

Characterization of transgenic *Arabidopsis thaliana* with metabolically engineered high levels of *p*-hydroxybenzylglucosinolate

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Abstract. The cytochrome P450 CYP79A1 catalyzes the conversion of L-tyrosine to *p*-hydroxyphenylacetaldoxime, the first step in the biosynthetic pathway of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. We have demonstrated that introduction of CYP79A1 into *Arabidopsis thaliana* (L.) Heynh. results in the production of the tyrosine-derived glucosinolate *p*-hydroxybenzylglucosinolate (*p*-OHBG), not found in wild-type *A. thaliana* (Bak et al., 1999, Plant J. 20: 663–671). In the present study, glucosinolate profiles and contents in various tissues (roots, leaves, stems, closed flower buds and green siliques) of *A. thaliana* plants expressing CYP79A1 were analyzed by high-performance liquid chromatography. The total glucosinolate content in these tissues was increased 3.5- to 4.5-fold in comparison with the level of the control plants. The increase was due solely to the production of *p*-OHBG, as the composition of the major endogenous aliphatic and indole glucosinolates was not affected. Conversely, in mature seeds the total glucosinolate content of CYP79A1 and control plants was similar, with *p*-OHBG accounting for ca. 30%. The transcript level of the post-oxime enzyme UDP-glucose:thiohydroximate glucosyltransferase in leaves of CYP79A1 plants was increased ca. 50% compared with control plants, indicating that the post-oxime enzymes in the biosynthetic pathway are up-regulated. Western blot analysis and activity measurements showed similar amounts and activities of myrosinase in CYP79A1 and control plants. Thus, the increase in glucosinolate content in CYP79A1 plants was not accompanied by an increase in content or activity of degradation enzyme. The present data dem-

onstrate that the high biosynthetic capacity of the post-oxime enzymes combined with a low substrate-specificity of the post-oxime enzymes in *A. thaliana* provide a highly flexible system for metabolic engineering of glucosinolate profiles, including new (non-endogenous) glucosinolates derived from oximes introduced into the plant, e.g. by transformation with CYP79 homologues.

Key words: *Arabidopsis* (glucosinolate) – Cytochrome P450 – Glucosinolate – Metabolic engineering – Myrosinase – Transgenic *Arabidopsis*

Introduction

Glucosinolates constitute a group of natural plant products found within the order Capparales (Rodman 1998), which includes agricultural important crop plants, such as oilseed rape and *Brassica* vegetables, and the model plant *Arabidopsis thaliana* (L.) Heynh. Upon tissue disruption, glucosinolates are hydrolyzed by endogenous β -thioglucohydrolases (myrosinases), yielding isothiocyanates, nitriles or other products via unstable thiohydroximate-O-sulfonates (Chew 1988; Agerbirk et al. 1998; Halkier 1999). In planta, glucosinolates and their hydrolysis products have a wide range of biological activities, such as inhibition of microbial growth (Mari et al. 1993; Manici et al. 1997), intermediacy in the biosynthesis of indole phytoalexins (Pedras et al. 2000), stimulation of oviposition and feeding by specialist insects (Stadler 1978; Nielsen 1988), as well as possible deterrent effects on non-crucifer specialist insects and generalist herbivores (e.g. Lazzeri et al. 1993). Also, in food and feed, anti-carcinogenic (Zhang et al. 1992; Fahey et al. 1997; Faulkner et al. 1998) and anti-nutritional (Fenwick et al. 1983) activities, respectively, have attracted attention.

More than 100 different glucosinolates have been identified to date (Sørensen 1990). Glucosinolates are derived from relatively few protein amino acids and their

Abbreviations: 3-msp = 3-methylsulphinylpropylglucosinolate; ind = indol-3-ylmethylglucosinolate; 4-OHind = 4-hydroxyindol-3-ylmethylglucosinolate; 4-meoind = 4-methoxyindol-3-ylmethylglucosinolate; 1-meoind = 1-methoxyindol-3-ylmethylglucosinolate; *p*-OHBG = *p*-hydroxybenzylglucosinolate; S-GT = UDP-glucose:thiohydroximate glucosyltransferase

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chain-elongated homologues (Chew 1988; Halkier 1999). In *A. thaliana*, 23 glucosinolates, derived from tryptophan and chain-elongated homologues of methionine and phenylalanine, have been identified (Hogge et al. 1988). The high chemical diversity is obtained by chain-elongation of precursor amino acids and by secondary side chain modifications such as, for example, hydroxylations and desaturations (Halkier 1999). Glucosinolates are related to cyanogenic glucosides by being derived from amino acids and having oximes as intermediates. In sorghum, the cytochrome P450 enzyme CYP79A1 catalyses the conversion of L-tyrosine to *p*-hydroxyphenylacetaldoxime as part of the biosynthesis of the cyanogenic glucoside dhurrin (Halkier et al. 1995; Koch et al. 1995). Recently, we reported that expression of CYP79A1 in *A. thaliana* yielded high levels of the glucosinolate *p*-hydroxybenzylglucosinolate (*p*-OHBG) (Bak et al. 1999), not previously found in *A. thaliana* (Hogge et al. 1988). In the transgenic plants, tyrosine was converted into *p*-hydroxyphenylacetaldoxime, which was efficiently channelled into the biosynthetic pathway of glucosinolates.

