Glucosinolates are plant natural products found throughout the Capparales order. Upon tissue disruption, the glucosinolates are hydrolyzed by specific enzymes called myrosinases to produce a wide range of biologically active compounds such as isothiocyanates, nitriles, and thiocyanates (for review, see Raybould and Moyes, 2001). In general, glucosinolates and their degradation products play a role in plant defense as attractants for generalist herbivores and microorganisms and as attractants for specialized insects (for review, see Raybould and Moyes, 2001). In human consumption, certain iso-...
Val- and Ile-metabolizing CYP79D1 and CYP79D2 from cassava (Manihot esculenta Crantz; Andersen et al., 2000).

The substrate-specific CYP79s constitute the first committed step in biosynthesis of protein amino acid-derived glucosinolates. This makes the CYP79 enzymes important tools for modifying glucosinolate profiles (for review, see Mikkelsen et al., 2002). Overexpression of endogenous CYP79s, e.g. CYP79B2 and CYP79A2, have resulted in high accumulation of Trp-derived indole glucosinolates and the Phe-derived benzylglucosinolate, respectively (Mikkelsen et al., 2000; Wittstock and Halkier, 2000). This shows that the CYP79s constituted the rate-limiting step and that the postaldoxime enzymes have a higher capacity for production of glucosinolates than what is required for biosynthesis at physiological levels. The postaldoxime enzymes have previously been shown to have high specificity for the functional groups of later intermediates, but low specificity for the side chain (for review, see Halkier, 1999). This indicates that exogenous aldoximes may be converted to the corresponding glucosinolates as has been shown for 2-nitrobenzaldoxime (Grootwassink et al., 1990) and p-hydroxyphenylacetaldoxime (Bak et al., 1999). In this article, we report metabolic engineering of the two novel Val- and Ile-derived glucosinolates in Arabidopsis expressing CYP79D2 from cassava and we show that this is achieved with no effect on the morphological phenotype or on the accumulation of endogenous glucosinolates.

RESULTS

Arabidopsis was transformed with the 35S::CYP79D2 construct by Agrobacterium tumefaciens-mediated DNA transfer. Transformants were selected by plating on gentamycin, and 31 independent T1 lines were identified. The 31 lines exhibited wild-type phenotype on soil, although when germinated on selective medium, growth was slightly retarded, possibly due to the presence of gentamycin. Glucosinolates were extracted from these lines and were analyzed by HPLC and liquid chromatography-mass spectrometry (LC-MS). The HPLC profile from 35S::CYP79D2 Arabidopsis plants contained two peaks that were not present in the wild type (Fig. 1A). The retention times for the peaks were 11.5 and 17.3 min, respectively, and both peaks had a UV spectrum characteristic of aliphatic glucosinolates (Fig. 1B). The LC profile of glucosinolates extracted from 35S::CYP79D2 Arabidopsis plants contained two peaks at 20.14 min and 26.15 min, respectively, that were not present in the wild-type (Fig. 2A). The mass spectrum of the peak at 20.14 min showed a major m/z of 303.9 corresponding to the sodium salt of the Val-derived desulphoglucosinolate molecular ion (Fig. 2B). Major fragmentary ions of m/z 185, 219, and 241 corresponded to, respectively, the sodium salt of the Glc moiety, the sodium salt of the thio-Glc moiety, and a desulphoglucosinolate structure lacking the amino acid side chain and with the N-C double bond reduced. The mass spectrum of the peak at 26.15 min showed a major m/z of 317.9, corresponding to the molecular ion of the sodium salt of the Ile-derived desulphoglucosinolate (Fig. 2C). A fragmentation pattern similar to that of the peak at 20.14 min was observed. This conclusively demonstrated that the 35S::CYP79D2 Arabidopsis plants produced i-prop and 1Me-prop.

