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Glucosinolate Profiles, Myrosinase and Peroxidase Activity in Horseradish (*Armoracia lapathifolia* Gilib.) Plantlets, Tumour and Teratoma Tissues

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Summary

In vitro grown plantlets, tumour and teratoma tissues of horseradish (*Armoracia lapathifolia* Gilib.) were compared with regard to glucosinolate profiles. Plantlets produced significantly higher amounts of total glucosinolates than tumour and teratoma tissues. The aliphatic glucosinolate sinigrin was quantitatively dominant. Plantlets also contained lower amounts of an aromatic glucosinolate, gluconasturtiin and indole compounds: glucobrassicin, 4-methylglucobrassicin and 4-hydroxyglucobrassicin. In tumour and teratoma tissues only these indole glucosinolates were detected. The activity of enzyme myrosinase (β-thioglucosidase) was significantly higher in plantlets than in teratoma. No myrosinase activity was recorded in tumour. Total peroxidase activity was 30–50 times higher in tumour and teratoma than in plantlets. The hypothesis that teratoma tissue with shoots is more similar to plantlets than to unorganised tumour has not been confirmed neither for glucosinolate profiles nor for peroxidase activity, but only for myrosinase activity.

Key words: *Armoracia lapathifolia* Gilib., glucosinolates, horseradish *in vitro* culture, myrosinase, peroxidase, teratoma, tumour

Introduction

Horseradish (*Armoracia lapathifolia* Gilib.), a popular spice with a pungent and lachrymatory odour, belongs to the family Brassicaceae. Roots of this plant species are the main source of peroxidase in the commercial production of this enzyme, widely used for different laboratory techniques. *In vitro* cultivated callus, hairy roots, tumour and teratoma tissues of horseradish have been proposed as an alternative source of peroxidase (*1–4*).

As all Brassicaceae, horseradish produces various sulphur-containing glucosides, *i.e.* glucosinolates. These compounds are classified in three classes, depending on the amino acid from which they were derived: (*i*) aliphatic/alkenyl glucosinolates, from methionine, (*ii*) aromatic glucosinolates from phenylalanine or tyrosine, and (*iii*) indole glucosinolates from tryptophan (*5,6*). Upon tissue disruption, glucosinolates, which are stored in vacuoles of specific phloem cells (*7*), are released and hydrolyzed immediately. The hydrolysis is catalyzed by myrosinase or β-thioglucosidase (EC 3.2.3.1). This enzyme is stored in separate cells (*8*). The nature of hydrolysis products and their biological activities depends on the structure of glucosinolate side chains, plant species and reaction conditions (*9,10*). A change of glucosinolate profiles by several environmental factors has brought forward differ-

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ent hypotheses regarding their potential role in plants. However, the most accepted opinion is that the glucosinolate-myrosinase system plays an important role in defense against herbivores and pathogens.

With mutant genes coding for glucosinolate biosynthesis, it has been demonstrated that glucosinolate compounds might be involved in plant developmental processes (*11–16*). Indole glucosinolates have been proposed as precursors of plant hormone indole-3-acetic acid (IAA). Indole-3-acetaldoxime, the first intermediate in the indole glucosinolate biosynthesis, is a product of the reaction catalyzed by enzymes CYP79B2 and CYP79B3. The compound was found to be a precursor of IAA and is considered to be the branch point between the two metabolic pathways (*12*). In addition, analysis of an *Arabidopsis* CYP79F1 knockout mutant showed that abolishing the formation of short-chain methionine-derived glucosinolates is accompanied by increased levels of IAA and cytokinin (*13,14,16*). Therefore, the possible interaction between glucosinolate metabolism and plant development is still unclear and opens a field for further research.

The aim of our study is to find out if tumour transformation and dedifferentiation of horseradish leaf cells is reflected on glucosinolate composition and if the backward cell differentiation and morphogenesis of teratoma shoots can reestablish the original glucosinolate pattern. Untransformed leaves, tumour and teratoma tissues are also compared with regard to myrosinase and peroxidase activities.