In the present study, we have determined the content of *p*-OHBG and the major endogenous glucosinolates in transgenic *A. thaliana* plants expressing CYP79A1 and found a significant accumulation of *p*-OHBG in all plant tissues investigated. Compared with control plants, concentrations of endogenous glucosinolates were either lowered or unchanged, depending on the tissue. In the seeds, the concentrations of endogenous glucosinolates were lowered proportionally to the concentration of *p*-OHBG. In all other tissues the significant accumulation of *p*-OHBG, which yielded a 3- to 4-fold increase in total glucosinolate content, still allowed unchanged accumulation of endogenous glucosinolates. In addition, we analyzed the effect of elevated glucosinolate content on the expression of an *A. thaliana* homologue of the glucosinolate biosynthetic UDP-glucose:thiohydroximate glucosyltransferase (S-GT) and on the glucosinolate-hydrolyzing enzyme myrosinase.

Materials and methods

Plant material and growth conditions

The transgenic CYP79A1 plants were made in the *A. thaliana* (L.) Heynh. ecotype Columbia (Cat. No. WT-02-17; Lehle seeds, Round Rock, Tex., USA) background, as described earlier (Bak et al. 1999). Briefly, the CYP79A1 gene from *Sorghum bicolor* (L.) Moench, under control of the cauliflower mosaic virus 35S (CaMV35S) promoter, was cloned into the pPZP111 vector (Hajdukiewicz et al. 1994), and then introduced by *Agrobacterium*-mediated transformation into *A. thaliana*. The control plants, designated 111, were transformed with an empty pPZP111 vector.

The plants were grown in peat for ca. 8 weeks in a controlled environment *Arabidopsis* Chamber (AR-60L; Percival, Boone, Iowa, USA) with a photoperiod of 8 h at a photosynthetic flux of 100–120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 20 °C, and 70% relative humidity. The short photoperiod was applied in order to suppress induction of flowering and thereby maximize the size of the rosette leaves. The onset of flowering was induced by changing the photoperiod to 12 h. Fully matured rosette leaves (ca. $3 \times 1 \text{ cm}^2$), roots, stems, closed flower buds and mature green siliques (ca. 1.5–

2 cm long) were harvested, frozen immediately in liquid nitrogen and stored at –80 °C. Segregation analysis of seeds of primary transformant plants on MS medium (Murashige and Skoog 1962) supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin, 2% sucrose, and 0.9% agar indicated one insertion for the CYP79A1 and control line used in this study. All plants were homozygous for the transgene. Mature seeds of *Brassica napus* L. cv. Polo were obtained from Danisco Seed, Holeby, Denmark.

Analysis of desulfoglucosinolates by HPLC

Tissue samples for HPLC analysis were freeze-dried for at least 48 h. Desulfoglucosinolates were obtained essentially as described in Minchinton et al. (1982) and analyzed as described in Sørensen (1990). Briefly, 100 mg DW plant material was extracted in $3 \times 3.5 \text{ ml}$ 70% boiling methanol-water (v/v). Benzyl-glucosinolate (Merck) was added as internal standard at the start of the extraction procedure. The crude extract was applied to 0.1 g DW of DEAE-Sephadex A25 (Pharmacia) equilibrated in 0.5 M sodium acetate (pH 5.0), and washed with H₂O. Desulfoglucosinolates were obtained by overnight treatment at room temperature with 2.5 mg ml^{-1} *Helix pomatia* sulphatase (EC 3.1.6.1; Sigma), dissolved in 0.02 M acetic acid (pH 5.0). Desulfoglucosinolates were eluted in 6 ml water and concentrated approximately 10-fold in vacuo. An aliquot of 100 μl concentrated eluate was injected into a Shimadzu LC-10ATvp equipped with a Supelcosil LC-ABZ 59142 C₁₈ column (25 cm \times 4.6 mm, 5 mm) from Supelco and a SPD-M10AVP (Shimadzu) diode array detector, flow rate 1 ml min^{-1} . The desulfoglucosinolates were eluted by the following gradient: H₂O (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). The elution volume from the DEAE column and pH of the sulfatase buffer were optimized to include all indolyl glucosinolates, which even on analytical-scale columns elute after the aliphatic glucosinolates with almost baseline separation between aliphatic glucosinolates and the last-eluting indolyl glucosinolate 4-hydroxyindol-3-ylmethylglucosinolate (4-OHind; Buchner 1987a; data not shown).

The individual glucosinolates were identified by (i) comparison to and spiking with authentic standard desulfoglucosinolates of *Barbarea* or *Brassica* species (3-methylsulphinylpropylglucosinolate (3-msp), methylsulphinylbutylglucosinolate (4-msb), indol-3-ylmethylglucosinolate (ind), 4-OHind, 4-methoxyindol-3-ylmethylglucosinolate (4-meoind), 1-methoxyindol-3-ylmethylglucosinolate (1-meoind), phenylethylglucosinolate; (ii) comparison to elution profiles of previously published chromatograms (Truscott et al. 1983; Hogge et al. 1988; Haughn et al. 1991; Magrath et al. 1994; Mithen et al. 1995); and (iii) on-column UV-diode array spectra recorded from 190 nm to 370 nm. The reference compounds were characterized by nuclear magnetic resonance analysis (data not shown). Quantification was obtained relative to the internal standard, using published relative response factors at 229 nm (Buchner 1987b; Sørensen 1990) and 225 nm (Haughn et al. 1991). Desulfophenylethylglucosinolate was assumed to have the same response factor as desulfobenzylglucosinolate (Buchner 1987b).

