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ORIGINAL RESEARCH PAPER

Transformation of *Nasturtium officinale*, *Barbarea verna* and *Arabis caucasica* for hairy roots and glucosinolate-myrosinase system production

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Abstract Hairy roots of *Nasturtium officinale*, *Barbarea verna* and *Arabis caucasica* with active glucosinolate-myrosinase system were obtained after transformation with *Agrobacterium rhizogenes*. Hairy roots of *N. officinale* produced phenylalanine-derived gluconasturtiin and glucotropaeolin (max. 24 and 7 mg g^{-1} DW). *B. verna* and *A. caucasica* hairy roots produced gluconasturtiin (max. 41 mg g^{-1} DW) and methionine-derived glucoiberverin (max. 32 mg g^{-1} DW), respectively. Treatment of the roots with amino acid precursors of glucosinolate or/and cysteine biosynthesis increased levels of glucosinolate production, combinations of phenylalanine with cysteine (for gluconasturtiin and glucotropaeolin) and methionine with *o*-acetylserine (for glucoiberverin) were the most effective.

Keywords Autolysis · Glucoiberverin · Gluconasturtiin · Glucotropaeolin · Isothiocyanates · Precursors

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Introduction

Glucosinolates are L-amino acid-derived β-thioglucoside-*N*-hydroxysulfates present in sixteen plant families, which upon hydrolysis by endogenous enzyme myrosinase (β-thioglucosidase, EC 3.2.3.1) are converted into bioactive compounds, including isothiocyanates. Isothiocyanates can be toxic or growth-suppressing to fungi, bacteria, insects, and nematodes (see Fahey et al. 2001 for review) and they have been shown to prevent the initiation of cancer in humans, especially induced by chemical carcinogens (see Hayes et al. 2008 for review). Since most plants produce the mixture of glucosinolates, the need for testing activity of pure compounds has increased interest in searching for rich sources of single glucosinolates both in plants and in plant in vitro cultures. We have already established hairy root cultures of *Tropaeolum majus* that are able to produce glucotropaeolin, myrosinase and benzyl isothiocyanate. We have also reported a method to stimulate glucotropaeolin production by precursor amino acids (Phe, Cys) and elicitors (Wielanek and Urbanek 2006). The aim of this present work was to obtain hairy root cultures of *Nasturtium officinale*, *Barbarea verna* and *Arabis caucasica* with active glucosinolate-myrosinase system, and to study the influence of individual or combined treatment with amino acid precursors of glucosinolates (Phe, Met, Cys) and cysteine (Ser, AOS) biosynthesis on glucosinolate and isothiocyanate production.

Materials and methods

Hairy roots were obtained after transformation of aseptically grown seedlings of *N. officinale* R.Br., *B. verna* (Mill.) Asch. and *Ar. caucasica* L. with *Agrobacterium rhizogenes* LBA 9402 (pRi 1855). The roots were cultured on B5 medium (Gamborg et al. 1968) containing 3% (w/v) sucrose and 0.02% (w/v) peptone from casein (pH 5.8), on the orbital shaker (120 rpm), in the dark, at 24°C, and subcultured every 4 weeks. Genomic DNA was isolated with the use of CTAB/PVP method (Pirtillä et al. 2001). To determine the presence of Ri T-DNA in the hairy roots genome, the polymerase chain reaction (PCR) was performed using as a target DNA isolated from untransformed and transformed roots of *N. officinale*, *B. verna*, *Ar. caucasica* (Królicka et al. 2001). To confirm that the hairy root cultures of *N. officinale*, *B. verna*, *Ar. caucasica* were free from *Agrobacterium rhizogenes*, a PCR with primers homologous to the sequence of *vir*G gene, which is localized beside T-DNA region of Ri plasmid, was performed (Aoyama et al. 1989).

Concentrations of the used precursors were selected on the basis of the previous experiments (data not shown). Phe, Cys, Met, Ser, OAS (*O*-acetyl-L-serine) were dissolved in water and sterilized (0.22 μM filter). The concentrated solutions (pH 5.8) were added, alone or in the mixture, to the 12-day-old cultures to obtain 0.5 mM of each precursor. The material from two flasks was collected on the 10th day after treatment (22nd day of culture) for analysis of glucosinolate content, myrosinase activity and isothiocyanate production.