As standards for i-prop and 1Me-prop, we analyzed the glucosinolate profiles of HPLC and LC-MS of two plant species known to contain predominantly branched aliphatic glucosinolates (Fahey et al., 2001). For Putranjiva roxburghii, the dominant peak in the LC profile had a retention time of 20.14 min, with an m/z of 303.9 corresponding to the sodium salt of the Val-derived desulphoglucosinolate. Using the HPLC system, the corresponding dominant peak had a retention time of 11.5 min. Similar to Capparis flexuosa, the dominant peak in the LC profile had a retention time of 26.15 min, with an m/z of 317.9 corresponding to the sodium salt of the Ile-derived desulphoglucosinolate. Using the HPLC, the corresponding peak had a retention time of 17.3 min. From the data, we concluded that i-prop has a retention time of 11.5 min and that 1Me-prop has a retention time of 17.3 min in the given HPLC system.

The glucosinolate profiles in 6-week-old rosette leaves of 31 independent T1 lines were analyzed by HPLC and quantified (Fig. 3). Line 28 accumulated the highest quantities of i-prop (5.4 nmol mg⁻¹ dry weight) and 1Me-prop (2.9 nmol mg⁻¹) with a total of 8.2 nmol mg⁻¹. In this line, i-prop and 1Me-prop accounted for approximately 35% of the total glucosinolate content. Other high-expressing lines included lines 5, 6, and 10, which contained, respectively, 5.1, 4.2, and 4.1 nmol mg⁻¹ i-prop plus 1Me-prop. All other lines accumulated lower amounts, and 16 lines contained a total of about 1 nmol mg⁻¹ or less. The data were produced from heterozygous T1 lines. It was expected that the homozygotes would accumulate higher levels of i-prop and 1Me-prop due to increased copy number. However, this was not the case (data not shown).

When 35S::CYP79D2 plants were germinated on gentamycin, the content of endogenous glucosinolates was significantly lower than in wild type (Figs. 1A and 4). The lower concentration of endogenous glucosinolates is not unexpected as the biosynthesis of many glucosinolates are under strict developmental control (Petersen et al., 2002). However, no differences in growth or in the profile of endogenous glucosinolates were seen between wild-type and 35S::CYP79D2 plants when germinated on soil. This indicates that the postaldoxime enzymes in the glucosinolate pathway are not rate limiting, and that the capacity for production and storage of glucosinolates can exceed the level found in uninduced wild-type plants.
When data was combined for all examined plants, i-prop accounted for 65% ± 8% (w/v) of the two novel glucosinolates produced. This is in accordance with the in vitro activity of the recombinant CYP79D2, of which the conversion rate of Ile is approximately 60% of that observed for Val (Andersen et al., 2000). The variation in the ratio of i-prop to 1Me-prop was less than 1% when multiple plants from any single line were examined (data not shown). The level of i-prop and 1Me-prop in old leaves were generally higher than in mature leaves (data not shown). This could be due to the longer time the plants have had to accumulate the novel glucosinolates. In old leaves, the novel glucosinolates accounted for 34% to 48% of the total glucosinolate content, and the percentage of i-prop decreased to approximately 55% ± 5% of the sum of i-prop and 1Me-prop. When the ratio of i-prop to 1Me-prop was compared with the total amount of i-prop and 1Me-prop, a correlation was found in which a higher total concentration of i-prop and
1Me-prop correlated with a lower ratio of i-prop to 1Me-prop (Fig. 5A). In general, the higher the concentration of i-prop and 1Me-prop, the higher the percentage of the novel glucosinolates were accounted for by 1Me-prop. This explains the very low variation between plants from the same line as they contain similar concentrations of i-prop and 1Me-prop. Furthermore, it explains the decreasing ratio of i-prop to 1Me-prop in old leaves, as the total concentration of the novel glucosinolates in this tissue in general was higher. The correlation was clearly visualized when data from lines containing 0–1.0, 1.0–2.0, 2.0–3.0, and more than 3.0 nmol mg⁻¹ of the novel glucosinolates were combined (Fig. 5B). These data indicated that i-prop accumulation at high concentrations was restricted due to slower metabolism of Val or its aldoxime or due to specific degradation of i-prop.