In roots, leaves, stems, closed flower buds and green siliques of the control plants, the major endogenous aliphatic glucosinolates 3-msp, 4-methylthiobutylglucosinolate (4-mtb), 4-msp, 8-methylsulphinylbutylglucosinolate (8-mso), and the endogenous indolyl glucosinolates (ind, 4-OHind, 4-meoind and 1-meoind), were estimated to account for more than 90% of the total glucosinolate content, when a response factor of 1 (as for allylglucosinolate) for minor unidentified peaks in the chromatograms was used. Using this criterion, the major glucosinolates in the seeds of the control plants were estimated to account for more than 90% of the total glucosinolate content, when the additional glucosinolates 3-hydroxypropylglucosinolate (3-OHp), 3-benzoyloxypropylglucosinolate (3-bzp), 4-hydroxybutylglucosinolate (4-OHb), 4-benzoyloxybutylglucosinolate (4-bzb), 7-methylthioheptylglucosinolate (7-mth) and 8-methylthiooctylglucosinolate

(8-mto) were included. The purity of commercial benzylglucosinolate used as internal standard was found to be 90% by UV ($\epsilon_{227.5} = 8,870 \text{ M}^{-1} \text{ cm}^{-2}$; Thies 1988). This was considered in the calculation of absolute glucosinolate concentrations. Analysis of each plant tissue was done in triplicate using different plants.

Northern blot analysis

A 725-bp fragment, corresponding to the second exon of the S-GT gene (EMBL accession number AC002396, *A. thaliana* I BAC F316) and a 1,585-bp fragment of the 25S rRNA gene (EMBL accession number X52530) were amplified by polymerase chain reaction (PCR) from 2 μg of *A. thaliana* ecotype Columbia genomic and cDNA, respectively.

Primers used in the PCR reactions were (P1-S-GT) 5'-GAT-TGTGAAAATGGTGAGTCTGAT-3', (P2-S-GT) 5'-TTACTTC-CCTAAACTCTATAAAC-3', (P1-25S) 5'-GAGGAAACTCTG-TGGGAAGCCCGCAGC-3', and (P2-25S) 5'-TCATTTCA-CAAAGTCGGACTAGAGTCA-3'. The PCR reactions were performed in 100 μl containing 200 M dNTPs, 100 pmol of each of the primers (P1 and P2), 5% dimethylsulfoxide, 2.5 units Taq polymerase (Pharmacia) in 1 \times PCR buffer [50 KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl_2 , 0.1% Triton X-100]. The cycle parameters were 97 $^\circ\text{C}$ for 3 min, addition of the primer-set, followed by 30 cycles of 95 $^\circ\text{C}$ for 1 min 30 s, 60 $^\circ\text{C}$ for 1 min, 70 $^\circ\text{C}$ for 2 min. The S-GT fragment was isolated, blunt-ended with Klenow enzyme (New England Biolabs), phosphorylated by T4-poly nucleotide kinase (New England Biolabs) and ligated into the *Sma*I site of pBluescriptSK⁻ (Stratagene). The 25S rRNA gene was cloned directly into pCR2.1-TOPO using the TOPO TA Cloning kit (Invitrogen, Groningen, The Netherlands). The identity of the cloned fragments was verified by sequencing and the inserts were used to label the probes.

Total RNA was extracted from 0.5 g FW of fully matured rosette leaves by use of Trizol Reagent (Gibco BRL). An aliquot of 25 g of total RNA was separated on formaldehyde agarose gels, blotted onto a Hybond-N (Amersham) filter and UV-crosslinked at 0.6 J cm^{-2} . Approximately 50 ng of the 750-bp S-GT fragment and the 1,585-bp 25S rRNA gene were labelled with α -[^{32}P]dCTP using the Megaprime labelling kit (Amersham). High-stringency washes were performed at 68 $^\circ\text{C}$ with 0.1 \times SSPE (1 \times SSPE: 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA) and 0.1% SDS in the final wash. The filter was first probed with the S-GT probe, stripped accordingly to Sambrook et al. (1989) and re-probed with the 25S rRNA probe. Autoradiography was visualized and quantified on a STORM 840 phosphorimager (Molecular Dynamics).

Protein extraction and myrosinase activity assay

Two fully mature rosette leaves were extracted in 0.5 ml isolation buffer [50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride] for 1 h, and spun at 10,000 g for 5 min. Glucosinolates and other components affecting the assay were removed by passing the supernatant through a 10-ml Sephadex G-25 column (Pharmacia) and the protein fraction was eluted in isolation buffer. Myrosinase activity was determined by monitoring the glucose-release after allylglucosinolate hydrolysis at 550 nm using a GOD-PAP kit (Randox, Cruclin, UK). The assay was carried out in 50 mM citrate buffer (pH 4.5) and 0.3 mM ascorbate, essentially as described by Bones and Slupphaug (1989). Total protein concentration was determined by the Bradford method using bovine serum albumin (BioRad) as standard.

Western blot analysis

For Western blot analysis, leaf extracts corresponding to 10 g of protein were separated on SDS polyacrylamide gels (8–25%) and blotted onto nitrocellulose paper. Myrosinase was detected by the monoclonal antibody 3D7 (Lenman et al. 1990) and visualized by

immuno-chemiluminescence as previously described (Chen and Halkier 1999), except that the developing reagent was from Pierce (New York, N.Y., USA). Equal loading was confirmed by visual inspection of Coomassie blue-stained SDS polyacrylamide gels run in parallel.