Glucosinolates were extracted, purified and analyzed according to Wielanek and Urbanek (2006). The identification of intact glucotropaeolin, gluconasturtiin and glucoiberverin was performed by HPLC. Glucosinolate content was expressed as mg g^{-1} dry weight (DW), based on the calibration curve for standards.

Myrosinase was extracted and analyzed according to the method of Wielanek and Urbanek (2006). Myrosinase activity was expressed in μmol min^{-1} (U) mg^{-1} protein determined by Bradford's method.

Endogenous hydrolysis and isothiocyanate extraction was performed according to the modified method of Sultana et al. (2002). Hairy roots (10 g fresh weight, FW) were homogenized with 50 mM sodium phosphate buffer pH 7.5 (1:20, w/v) then placed on the rotary shaker in 37°C; autolysis was conducted for 30 min after that liquid paraffin was added and hydrolysis was continued for the next 30 min. The paraffin oil extraction was repeated two times. The collected paraffin layers were partitioned three times against methanol not shaking (30 min). The methanol layers were evaporated under reduced pressure, the residues were dissolved in acetonitrile, and the identification of benzyl phenylethyl and 3-(methylthiopropyl) isothiocyanates was performed by HPLC. Isothiocyanate content was expressed as mg 10 g^{-1} FW based on the calibration curve for standards.

Results and discussion

Several clones of *N. officinale*, *B. verna* and *Ar. caucasica* hairy roots were obtained (Table 1). DNA isolated from the root clones was used as a target in PCR with primers designed to be complementary to *rol*B and *rol*C genes (Królicka et al. 2001). The primers generated amplification products of approximately 423 and 626 bp with target DNA isolated from all hairy roots whereas DNA from untransformed roots did not act as a template for these primers (Fig. 1). The amplification product of *vir*G gene was observed only in the case of DNA isolated from *Ag. rhizogenes* LBA 9402 strain (Fig. 1, line 19). The results indicate that *rol*B and *rol*C genes from Ri plasmid of *Ag. rhizogenes* LBA 9402 became integrated with the genome of *N. officinale*, *B. verna* and *Ar. caucasica* hairy roots.

HPLC analyses of intact glucosinolate indicated that the obtained hairy roots were able to synthesise gluconasturtiin (*N. officinale* and *B. verna*), glucotropaeolin (*N. officinale*), and glucoiberverin (*Ar. caucasica*) (Fig. 2) and glucosinolate biosynthesis was associated with myrosinase activity (Table 1). In all of the cultures increase in glucosinolate content and myrosinase activity was accompanied by the phase of biomass growth reaching the maximum on the 22nd day when the roots started to enter stationary phase of growth (Fig. 3).

Our experiments with *T. majus* hairy roots (Wielanek and Urbanek 2006), as well as data from the studies with suspension cultures of *Nasturtium montanum* and *Cleome chelidonii* (Songsak and Lockwood 2004) indicated the stimulating effect of the amino acid precursors on glucosinolate and isothiocyanate

Table 1 Glucosinolate content and myrosinase activity on the 22nd day of culture of the obtained clones of *N. officinale*, *B. verna* and *Ar. caucasica* hairy roots

Hairy root clones	Glucosinolate content (mg g^{-1} DW)			Myrosinase activity (U mg^{-1} protein)
	Gluconasturtiin	Glucotropaeolin	Glucoiberverin	
N. officinale				
NO-1	16.2 ± 1.35	2.25 ± 0.21	–	0.65 ± 0.084
NO-2	8.26 ± 0.91	0.73 ± 0.12	–	0.12 ± 0.016
NO-3	21.8 ± 2.06	3.44 ± 0.29	–	0.32 ± 0.035
NO-4	18.4 ± 1.44	3.63 ± 0.41	–	0.50 ± 0.013
NO-5	9.03 ± 0.86	0.89 ± 0.09	–	0.21 ± 0.028
NO-6	**24.3 ± 2.31**	**7.03 ± 0.65**	–	0.56 ± 0.047
NO-7	19.1 ± 2.03	5.11 ± 0.62	–	0.43 ± 0.057
NO-8	16.3 ± 1.33	1.78 ± 0.19	–	0.49 ± 0.064
B. verna				
BV-1	12.4 ± 1.03	–	–	2.74 ± 0.36
BV-2	35.1 ± 3.24	–	–	2.43 ± 0.27
BV-3	33.3 ± 3.11	–	–	3.01 ± 0.11
BV-4	**40.6 ± 3.91**	–	–	3.48 ± 0.09
BV-5	24.6 ± 2.27	–	–	2.94 ± 0.31
Ar. caucasica				
AC-1	–	–	**32.3 ± 3.05**	1.47 ± 0.174
AC-2	–	–	24.7 ± 2.73	1.05 ± 0.148
AC-3	–	–	13.3 ± 1.09	1.33 ± 0.161

