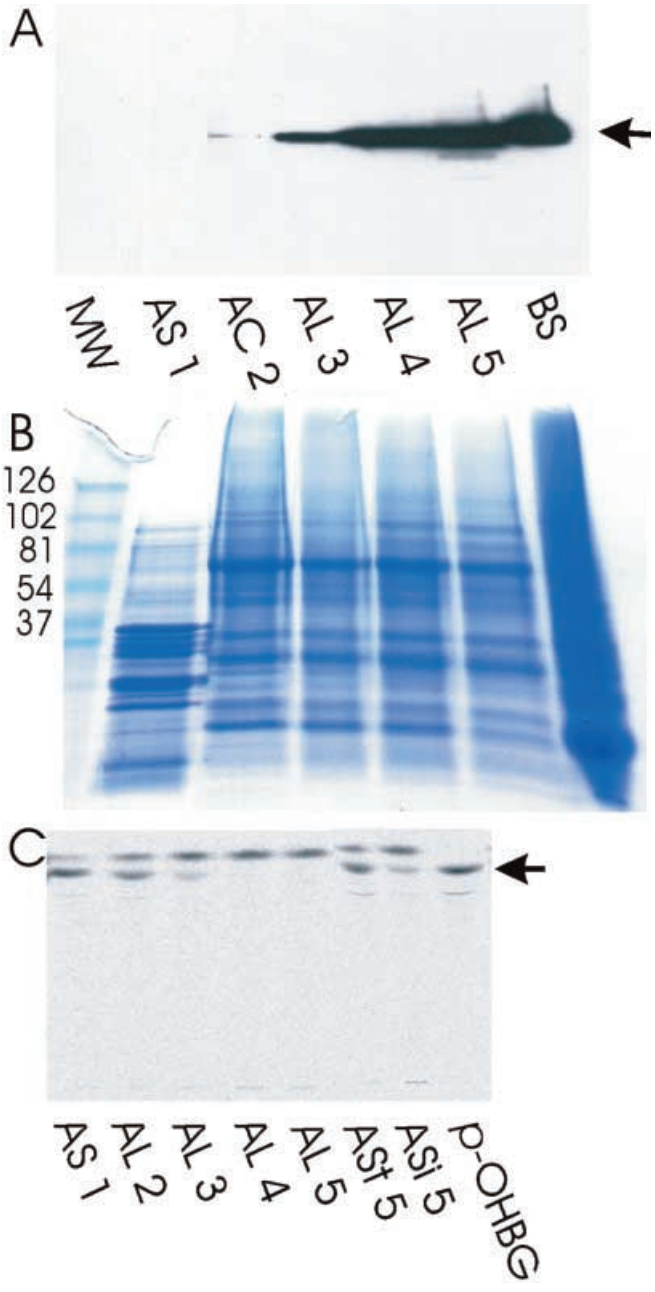

Fig. 4a–c Analysis of myrosinase levels in various tissues of *A. thaliana* and in seeds of *Brassica napus*. **a** Western blot analysis of myrosinase content in seeds (*AS*), cotyledons (*AC*) and leaves of stages 3–5 (*AL*) of *A. thaliana* and in seeds of *B. napus* (*BS*). 10 µg of total protein was applied in each lane. **b** Coomassie brilliant blue-stained SDS-PAGE gel identical to the one blotted. **c** TLC analysis of myrosinase activity measurements in seeds (*AS*), cotyledons (*AC*), leaves (*AL*), stems (*ASt*), and siliques (*ASi*) of *A. thaliana*. Myrosinase activity was measured as the amount of hydrolyzed [^{14}C]*p*-OHBG after 15 min incubation. The *arrow* depicts the position of authentic [^{14}C]*p*-OHBG