Results

Total glucosinolate content in various tissues

We have used HPLC analysis to determine the glucosinolate composition and content in transgenic *A. thaliana* plants expressing CYP79A1. The analyses were done on plants at the time of bolting. In CYP79A1 plants, the metabolically engineered production of *p*-OHBG resulted in a total glucosinolate content in roots, leaves, stems, closed flower buds and green siliques of (mean \pm SD) 46 \pm 4, 56 \pm 12, 41 \pm 4, 33 \pm 3, 49 \pm 3 μmol glucosinolate (g DW)⁻¹, respectively. This corresponded to a 3.5- to 4.5-fold increase in the total glucosinolate content, which was solely attributable to the accumulation of *p*-OHBG as the level of the major endogenous glucosinolates was unaffected by the production of *p*-OHBG (Fig. 1).

In mature seeds of CYP79A1 and control plants, the total glucosinolate content was 81 \pm 4 and 75 \pm 10 μmol (g DW)⁻¹, respectively (Fig. 1). The similar level of total glucosinolate content in the seeds is in contrast to the pattern seen in the other tissues. Whereas the amount of *p*-OHBG [29 \pm 8 μmol (g DW)⁻¹] in the seed corresponded to ca. 30% of the total glucosinolate content, the amount of *p*-OHBG in the other tissues corresponded to ca. 75% of the total glucosinolate

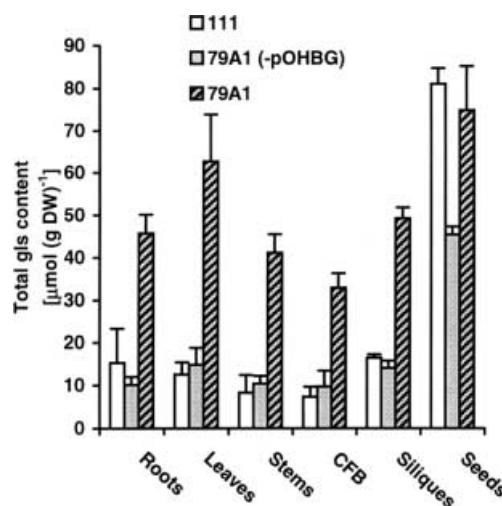


Fig. 1. Total glucosinolate content in various tissues. Methanol extracts of roots, leaves, stems, closed flower buds, green siliques and mature seeds of *Arabidopsis thaliana* control (111) and CYP79A1 (79A1) plants were treated with sulfatase and the content of desulfoglucosinolates was analysed by HPLC. The columns designated 111 and 79A1 (-pOHBG) represent glucosinolates endogenous to *A. thaliana* ecotype Columbia. The columns designated 79A1 represent the total glucosinolate content in transgenic 79A1 plants, including the novel glucosinolate *p*-OHBG. CFB Closed flower buds. Mean values \pm SD ($n = 3$)

content. The major endogenous glucosinolates in the CYP79A1 seeds were approximately equally reduced (Fig. 2F).

Individual endogenous glucosinolates

Phenylethylglucosinolate was identified in minute amounts in mature seeds of CYP79A1 and control plants. Previous HPLC analyses of glucosinolate content in leaves, seeds (Haughn et al. 1991) and roots (Ludwig-Müller et al. 1999) of *A. thaliana* ecotype Columbia have not identified phenylalanine-derived glucosinolates such

as benzyl- and phenylethylglucosinolate. In mature rosette leaves of *A. thaliana* ecotype Columbia at the stage just before bolting, 4-msb has been identified as the major glucosinolate (Haughn et al. 1991). With the exceptions of roots and seeds, 4-msb was identified as the major glucosinolate in both CYP79A1 and control plants, accounting for 40–50%, 55–80%, 54–76% and 62–77% of the total endogenous glucosinolate content in leaves, stems, closed flower buds and siliques, respectively. The level of the indolylglucosinolate 1-meind in roots of CYP79A1 and control plants was in the same range as previously reported for the roots of *A. thaliana* ecotype Columbia, i.e. 2–4 (Fig. 2B) versus ca. 2 μmol glucosin-