The effect of growth conditions on the accumulation of i-prop and 1Me-prop in 35S::CYP79D2 plants was investigated by growing the plants in 8, 12, and 16 h of light followed by analysis of the glucosinolate content...
in mature rosette leaves 4 weeks after germination. Only small differences were seen in mature leaves from 35S::CYP79D2 plants grown at 8-, 12-, or 16-h light periods (data not shown). Furthermore, no physiological phenotype was observed under any of these conditions, and the levels of endogenous glucosinolates were unchanged compared with wild type. No significant difference was seen between wild type germinated on nonselective medium or soil.

**DISCUSSION**

Arabidopsis transformed with the exogenous cassava CYP79D2 under control of the 35S promotor was shown to produce two novel glucosinolates, i-prop and 1Me-prop, which are not natural constituents in this ecotype. This shows that the Val- and Ile-derived aldoximes produced by CYP79D2 are efficiently converted by the postaldoxime enzymes to the corresponding glucosinolates. Metabolic engineering of Arabidopsis with 35S-driven CYP79A1, CYP79A2, or CYP79B2 have previously been shown to produce approximately 52, 18, and 24 nmol mg\(^{-1}\) dry weight of Tyr-, Phe-, and Trp-derived glucosinolates, respectively (Bak et al., 1999; Mikkelsen et al., 2000; Wittstock and Halkier, 2000; Petersen et al., 2001). This is significantly more than the 8.2 nmol mg\(^{-1}\) found in mature leaves of the 35S::CYP79D2 plants. The relatively low accumulation of i-prop and 1Me-prop is unlikely to be due to position effect of the transgene as 31 different lines were examined. A possible explanation could be that CYP79D2 is a less efficient or less stable enzyme. The \(K_m\) values of CYP79A1, CYP79A2, and CYP79B2 are 220 \(\mu\)M (Halkier et al., 1995), 6.7 \(\mu\)M (Wittstock and Halkier, 2000), and 21 \(\mu\)M (Mikkelsen et al., 2000), respectively, whereas the \(K_m\) values for CYP79D1 is 2.2 and 1.3 \(\mu\)M for Val and Ile, respectively (Andersen et al., 2000). The \(K_m\) values for CYP79D2 have not been determined, but are likely to be in the same range as those of CYP79D1 as the two recombinant enzymes show similar conversion rates of Val and Ile (Andersen et al., 2000). High \(K_m\) values of CYP79D2 may have limited aldoxime production and thereby accumulation of i-prop and 1Me-prop. In an alternate manner, the availability of the substrates for CYP79D2 may have been reduced if the Val and Ile pools were not efficiently feedback up-regulated in response to the increased draw from the pool. Acetoxyhydroxy acid synthase is the first common enzyme in biosynthesis of branched chain amino acids. Leu, Val, and Ile are each able to inhibit acetoxyhydroxy acid synthase, although the most efficient inhibition is caused by the combination of excess Leu and Val (Lee and Duggleby, 2001). Therefore, the application of excess Leu and Val may create a condition of Ile starvation, which results in growth inhibition. In accordance with this, depletion or reduction of the Val and Ile pools may result in starvation if Leu inhibits Val and Ile biosynthesis. However, in this case, one would expect to see inhibition of growth in these conditions, and the levels of endogenous glucosinolates were unchanged compared with wild type.

![Figure 4](image_url) **Figure 4.** Glucosinolate content in wild-type and 35S::CYP79D2 grown on selective medium or soil. Plants were germinated on soil or on Murashige and Skoog plates containing no antibiotic or gentamycin for wild-type and 35S::CYP79D2 plants, respectively. Two weeks after germination, the plants on Murashige and Skoog plates were transferred to soil. After 6 weeks, glucosinolates were extracted from rosette leaves and were analyzed by HPLC. In 35S::CYP79D2 plants grown in the presence of gentamycin, the concentration of endogenous glucosinolates was significantly lower compared with wild type. However, in soil-grown 35S::CYP79D2 plants, the content of endogenous glucosinolates was virtually identical to that of wild type. No significant difference was seen between wild type germinated on nonselective medium or soil.