Material and Methods

Horseradish in vitro culture

Horseradish (*Armoracia lapathifolia* Gilib.) plantlets, teratoma and tumour tissues (*17,18*) were grown on the solid Murashige and Skoog (MS) (*19*) medium without any growth regulator. Primary tumours were induced by infecting leaf fragments of *in vitro* grown plantlets with a wild strain *Agrobacterium tumefaciens* B6S3. Tumour-inducing bacteria were eliminated by an antibiotic (Pyopen, Pliva, Croatia) treatment. Transformed tissues were subcultured on the solid MS medium every three weeks. Culture conditions were: 24 °C, 16-hour light, 8-hour dark period and irradiation of 33 μmol/(m^2·s).

Glucosinolate extraction

The extraction, isolation and desulphation of glucosinolates were carried out according to the procedure of the ISO method (*20*). Glucosinolates were extracted from 6–10 cm high plantlets and from tumour and teratoma tissues in their exponential phase of growth (10 days after subculturing). Plant samples were collected, pooled together, immediately freeze-dried and lyophilized. Five samples were analyzed for each of the three independent experiments. Lyophilized plant tissue (1 g) was homogenised and extracted twice with 70 % methanol in the water bath at 75 °C for 15 min by the addition of an internal standard benzyl glucosinolate (200 μL of 20 mM benzyl glucosinolate, *i.e.* glucotropeolin). Combined supernatants were concentrated on a rotary evaporator to approx. 2 mL and water was added to the volume of 5 mL. A volume of 1 mL of glucosinolate extract was treated with 100 mL of 1:1 0.5 M solution of lead acetate and 0.5 M solution of barium acetate. The supernatant was passed through an ion-exchange resin Fast DEAE Sepharose CL-6B microcolumn for desulphation with purified sulphatase (EC 3.1.6.1, type H-1, from *Helix pomatia*, Sigma, St. Louis, USA) (*21*). Desulphoglucosinolates were eluted with 1.5 mL of deionised water, usually in 3 washes of 0.5 mL.

HPLC analysis

Desulphoglucosinolate extracts were separated on Supelcosil C18 reversed-phase column (150×4.6 mm i.d, 5 μm) (Supelco Park, Belafonte, USA) using a Varian LC Star System equipped with a Star Solvent Delivery System 9010, Injector Rheodine 7125, Polychrom 9065 (UV--Diode Array Detector). The column was let at the ambient temperature. A two-component solvent system consisting of water (A) and 20 % acetonitrile in water (B) was used. A constant flow rate of 1 mL/min was employed with gradient elution: 0–1 min 100 % A, 1–20 min linear gradient change to 100 % B, 20–25 min linear gradient change to 100 % A and 25–30 min 100 % A. Detection was performed with UV-Diode Array Detector (UV-DAD) at 229 nm. Positive identification of desulphoglucosinolates (DSGSL) was accomplished by comparing the elution order (*20*) and UV-DAD peak spectral analyses. In order to calculate molar concentration of individual DSGSL, relative response factors were used to correct the absorbance differences between the internal standard (glucotropeolin) and other components in the extract.

Protein extraction and quantification

Soluble proteins were extracted from the leaves of 6–10 cm high plantlets and from tumour and teratoma tissues in their exponential phase of growth (10 days after subculturing). Five samples were analyzed for each of the three independent experiments. Tissue samples were homogenised in ice cold 0.1 M Tris/HCl buffer (pH=8.0) containing 17.1 % sucrose, 0.1 % ascorbic acid and 0.1 % cystein/HCl. Tissue mass (g) to buffer volume (mL) ratio was 1:5 for leaves, 1:1.2 for teratoma and 1:0.9 for tumour tissue. To remove phenols, insoluble polyvinylpyrrolidone (approx. 50 mg) was added to tissue samples before grinding. The homogenates were centrifuged for 15 min at 20 000×*g* and 4 °C. The supernatants were ultracentrifuged for 90 min at 120 000×*g* and 4 °C. Protein content of the supernatants was determined according to Bradford (*22*) using bovine serum albumin (BSA) as a standard. Supernatants were used for myrosinase and peroxidase activity measurements.