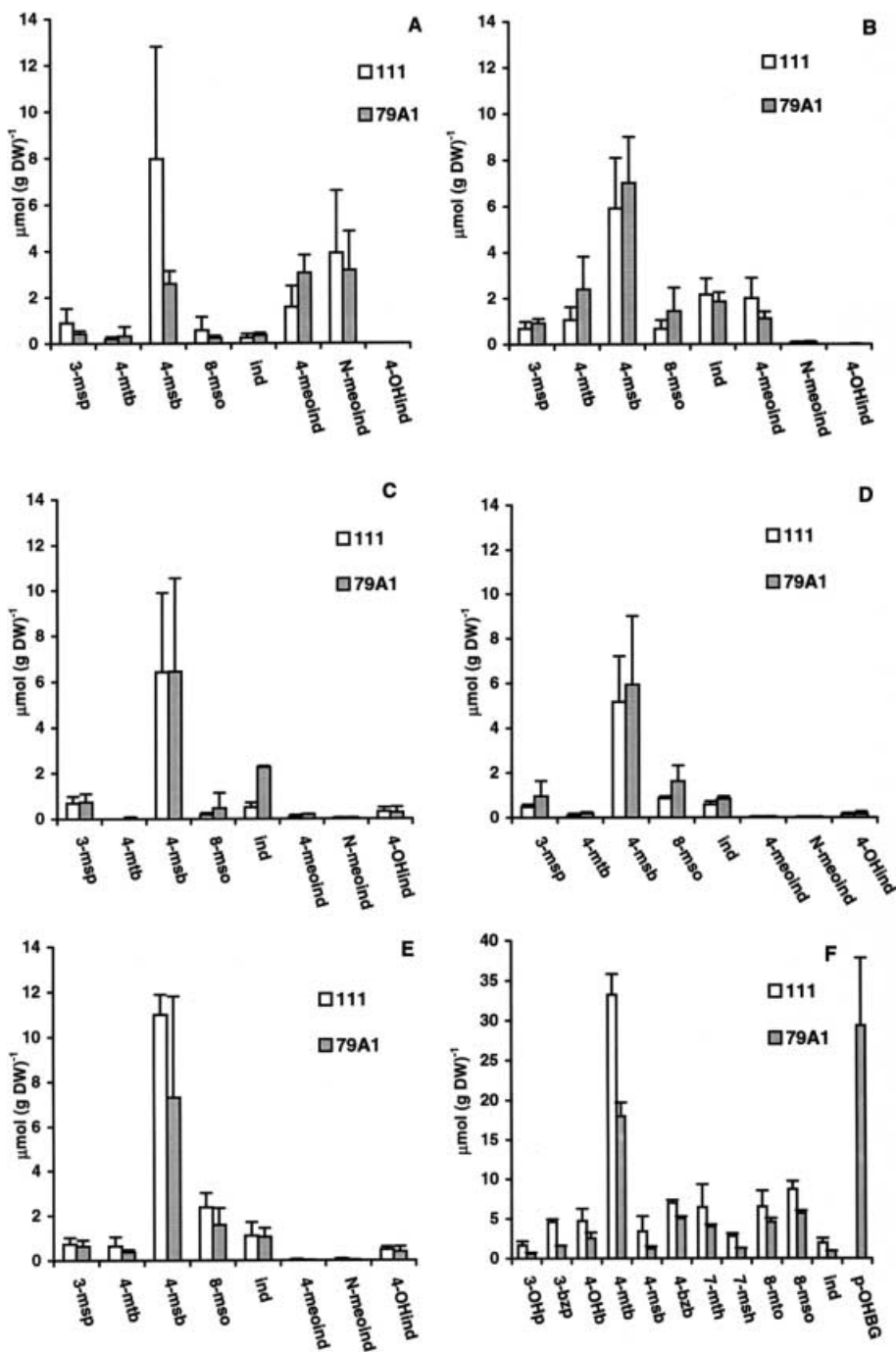


Fig. 2A–F. Major endogenous glucosinolates in various tissues. Methanol extracts of roots (A), leaves (B), stems (C), closed flower buds (D), green siliques (E) and mature seeds (F) of *A. thaliana* control (111) and CYP79A1 (79A1) plants were treated with sulfatase and analysed by HPLC. In the case of seeds (F), *p*-OHBG is included. Abbreviations: *gls* glucosinolate; *3-msp* 3-methylsulphinyl-propylglucosinolate; *3-OHp* 3-hydroxy-propylglucosinolate; *3-bzp* 3-benzoyloxypropylglucosinolate; *4-mtb* 4-methylthiobutylglucosinolate; *4-msp* 4-methylsulphinylbutylglucosinolate; *4-OHb* 4-hydroxybutylglucosinolate; *4-bzb* 4-benzoyloxybutylglucosinolate; *7-mth* 7-methylthioheptylglucosinolate; *8-mto* 8-methylthiooctylglucosinolate; *8-mso* 8-methylsulphinyloctylglucosinolate; *ind* indol-3-ylmethylglucosinolate; *4-OHind* 4-hydroxyindol-3-ylmethylglucosinolate; *4-meoind* 4-methoxyindol-3-ylmethylglucosinolate; *1-meind* 1-methoxyindol-3-ylmethylglucosinolate; *p-OHBG* *p*-hydroxybenzylglucosinolate. Mean values \pm SD ($n=3$)

olate (g DW)⁻¹ (Ludwig-Müller et al. 1999). The levels of ind and 4-meoind, however, were found to be approximately 10-fold higher in the CYP79A1 and control plants compared to previously reported investigations (Ludwig-Müller et al. 1999). Indolylglucosinolates, methoxylated at either the 1 or 4 position on the indolyl ring, were found in only trace amounts in green siliques. The indole glucosinolate 4-OHind, not previously identified in *Arabidopsis*, was found in stems, closed flower buds and siliques (Fig. 2C–E) and in trace amounts in seeds. The 4-OHind was identified from the retention time, spiking with desulfoglucosinolates obtained from seeds of *Brassica napus* cv. Polo (a double low variety) where 4-OHind constitutes > 65% of the total glucosinolate content (data not shown), and the characteristic UV diode spectrum. Identical ¹H nuclear magnetic resonance spectra of 4-OHind purified from green siliques of *A. thaliana* ecotype Columbia and *B. napus* cv. Polo seeds have confirmed the identity of this compound (data not shown). While the discrepancy on indolylglucosinolate levels between the present and earlier studies may be due to differences in growth conditions and/or stage of tissue, the elution volume of the DEAE columns and pH of the sulfatase buffer may also substantially influence the yield, especially of ind, 4-meoind and 4-OHind (see *Materials and methods*).

