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Carbohydrase activities of the *rolC*-gene transformed and non-transformed ginseng cultures

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Abstract

The levels of activity of β-D-glucosidase, α-D-mannosidase, α- and β-D-galactosidase, 1,3-, 1,6-, 1,4-β-D-glucanases, 1,4-α-D-glucanase, fucoidanhydrolase and agarase were measured in extracts of non-transgenic and transgenic *Panax ginseng* cultures transformed with the *rolC* gene. Significantly increased levels of activity of β- and α-D-galactosidases and 1,3-β-D-glucanase were detected in *rolC*-gene transformed cells, compared to the control non-transformed cells, while levels of activity of other enzymes were unchanged. These, as well as the gel-permeation experiments, revealed that transformation of ginseng cells by the *rolC* gene could significantly affect activity of some carbohydrases and production of their molecular forms.
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1. Introduction

The *rolC* gene of *Agrobacterium rhizogenes* has been shown to be particularly efficient in stimulating ginsenoside production in *Panax ginseng* transformed cultures

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[1]. In tests with *rolC*-transgenic *Nicotiana tabaccum* and *Catharantus roseus* cultures, it was also shown that the total yield of secondary compounds could be greatly increased [2,3]. The mechanism by which the *rolC* gene influences secondary metabolism is unclear. The relevance of *rolC*-gene transformation with respect to primary metabolite production in cultured plant cells has never been demonstrated.

We wanted to investigate the effect of the *rolC* gene integration on enzyme activity in ginseng transgenic cells. For this work we applied our model system, which consists of non-transformed cells of *P. ginseng* (1c cell culture) and 1c-derived transgenic cultures (1c-*rolC*) [1]. Enzymes for carbohydrate metabolism were chosen because of their potential use for human treatment. For example, plant α-D-galactosidases that can remove the α-1,3-bond galactose residues from glycoproteins and erythrocytes of group B(III) blood, thus, converting them into the substances and erythrocytes of group O(H), are of great practical interest [4]. Another aspect concerning potential of carbohydrase enzymes for practical purposes has recently been reported. Some carbohydrases, such as an endo-1→3-β-D-glucanase from Pacific Coast algae, have been shown to cleave non-active laminaran polysaccharides to its active glucan derivatives, possessing a strong immunomodulatory activity [5].

2. Experimental

2.1. Chemicals

Amylose, agarose, CM-cellulose, as well as *p*-nitrophenyl derivatives of β-D-gluco-, α-D-manno-, α- and β-D-galactopyranosides, were purchased from Serva. Laminaran, fucoidan and pustulan were obtained from the brown seaweeds *Laminaria cichorioides* and *Fucus evanescens* and the lichen *Umbellicaria russica*, respectively [6]. Standard proteins from Pharmacia and Sigma were used for column calibration. Sephadex G-25 and Toyopearl HW 65 were obtained from Pharmacia and Toyo Soda (Japan), respectively.

2.2. Cell cultures and media

The origin of the initial non-transformed culture 1c of *P. ginseng* C.A. Meyer, the transformation of 1c culture and the establishment of the 1c-*rolC*-root cultures has been described in the previous paper [1]. The 1c-*rolC*-II line 2c3, which is incapable of forming roots, was obtained from the 1c-*rolC*-II callus culture via selection of root-free aggregates in several passages [7]. Cultural conditions were identical for both transgenic and control cultures; they were grown in liquid W_{IBA} medium in the dark for 21 days at 25 °C [1].

2.3. Enzyme activity determination

Ginseng tissues (0.5 g) were frozen at −20 °C, crushed in a mortar and extracted with 1 ml of 0.05 M Na-succinate buffer, pH 5.2. The extracts were centrifuged

and supernatant portions (1 ml) were passed through a Sephadex G-25 column (1×8 cm) to remove low-molecular weight substances.

Glycosidase activities were tested using the corresponding *p*-nitrophenyl-glucopyranosides as substrate [8]. The reaction mixture containing 50 μl of the extract and 450 μl of the corresponding substrate (1 mg/ml) was dissolved in 0.05 M Na-succinate buffer (pH 5.2), and incubated at 37 °C for 2 h. The reaction was stopped by addition of 2 ml of 1 M Na_2CO_3, and the absorbance of *p*-nitrophenol was measured at 410 nm.