Presented data are means (±SD) of three subcultures day after treatment

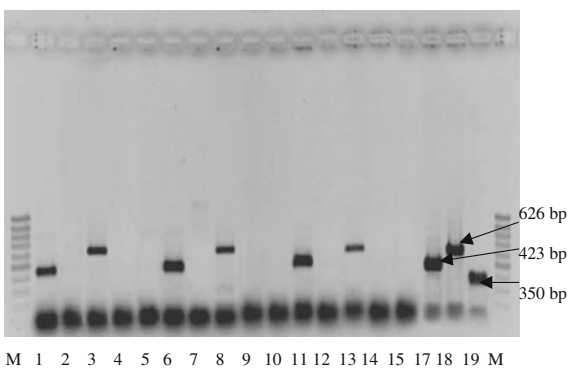


Fig. 1 PCR analysis of *N. officinale* (lanes 1–5), *B. verna* (6–10) and *Ar. caucasica* (11–15) roots transformed by *A. rhizogenes* LBA9402 (1, 3, 6, 8, 11, 13) and untransformed (2, 4, 7, 9, 12, 14); *A. rhizogenes* LBA9402 as a positive control (17–19). Lanes 5, 10, 15: hairy roots + *vir*G as a negative control. Lanes M: GeneRuler^{TM} 100 bp DNA ladder. *Arrows* show amplified fragments of *rol*B (423 bp), *rol*C (626 bp) and *vir*G (350 bp) genes

production. Biosynthesis of glucotropaeolin and gluconasturtiin depends on Phe, glucoiberverin is Met-derived, Cys is a source of sulfur in β-D-thioglucoside moiety of all glucosinolates (Du and Halkier 1996; Wallsgrove et al. 1999; Campos de Quiros et al. 2000). Cys is the product of cysteine synthase complex catalyzing sulfide incorporation into Ser-derived *O*-acetyl-L-serine (OAS), and then Met, via a trans-sulfuration, is derived from Cys (Wirtz and Droux 2005). The influence of the abovementioned amino acid on glucosinolate and isothiocyanate production was studied in NO-6, BV-4 and AC-1 clones with the highest glucosinolate content (Table 1). Treatment of the cultures with the precursors, either individually or in combination, enhanced glucosinolate content (see Fig. 4), the most effective were Phe + Cys and Met + OAS combinations, which resulted in increased isothiocyanates production in the autolysates of the treated hairy roots.

Hairy roots of *B. verna* and *Ar. caucasica* with active glucosinolate-myrosinase system can be used both as experimental models and as a rich sources of gluconasturtiin and glucoiberverin, respectively as the single glucosinolate, and of their bioactive