Expression of S-GT

The ca. 4-fold increase in glucosinolate content in the CYP79A1 plants led us to investigate whether this increase was accompanied by an increased expression of post-oxime enzymes in the glucosinolate biosynthetic pathway. When a cDNA sequence, encoding the *B. napus* S-GT (EMBL accession number CAA03659), was used in a BLAST search, the highest scoring *A. thaliana* gene (EMBL accession number AC002396) displayed 83% identity at the amino acid level with more than 85% of the *A. thaliana* genome sequenced. The next highest score was an indole-3-acetate β -glucosyltransferase with an amino acid identity of 42%. This strongly indicated that AC002396 encodes a homologue of S-GT in *A. thaliana*. Northern analysis on fully matured rosette leaves showed that the *A. thaliana* S-GT homologue was expressed at a low level (Fig. 3A) and that the level of the putative S-GT mRNA was approximately 50% higher in the CYP79A1 plants compared to the control plants (Fig. 3B). Care had been taken to use plants of the same developmental stage that had grown under the same culture conditions. The analysis had been repeated at least twice and yielded similar results.

Expression of myrosinase

The level of myrosinase activity and the content of myrosinase protein were analyzed in order to investigate whether the 4-fold increase in glucosinolate content in the CYP79A1 plants was accompanied by an increase in content or activity of the hydrolytic enzyme. Myrosin-

ase activity measured in fully mature rosette leaves of CYP79A1 and wild-type plants was 1.1 ± 0.2 and 1.1 ± 0.3 mol (g protein)⁻¹ min⁻¹, respectively (Fig. 4A; *P* 0.47, mean \pm SD, 1-tailed *t*-test). Western blot analysis showed the presence of a strong band of ca. 70 kDa of similar intensity in protein extracts of fully mature rosette leaves of CYP79A1 and control plants (Fig. 4B). These data show that the level of myrosinase activity and expression of myrosinase protein in the wild type and CYP79A1 plants is not substantially affected by the 4-fold increase in total glucosinolate content in leaves of CYP79A1 plants.

Discussion

Introduction of CYP79A1 into *A. thaliana* increased the total glucosinolate content 3.5- to 4.5-fold in roots, leaves, stems, closed flower buds and green siliques. The composition and level of individual, major endogenous aliphatic and indole glucosinolates were not significantly different in CYP79A1 and control plants. This shows that the capacity for biosynthesis of glucosinolates has not yet reached its maximum, and that there is no feedback inhibition on the pre-oxime biosynthetic genes. In contrast, the level of total glucosinolates in the seeds was similar in CYP79A1 and control plants. It is an open question to what extent the accumulated glucosinolates in the seeds are synthesized de novo in the siliques and/or possibly other parts of the plant, and subsequently transported to the seeds (Haughn et al. 1991; Magrath et al. 1994; Du and Halkier 1998). In all of the investigated tissues the CaMV35S-driven expression of CYP79A1 resulted in addition of *p*-hydroxyphenylacetaldoxime to the pool of endogenous oximes. The relatively equal reduction in the level of endogenous glucosinolates in seeds of CYP79A1 plants combined with the similar level of total glucosinolates in seeds of CYP79A1 and control plants indicate that *p*-hydroxyphenylacetaldoxime competes with the endogenous

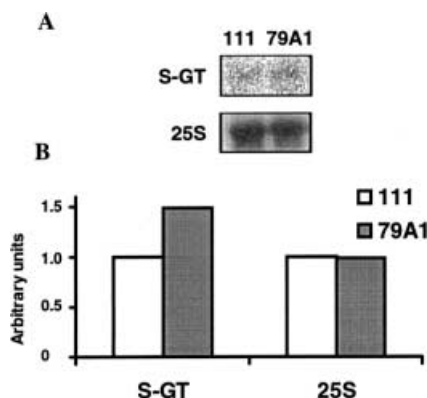


Fig. 3A, B. Expression of an *A. thaliana* S-GT homologue. Northern blot analysis of the gene encoding S-GT in leaves of control (111) and CYP79A1 (79A1) plants. A ³²P-labelled fragments of the second exon of the S-GT and the 25S rRNA ribosomal gene of *A. thaliana* were used as probes. B Autoradiography was quantified using a STORM 840 phosphoimager. The lower of the two values in each of the two data sets was normalized to the value of 1

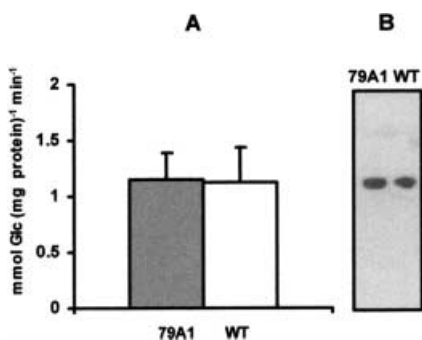


Fig. 4. Measurement of myrosinase activity (A) and Western blot analysis of myrosinase content (B) in leaves of CYP79A1 (*79A1*) and wild type (*WT*) plants. Myrosinase activity (mean \pm SD; CYP79A1, $n = 5$; WT, $n = 6$) was determined by monitoring the glucose release [mmol (mg total protein)⁻¹ min⁻¹] after hydrolysis of allylglucosinolate. The monoclonal anti-myrosinase antibody 3D7 (Lenman et al. 1990) was used as primary antibody

oxime pool in the glucosinolate biosynthesis during the development of the silique. Possible explanations for this observation could be that there is a maximum storage capacity or a limiting translocation capacity or a limiting biosynthetic capacity (specifically in the siliques).