Myrosinase and peroxidase activities

Myrosinase activity was determined by measuring a degradation of sinigrin (Sigma, St. Louis, USA) by following the decrease in absorbance at 227 nm (*23*). The reaction mixture was incubated at 35 °C for 5 min before adding the protein extract. The activity was determined from a molar absorption coefficient of sinigrin ε_{227nm}=6784 M^{-1}·cm^{-1} and the enzyme activity was reported in μmol/(min·mg) protein.

The reaction mixture for peroxidase activity measurement contained 5 mM guaiacol and 5 mM H_2O_2 as substrates according to Siegel and Galston (*24*). Peroxidase activity was determined spectrophotometrically at 470 nm. The obtained rate of change in absorbance was then used to quantify the enzyme activity in the mixture using the molar absorption coefficient of tetraguaiacol (the oxidized product) (26.6 $mM^{-1}\cdot cm^{-1}$). The enzyme activity was reported in μmol/(min·mg) protein.

Statistical analysis

Statistical analysis was done by using the STATISTICA 7.1 software. Differences between the mean values were analyzed by ANOVA test ($p<0.05$).

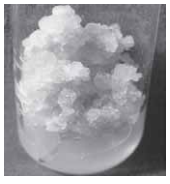
Results and Discussion

Glucosinolate patterns of horseradish plantlets and transformed tissues

The RPLC analysis of desulphoglucosinolates showed significantly higher total glucosinolate contents in plantlets than in tumour and teratoma tissues (Table 1).

An aliphatic glucosinolate 2-propenyl glucosinolate (sinigrin), making more than 80 % of total glucosinolates, was quantitatively dominant in plantlets. They also contained lower amounts of phenylethyl glucosinolate (gluconasturtiin), and three indole compounds, 3-indolylmethyl glucosinolate (glucobrassicin), 4-methoxy-3-indolylmethyl glucosinolate (4-methoxyglucobrassicin) and 4-hydroxy-3-indolylmethyl glucosinolate (4-hydroxyglucobrassicin. Neither sinigrin nor gluconasturtiin were detected in teratoma and tumour tissues. Certain degree of cell differentiation and tissue pattern formation in teratoma shoots was not sufficient to re-establish the sinigrin or gluconasturtiin biosynthesis. It might be blocked by genetic transformation due to T-DNA integration into horseradish genome. Transformed tissues produced only indole glucosinolates and their content was higher in unorganised tumour than in teratoma shoots. Among indole compounds, glucobrassicin was dominant and its level was approx. 4 times higher in tumour than in the leaves and twice higher than in teratoma shoots. Mevy *et al.* (*25*) compared glucosinolate contents in horseradish regenerants, suspension cells, embryoids and calli. They measured the highest level of glucobrassicin in calli and concluded that its production was the result of biochemical differentiation rather than of the medium composition. In contrast to calli, which are hormone dependent, tumour and teratoma are hormone autonomous. Rapid cell proliferation of tumours is promoted

Table 1. Glucosinolate content in horseradish plantlet and transformed tissues*

Tissue	Total content/(μmol/g dry mass)	Individual content/(μmol/g dry mass)**				
		SIN	GNT	GBS	4-CH_3O-GBS	4-OH-GBS
Leaves 	15.337±0.830	12.277±0.796	1.753±0.121	0.439±0.033	0.473±0.035	0.395±0.035
Teratoma 	1.686±0.044	0	0	0.842±0.077	0.596±0.036	0.247±0.004
Tumour 	3.371±0.085	0	0	1.682±0.060	1.107±0.066	0.582±0.031