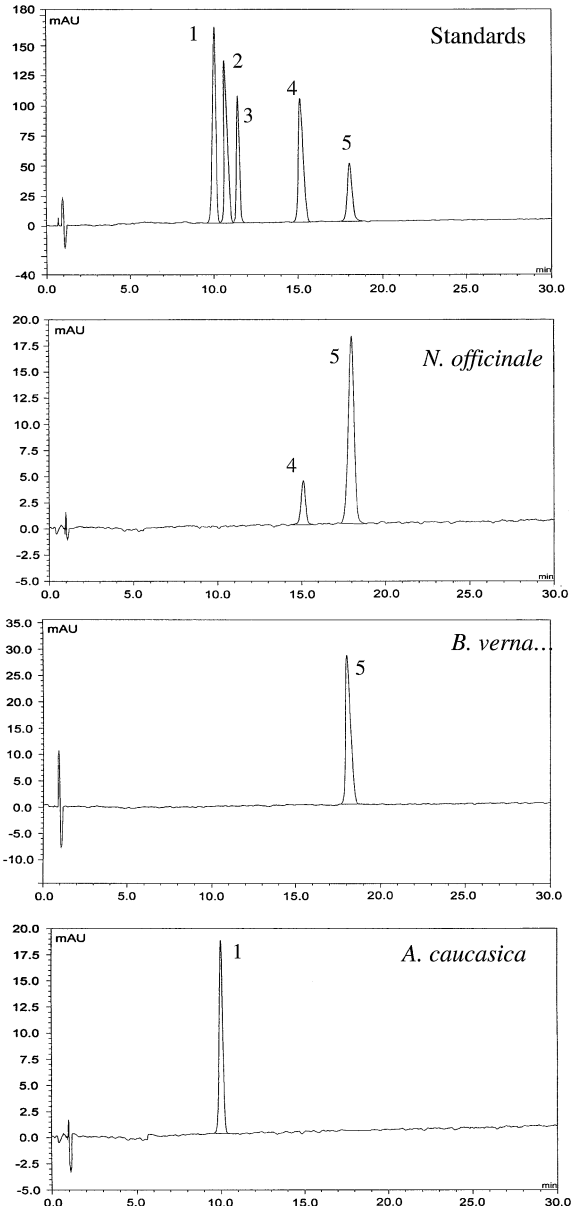


Fig. 2 Paired-ion chromatography of intact glucosinolates from hairy roots. Standards: 1, glucoiberverin; 2, sinigrin; 3, sinalbin; 4, glucotropaeolin; 5, gluconasturtiin. HPLC performed on Hypersil Gold C18 RP column (250 × 4.6 mm, 5 μm) coupled to an ODS Hypersil guard column (10 × 3 mm, 5 μm), developed isocratically with H_2O/acetonitrile (50:50, v/v) containing 5 mM tetradecyloammonium bromide, flow rate 3 cm^3 min^{-1}, at 229 nm