The indolylglucosinolate 4-OHind was identified in *Arabidopsis* for the first time. Previous studies on the glucosinolate content in *A. thaliana* have been confined to mature seeds and rosette leaves (Hogge et al. 1988; Haughn et al. 1991) and roots (Ludwig-Müller et al. 1999). The identification of 4-OHind in stems, closed flower buds and siliques emphasizes the importance of yield optimization, especially of the desulfo-indolylglucosinolates, and of including all plant parts in the investigation of glucosinolate composition.

The total glucosinolate content in *A. thaliana* leaves immediately prior to bolting has been reported to vary greatly from plant to plant (Haughn et al. 1991). Also, high intra-specific variations in the aliphatic glucosinolate content have been reported both within and between natural populations of *A. thaliana*, *Brassica* and other crucifers (Mithen et al. 1995; Kushad et al. 1999). In the present study the total content of endogenous glucosinolates was found to be relatively constant with the major glucosinolate 4-msb giving rise to the highest variation (cf. Fig. 2).

The high increase in glucosinolate content shows that the post-oxime enzymes possess the biosynthetic capacity to produce substantially more glucosinolates. The 50% increase in the expression of the S-GT transcript indicates that the post-oxime enzymes in the CYP79A1 plants are up-regulated, i.e. that the transgenic CYP79A1 plants sense the increased flux of oximes in the pathway and regulate the expression level of the various post-oxime enzymes accordingly.

In the present study, Western analysis and activity measurements of myrosinase indicated that myrosinase, at the level of activity and protein, is not appreciably different in the CYP79A1 and control plants. Thus, a coupling between the total glucosinolate level and the expression of myrosinase was not found. This is in

agreement with a previous study on the distribution of myrosinase activity in *B. napus* (Bones 1990). However, based on the myrosinase activity (ca. 1 mmol glucose (mg protein)⁻¹ min⁻¹; Fig. 4A) and total glucosinolate content (ca. 6 μ mol glucosinolates (g FW)⁻¹; Fig. 1.) combined with a roughly estimated protein concentration of ca. 10 mg protein (g FW)⁻¹ in mature rosette leaves of CYP79A1 plants, the hydrolytic capacity of the myrosinase present is clearly in vast excess when compared to the substrate present.

In conclusion, our data show that the high biosynthetic capacity of the post-oxime enzymes in *A. thaliana* combined with the low substrate specificity of the post-oxime enzymes (Grootwassink et al. 1990) provide a very flexible system for metabolic engineering of *A. thaliana* plants with altered glucosinolate profiles. This includes modification of the production of endogenous glucosinolates by modification of the endogenous pre-oxime enzymes, of which several have been identified in *A. thaliana* (Hull et al. 2000; Wittstock and Halkier 2000). As exemplified in the present study, this also includes metabolic engineering of novel glucosinolates derived from non-endogenous oximes introduced into the plant by, for example, transformation with CYP79 homologues with substrate specificities different from those of the endogenous CYP79s.

References

- Agerbirk N, Olsen CE, Sørensen H (1998) Initial and final products, nitriles and ascorbigens, produced in myrosinase-catalyzed hydrolysis of indole glucosinolates. *J Agric Food Chem* 46: 1563–1571
- Bak S, Olsen CE, Petersen BL, Møller BL, Halkier BA (1999) Metabolic engineering of *p*-hydroxybenzylglucosinolate in *Arabidopsis* by expression of the cyanogenic CYP79A1 from *Sorghum bicolor*. *Plant J* 20: 663–671
- Bones A (1990) Distribution of β -thioglucosidase activity in intact plants, cell and tissue cultures and regenerant plants of *Brassica napus*. *J Exp Bot* 41: 737–744
- Bones A, Slupphaug G (1989) Purification, characterization and partial amino acid sequencing of β -thioglucosidase from *Brassica napus* L. *J Plant Physiol* 134: 722–729
- Buchner R (1987a) Comparison of procedures for optimum extraction, purification and analysis of desulfo indole glucosinolates. In: Wathelet J-P (ed) *World crops: production, utilization, description*, vol 13. *Glucosinolates in rapeseeds: analytical aspects*. Kluwer, Dordrecht, p 76
- Buchner R (1987b) Approach to determination of HPLC response factors for glucosinolates. In: Wathelet J-P (ed) *World crops: production, utilization, description*, vol 13. *Glucosinolates in rapeseeds: analytical aspects*. Kluwer, Dordrecht, pp 50–58
- Chen S, Halkier BA (1999) Functional expression and characterization of the myrosinase MYR1 from *Brassica napus* in *Saccharomyces cerevisiae*. *Protein Expr Purif* 17: 414–420
- Chew FS (1988) Biological effects of glucosinolates. In: Cutler EG (ed) *Biologically active natural products: potential use in agriculture*. American Chemical Society, Washington DC, pp 155–181
- Du L, Halkier BA (1998) Biosynthesis of the glucosinolates in the developing silique walls and seeds of *Sinapis alba*. *Phytochemistry* 48: 1145–1150
- Fahey JW, Zhang Y, Talalay (1997) Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci USA* 94: 10367–10372