*Values indicate mean±SD; *N*=5; LSD, least significant difference at $p>0.05$

**SIN=sinigrin (2-propenyl glucosinolate); GNT=gluconasturtiin (phenylethyl glucosinolate); GBS=glucobrassicin (3-indolylmethyl glucosinolate); 4-OCH_3-GBS=4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl glucosinolate); 4-OH-GBS=4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl glucosinolate)

by high concentrations of cytokinin and auxin, which are synthesized by enzymes encoded by genes of the T-DNA (*26*). In these transformed tissues indole glucosinolates might be involved in the pathway of indole-3--acetic acid (IAA) biosynthesis. Recently, several studies have brought more evidence of a link between indole glucosinolates and IAA. Bak *et al.* (*12*) demonstrated that cytochrome P450 CYP83B1 was a regulator of auxin production in *Arabidopsis* by controlling the flux of indole-3-acetaldoxime into IAA and indole glucosinolate biosynthesis. This enzyme is primarily involved in biosynthesis of indole glucosinolates (*27*). *Arabidopsis* overexpressing the CYP79B2 showed a significant increase of the two most abundant indoles glucobrassicin and 4-methoxyglucobrassicin (*28*). It remains to elucidate if indole glucosinolates recorded in horseradish transformants resulted from the similar biosynthetic pathway as the ones established for *Arabidopsis*.

In the recent study, a functional genomics approach has been applied to measure the changes in gene expression between *Arabidopsis thaliana* tumours and an uninfected plant. The most expressed changes were 56-fold upregulation of an auxin-responsive GH3 family gene and 49-fold downregulation in tumour tissues of the branched chain amino acid aminotransferase gene (*BCAT4*) (*29*). The BCAT4 catalyzes the transamination step initiating the chain elongation pathway of aliphatic glucosinolate biosynthesis (*30*). In addition, all genes involved in biosynthesis of glucosinolates were strongly down regulated, except the *CYP79B1*, which was unchanged. Drastic downregulation of *BCAT4* and other glucosinolate genes in *Arabidopsis* might be common gene regulation after a tumour formation induced by virulent agrobacteria, which could explain the lack of glucosinolates, especially the aliphatic ones, in transformed horseradish tissue.

Myrosinase and peroxidase activity

The soluble protein content per dry tissue mass was higher in the leaves than in teratoma or tumour tissues (Table 2). The specific myrosinase activity was approx. 7 times higher in the leaves than in teratoma tissue. No myrosinase activity was detected in the tumour. Peroxidase activity was 30–50 times higher in teratoma and tumour tissues than in the leaves.

As our previous results had shown (*3*), horseradish teratoma and tumour tissues had significantly higher peroxidase activity than leaves. Soudek *et al.* (*4*) also established the highest peroxidase activity in horseradish tumour tissue on the medium without growth regulators. Contrary to peroxidase, myrosinase activity was higher in the leaves than in teratoma and it was not measurable in the tumour. Both enzymes could be involved in auxin metabolism, peroxidase acting as IAA oxidase and myrosinase catalysing 3-indolylmethyl glucosinolate conversion to IAA. According to the literature data, glucosinolates and myrosinase are always present in plant tissues together (*9,10*). The lack of activity in the tumour was probably a consequence of tissue disorganisation, although it might also be that myrosinase was present but in an inactive form, as Western blot indicated (data not shown).

Table 2. Protein content, myrosinase and peroxidase activity in plantlet leaves and transformed tissue*

Tissue	Protein content/ (mg/g dry mass)	Myrosinase specific activity/ (μmol/(min·mg protein))	Peroxidase specific activity/ (μmol/(min·mg protein))
Plantlet leaves	95.73±3.62	0.732±0.0180	326.69±23.32
Teratoma	75.59±2.86	0.106±0.0029	16174.43±530.82
Tumour	54.92±1.26	–	10977.44±423.15

*Values indicate mean±SD; *N*=5; LSD, least significant difference at p>0.05

Conclusion

The obtained results show that *in vitro* grown horseradish tumour and teratoma tissues differ from the leaf in glucosinolate profiles and in myrosinase and peroxidase activities. Unorganised tumour and teratoma with certain degree of tissue pattern formation seem to be an interesting *in vitro* model to study indole glucosinolates and IAA biosynthesis as well as the glucosinolate-myrosinase system.

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