![Figure 5](image_url) **Figure 5.** The ratio of i-prop to 1Me-prop in relation to total content of i-prop and 1Me-prop in leaves from 35S::CYP79D2 lines. A, Scatterplot of ratio of i-prop to 1Me-prop versus total amount of i-prop and 1Me-prop. The plot comprises data from the 31 independent lines in the T<sub>1</sub> generation, mature, and old leaves of homozygous lines 5, 6, 8, 10, and 28. One data point from the T<sub>1</sub> generation highest expressing line (28) has been omitted for clarity. B, Grouping of 35S::CYP79D2 lines containing, respectively, less than 1.0, 1.0 to 2.0, 2.0 to 3.0, or more than 3.0 nmol mg\(^{-1}\) i-prop and 1Me-prop. Columns 0 through 1.0 through 3.0+ represent 15, 20, 12, and 23 independent measurements, respectively.
plants, which was not observed. The expected $K_m$ values of CYP79D2 suggest that the size of amino acid pools may never be reduced further than to a level where CYP79D2 is unable to efficiently bind and metabolize the substrates. It has been suggested that the relatively high $K_m$ values of CYP79D1 function to impede the chance of amino acid starvation (Andersen et al., 2000). However, depletion of the amino acid pools could explain the decreasing ratio of i-prop to 1Me-prop with increasing overall i-prop and 1Me-prop concentrations, if the Val pool was depleted first.

An interesting feature of the 35S::CYP79D2 plants was the almost identical ratio of i-prop to 1Me-prop in several plants representing the same line, whereas a larger variation was observed between different transgenic lines. In general, the ratio of i-prop to 1Me-prop was similar for lines containing comparable quantities of the novel glucosinolates, whereas the i-prop concentration decreased relative to the 1Me-prop concentration at increasing levels of the novel glucosinolates. The reason for this is not understood. It may be that the difference in the i-prop to 1Me-prop ratio is due to differences in substrate availability or due to increased turnover of specifically i-prop at increasing i-prop concentration.

Different approaches for metabolic engineering of glucosinolate profiles are required depending on which glucosinolates are the target. The condensing enzymes in the chain elongation pathway are likely to be rate limiting for engineering of glucosinolates derived from chain-elongated protein amino acids. At present, the substrate specificity and number of condensing enzymes have not been determined (Campos et al., 2000; Kroymann et al., 2001). For glucosinolates derived from protein amino acids, the CYP79s are the first committed step and the rate-limiting step. This makes the CYP79s particularly powerful tools for (over-) expression and knockout strategies (for review, see Mikkelsen et al., 2002). Secondly, modified glucosinolate side chains are often the determining factor for the biological activity of the glucosinolate degradation products. However, the outcome of engineering of modifying enzymes is not easily predicted, as a knockout mutant will result in accumulation of the preceding intermediate, and overexpression might not have any effect if it is not a rate-limiting step. In the present study, we have generated transgenic Arabidopsis plants that produce two novel glucosinolates derived from Val and Ile while maintaining wild-type morphological and glucosinolate phenotype. Furthermore, although iso-thiocyanates with small side chains are generally less toxic than those with larger side chains (Borek et al., 1998), the Val- and Ile-derived isothiocyanates have potentially beneficial effects as insect repellents and biofumigants due to their high volatility. In general, it is difficult to predict the success of metabolic engineering as $K_m$ values, turnover, pool rebuilding, enzyme stability, and other presently unknown factors influence the outcome. However, with the recent advances in our understanding of the glucosinolate biosynthesis (Wittstock and Halkier, 2002), it has become a realistic goal to produce custom-designed crop plants enriched in desirable glucosinolates and without unwanted glucosinolates. This will ultimately improve nutritional value, including cancer-preventing properties, as well as increase resistance to herbivores and pathogens.