- Faulkner K, Mithen R, Williamson G (1998) Selective increase in the potential anticarcinogen 4-methylsulphinylbutyl glucosinolate in broccoli. *Carcinogenesis* 19: 605–609
- Fenwick GR, Heaney RK, Mullin WJ (1983) Glucosinolates and their breakdown products in food and food plants. *Crit Rev Food Sci Nutr* 18: 123–201
- Grootwassink JWD, Balsevich JJ, Kolenovsky AD (1990) Formation of sulfatoglucosides from exogenous aldoximes in plant cell cultures and organs. *Plant Sci* 66: 11–20
- Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile *pPZP* family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25: 989–994
- Halkier BA (1999) Glucosinolates. In: Ikan R (ed) Naturally occurring glycosides. Wiley, New York, pp 193–223
- Halkier BA, Nielsen HL, Koch B, Møller BL (1995) Purification and characterisation of recombinant cytochrome p450_{TYR} expressed at high levels in *Escherichia coli*. *Arch Biochem Biophys* 322: 369–377
- Haughn GW, Davin L, Giblin M, Underhill EW (1991) Biochemical genetics for plant secondary metabolites in *Arabidopsis thaliana*. *Plant Physiol* 97: 217–226
- Hogge LR, Reed DW, Underhill EW (1988) HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography/mass spectrometry. *J Chrom Sci* 26: 551–556
- Hull AK, Rekha V, Celenza JL (2000) *Arabidopsis* cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *Proc Natl Acad Sci USA* 97: 2379–2384
- Koch B, Sibbesen O, Halkier BA, Møller BL (1995) The primary sequence of cytochrome P450_{TYR}, the multifunctional N-hydroxylase catalyzing the conversion of L-tyrosine to p-hydroxyphenylacetaldoxime in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. *Arch Biochem Biophys* 323: 177–186
- Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wallig MA, Jeffery EH (1999) Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *J Agric Food Chem* 47: 1541–1548
- Lazzeri L, Tacconi R, Palmieri S (1993) In vitro activity of some glucosinolates and their reaction products toward a population of the nematode *Heterodera schachtii*. *J Agric Food Chem* 41: 825–829
- Lenman M, Rödin J, Josefsson L-G, Rask L (1990) Immunological characterization of rapeseed myrosinase. *Eur J Biochem* 194: 747–753
- Ludwig-Müller J, Pieper K, Ruppel M, Cohen JD, Epstein E, Kiddle G, Bennett R (1999) Indole glucosinolate and auxin biosynthesis in *Arabidopsis thaliana* (L.) Heynh. Glucosinolate mutants and the development of clubroot disease. *Planta* 208: 409–419
- Magrath R, Bano F, Morgner M, Parkin I, Sharp A, Lister C, Dean C, Turner J, Lydiat D, Mithen R (1994) Genetics of aliphatic glucosinolates. I. Side chain elongation in *Brassica napus* and *Arabidopsis thaliana*. *Heredity* 72: 290–299
- Manici LM, Lazzeri L, Palmieri S (1997) In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products toward pathogenic fungi. *J Agric Food Chem* 45: 2768–2773
- Mari M, Lori R, Leoni O, Marchi A (1993) In vitro activity of glucosinolate-derived isothiocyanates against postharvest fruit pathogens. *Ann Appl Biol* 123: 155–164
- Minchinton I, Sang J, Truscoscott RW (1982) Separation of desulfoglucosinolates by reversed-phase high-performance liquid chromatography. *J Chromatogr* 247: 141–148
- Mithen R, Clake J, Lister C, Dean C (1995) Genetics of aliphatic glucosinolates. III. Side chain structure of aliphatic glucosinolates in *Arabidopsis thaliana*. *Heredity* 74: 210–215
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Nielsen JK (1988) Crucifer-feeding Chrysomelidae: mechanisms and of host plant finding and acceptance. In: Jolivet P, Petitpierre E, Hsiao TH (eds) *Biology of Chrysomelidae*. Kluwer, Dordrecht, pp 25–40
- Pedras MSC, Okanga FI, Zaharia IL, Khan AQ (2000) Phytoalexins from crucifers: synthesis, biosynthesis, and biotransformation. *Phytochemistry* 53: 161–176
- Rodman JE, Soltis PS, Soltis DE, Sytsma KJ, Karol KG (1998) Parallel evolution of glucosinolate biosynthesis inferred from congruent nuclear and plastid gene phylogenies. *Am J Bot* 85: 997–1006
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York
- Sørensen H (1990) Glucosinolates: structure-properties-function. In: Shahidi F (ed) *Canola and rapeseed: production, chemistry, nutrition and processing technology*. Reinhold, New York, p 149
- Stadler E (1978) Chemoreception of host plant chemicals by oviposition females of *Delia (Hylemia) Brassicae*. *Entomol Exp Appl* 24: 711–720
- Thies W (1988) Isolation of sinigrin and glucotropaeolin from cruciferous seeds. *Fat Sci Technol* 90: 311–314
- Truscott RW, Johnstone PK, Michinton IR, Sang JP (1983) Indole glucosinolates in swede (*Brassica napobrassica* L. Mill). *J Agric Food Chem* 31: 863–967
- Wittstock U, Halkier B (2000) Cytochrome P450 CYP79A2 from *Arabidopsis thaliana* L. catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. *J Biol Chem* 275: 14659–14666
- Zhang Y, Talalay, Cho C-G, Posner GH (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA* 89: 2399–2403