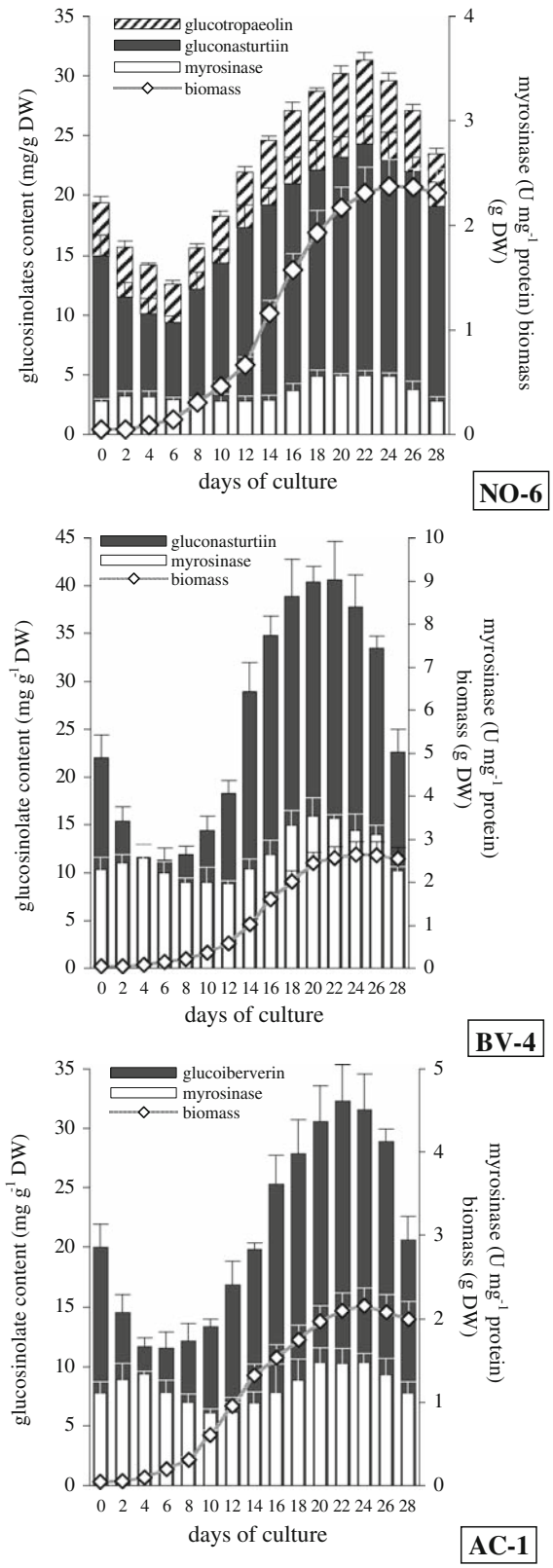
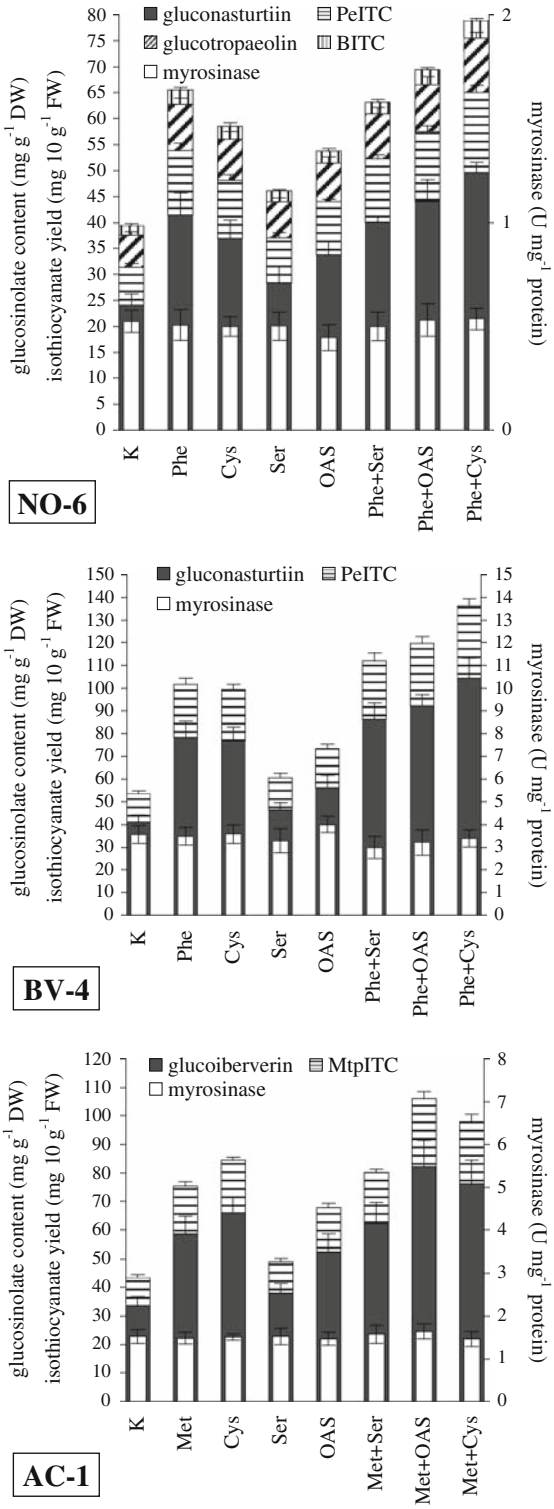


Fig. 3 Glucosinolate content, myrosinase activity and biomass growth in 28-day cultures of hairy root clones of *N. officinale* (NO-6), *B. verna* (BV-4) and *Ar. caucasica* (AC-1). Presented data are means (±SD) of three subcultures: 8th–10th day after treatment



derivatives—isothiocyanates. Availability of precursor amino acids seemed to be a factor limiting glucosinolate and isothiocyanate production in the studied hairy roots.

◀**Fig. 4** Effect of amino acid precursors (0.5 mM applied alone or in combination) on glucosinolate content, isothiocyanate yield and myrosinase activity on the 22nd day of culture (10 days after treatment) of hairy root clones of *N. officinale* (NO-6), *B. verna* (BV-4) and *Ar. caucasica* (AC-1): BITC, benzyl isothiocyanate; MtpITC, 3-(methylthio)propyl isothiocyanate; PeITC, 2-phenylethyl isothiocyanate. HPLC determination of isothiocyanates was performed on a Hypersil Gold C18 RP column (250 × 4.6 mm, 5 μm) coupled to an ODS Hypersil guard column (10 × 3 mm, 5 μm), with H_2O/acetonitrile (49:51v/v), at 0.8 cm^3 min^{-1}, and monitoring at 246 nm. Presented data are means (±SD) of three independent experiments

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