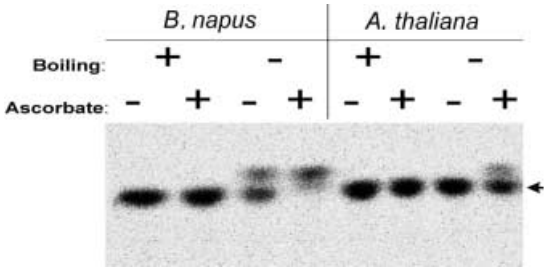

Fig. 5 Ascorbate dependent myrosinase activity in seeds of *A. thaliana* and *B. napus*. Myrosinase activity was measured as the amount of hydrolyzed [^{14}C]*p*-OHBG after 20 min incubation in the presence (+) or absence (–) of 0.3 mM ascorbate. The *arrow* depicts the position of authentic [^{14}C]*p*-OHBG

The similar glucosinolate profiles in mature seeds and cotyledons indicate that seed-derived glucosinolates to some extent are retained in the expanding cotyledons. At the end of the cotyledon stage (stage 2) or very early thereafter a net synthesis of glucosinolates (as determined on a per-unit basis) is evident.

Several studies have suggested that glucosinolates are turned over in vivo (Cole 1978, 1980; Svanem et al. 1997). For example, diurnal variation in glucosinolate content of leaves of cabbage indicates continuous degradation as well as de novo synthesis of glucosinolates (Rosa 1997). In developing seedlings of single low varieties of *B. napus* the content of aliphatic glucosinolates was found to diminish markedly (McGregor 1988). However, it is not clear whether glucosinolates are turned over during the germination processes or merely diluted as a consequence of growth (e.g. McGregor 1988; Clossais-Besnard and Larher 1991). In watercress, feeding of radiolabelled desulphophenylethylglucosinolate, resulted in the formation of substantial levels of the corresponding intact glucosinolate which was accompanied by a low but measurable rate of turnover (identified as the corresponding isothiocyanate) (Svanem et al. 1997). In *A. thaliana*, the seed-derived non-endogenous radiolabelled glucosinolate, *p*-OHBG, as well as at least some seed-derived endogenous glucosinolates, seemed to be turned over at a low rate during germination of the seeds and probably at a higher rate thereafter. The low turnover from seeds to the cotyledon stage (stage 2) is consistent with the low turnover of radiolabelled 2-hydroxy-3-butenyl- and 3-butenylglucosinolate in germinating seedlings of *B. napus* (Rossiter and James 1990). The turnover coincided with the expression of myrosinase, which at the protein level was barely detectable (a single weak but distinct band) in cotyledons at the seedling stage, but which dramatically increased both in amount and activity in leaves of young rosette plants and of subsequent developmental stages (stages 3–5; Fig. 4b). In *B. napus*, a cotyledon-specific myrosinase isoform (myrosinase II), not present in dry seeds, was found together with an isoform (myrosinase I), present throughout the seedling during early seedling growth (James and Rossiter 1991). Myrosinase-mediated degradation of glucosinolates results in the release of glucose and sulphate, which have been suggested to be of nutritional value for developing seedlings (James and Rossiter 1991), and additionally an array of more or less

toxic compounds with various biological effects (Chew 1988). Whereas myrosinase protein was undetectable and myrosinase activity was low in the seeds of *A. thaliana*, myrosinase protein and activity levels were high in seeds of *B. napus* (Figs. 4, 5), a finding which is in agreement with comparative immunocalization studies in *A. thaliana* and *B. napus* (Andreasson 2000). The apparent differences in the distribution of myrosinase between the two species might reflect that *A. thaliana* and *B. napus* are high and low seed producers, respectively, i.e. that the high number of seeds of *A. thaliana* is obtained at the cost of, for example, resistance.

It is puzzling how the plant controls turnover of glucosinolates in a physiologically safe manner. *Arabidopsis thaliana* myrosinase has been shown to be located in idioblastic structures in the parenchymal cells of the phloem (Andreasson 2000) whereas myrosinase in *B. napus* and *B. juncae* is also found in idioblasts in the ground tissue (Thangstad et al. 1990; Xue et al. 1992; Kelly et al. 1998; Andreasson 2000). Accordingly, in order to come into contact, either glucosinolates or myrosinases have to be transported, or contact might be obtained upon tissue damage during germination. Further studies are needed to elucidate where glucosinolates and myrosinases are compartmentalized at the cellular and subcellular level and how the delicate balance between de novo synthesis and degradation of glucosinolates is maintained.

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