**MATERIALS AND METHODS**

**Generation of Transgenic Arabidopsis Expressing CYP79D2**

The full-length CYP79D2 cDNA was amplified by PCR using primers CYP79D2forward (5′-ATGCTCGGACCATGCTGGATCATGAACGTCTCC-3′) and CYP79D2reverse (5′-CGCTCTGACTCTGCTACAAAGGGAAGG-3′) to incorporate BamHI/Xhol restriction sites. The PCR product was cloned into BamHI/Xhol-digested pRT101 (Topfer et al., 1987) and sequenced. The expression cassette, including the cauliflower mosaic virus 35S promoter, was excised from pRT101 by HindIII digestion and was transferred to pZIP221 (Hajdukiewicz et al., 1994). Agrobacterium tumefaciens C58C1/pGV3850 (Zambryski et al., 1983) was transformed with the pZIP221 cauliflower mosaic virus 35S::CYP79D2 construct by electroporation and was used to transform Arabidopsis ecotype Colombia by A. tumefaciens-mediated DNA transfer. This was accomplished using the floral dip method (Clough and Bent, 1998) with 0.005% (v/v) Silwet L-77 and 5% (w/v) Suc in 10 mM MgCl₂. Seeds were germinated on one-half-strength Murashige and Skoog medium supplemented with 100 μg mL⁻¹ gentamycin, 3% (w/v) Suc, and 0.9% (w/v) agar. Transformants were selected after 2 to 4 weeks and were transferred to soil.

**Sequencing and Sequence Analysis**

Sequence analysis was performed using Thermo Sequenase Fluorescent-labeled Primer cycle sequencing kit (Amersham Pharmacia Biotech). Sequence computer analysis was accomplished using programs of the Wisconsin Sequence Analysis Package.

**Growth of Plants**

Arabidopsis ecotype Colombia was used for all experiments. Plants were grown in a controlled environment Arabidopsis chamber (AR-60 I; Percival, Boone, IA) at a photosynthetic flux of 100 to 120 nmol photons m⁻² s⁻¹ at 20°C and 70% relative humidity. The photoperiod was 8, 12, or 16 h. Leaves from *Capparis flexuosa* and *Putranjiva rosularis* were kindly supplied by the Copenhagen Botanical Garden.

**HPLC Analysis of Glucosinolates**

Glucosinolates were extracted from approximately 20 mg of slightly homogenized freeze-dried rosette leaves by boiling for 4 min in 4 mL of 70% (v/v) methanol. The supernatant was collected and the plant material was homogenized freeze-dried rosette leaves by boiling for 4 min in 4 mL of 70% (v/v) methanol. The supernatant was collected and the plant material was homogenized freeze-dried rosette leaves by boiling for 4 min in 4 mL of 70% (v/v) methanol. The supernatant was collected and the plant material was homogenized freeze-dried rosette leaves by boiling for 4 min in 4 mL of 70% (v/v) methanol. The supernatant was collected and the plant material was homogenized freeze-dried rosette leaves by boiling for 4 min in 4 mL of 70% (v/v) methanol. The supernatant was collected and the plant material was homogenized freeze-dried rosette leaves by boiling for 4 min in 4 mL of 70% (v/v) methanol.
LC-MS Analysis of Glucosinolates

Desulphoglucosinolates obtained as described above were subjected to LC-MS analysis. LC-MS was performed using an HP1100 LC (CMI, Albertville, MN) coupled to an iontrap mass spectrometer (Esquire-LC; Bruker Daltonik, Bremen, Germany). The reversed-phase LC conditions were as follows: A C18 column (Chrompack Inertsil 3 ODS-3 S15 × 3 COL CP 29126, Analytical Instruments A/S, Vælæne, Denmark) was used. The mobile phases were: A: water doped with sodium acetate (50 μm), and B: methanol. The flow rate was 0.25 mL min⁻¹ and the gradient program was 0 to 2 min: isocratic 100% A; 2 to 40 min: linear gradient 0% to 60% B; 40 to 45 min: linear gradient 60% to 100% B; and 45 to 50 min: isocratic 100% B. The mass spectrometer was run in positive-ion mode. A 15-μL aliquot of each glucosinolate preparation was injected. Total ion currents and UV traces were used to locate peaks, and the [M + Na]⁺ adduct ions in conjunction with diode array UV spectra were used for identification.

Received August 21, 2002; returned for revision October 1, 2002; accepted November 13, 2002.

LITERATURE CITED