Glycanase activities were measured by determination of reducing sugars released from corresponding substrates [8]. The reaction mixture, containing 100 μl of the extract, 200 μl of the substrate (1 mg/ml) and 200 μl 0.05 M Na-succinate buffer (pH 5.2), was incubated for 18 h at 37 °C.

Molecular masses of the enzymes were evaluated by gel-filtration chromatography using Toyopearl HW 65 column (1×70 cm), equilibrated with 0.05 M Na-succinate buffer (pH 5.2). A 1-ml portion of extract was placed on a column and eluted with 0.05 M Na-succinate buffer (pH 5.2). The enzymes were detected by determination of their activities.

3. Results and discussion

Extracts from cultured ginseng tissues were prepared and assayed for glycosidase (β-D-glucosidase, α-D-mannosidase, β- and α-D-galactosidase) and glycanase (1,3-β-D-glucanase, 1,6-β-D-glucanase, 1,4-β-D-glucanase, 1,4-α-D-glucanase, fucoidan-hydrolase and agarase) activities in several subsequent experiments, which have been performed to ensure statistical validity of the results. The α-D-mannosidase level in ginseng tissues was found to be the highest among all activities tested (Table 1). High levels of β- and α-D-galactosidase activities were found in the transgenic root cultures. Transgenic tissues showed an increase of β-D-glucosidase activity compared with the 1c control culture (Table 1). However, the increase was statistically insignificant for two transgenic root cultures because of the high variability of their activities from one subculture to another. Our data are in a good agreement with studies describing *exo-O*-glycosylhydrolase activities of the natural ginseng roots: among 16 activities tested, only α-, β-D-galactosidase, α-L-arabinosidase, β-D-fucosidase, α-D-mannosidase and *N*-acetyl-β-D-glucosaminidase were detected [9].

To test whether a polysaccharide-hydrolase activity was changed in transgenic cultures compared with the non-transformed culture, we used laminaran, pustulan, CM-cellulose, amylose, fucoidan and agarose as substrates. As a rule, the ability of ginseng tissue extracts to hydrolize these substrates was low, although moderate activities of 1,3-β-D-glucanase and 1,4-α-D-glucanase were detected (Table 1). The only polysaccharide-hydrolase activity that was increased as a result of transformation was the 1,3-β-D-glucanase activity (Table 1).

The *rolC*-callus culture was included in the analysis to investigate whether the enzyme activity would be dependent upon the level of cell differentiation. We have found some differences between 1c-*rolC*-root lines, as well as between transgenic

Table 1
Carbohydrase activities of 1c calluses and 1c-derived transgenic ginseng tissues

Carbohydrase	Substrate	Activity, nM h^{-1} g^{-1} FW[a]				
		1c	1c-*rolC*-II roots	1c-*rolC*-III roots	1c-*rolC*-IV roots	1c-*rolC*-II callus (2c3 line)
Glycosidases						
β-D-Glucosidase	*p*-Nitrophenyl-β-D-glucopyranoside	140±60	470±120*	360±190	200±90	400±60*
β-D-Galactosidase	*p*-Nitrophenyl-β-D-galactopyranoside	160±70	1030±100**	1340±180**	1000±310*	590±110*
α-D-Galactosidase	*p*-Nitrophenyl-α-D-galactopyranoside	400±50	820±50*	1140±220*	890±180*	750±290*
α-D-Mannosidase	*p*-Nitrophenyl-α-D-mannopyranoside	1370±900	1400±400	3380±1630	2370±1800	2250±730
Glycanases						
1,3-β-D-Glucanase	Laminaran	120±30	260±40**	310±40**	370±20**	230±20**
1,6-β-D-Glucanase	Pustulan	60±20	35±12	30±7	30±10	40±20
1,4-β-D-Glucanase	CM-cellulose	40±10	22±8	60±50	50±40	30±20
1,4-α-D-Glucanase	Amylose	320±70	290±170	260±40	210±40	190±60
Fucoidanhydrolase	Fucoidan	60±20	35±8	70±40	60±30	30±20
Agarase	Agarose	35±8	65±20	70±15	50±20	nd[b]

[a] Mean values ± S.E.M. based on three separate experiments (with three replications each).
[b] nd: Not detectable.
* $P < 0.05$ vs. control 1c culture, Student's *t*-test.
** $P < 0.01$ vs. control 1c culture, Student's *t*-test.

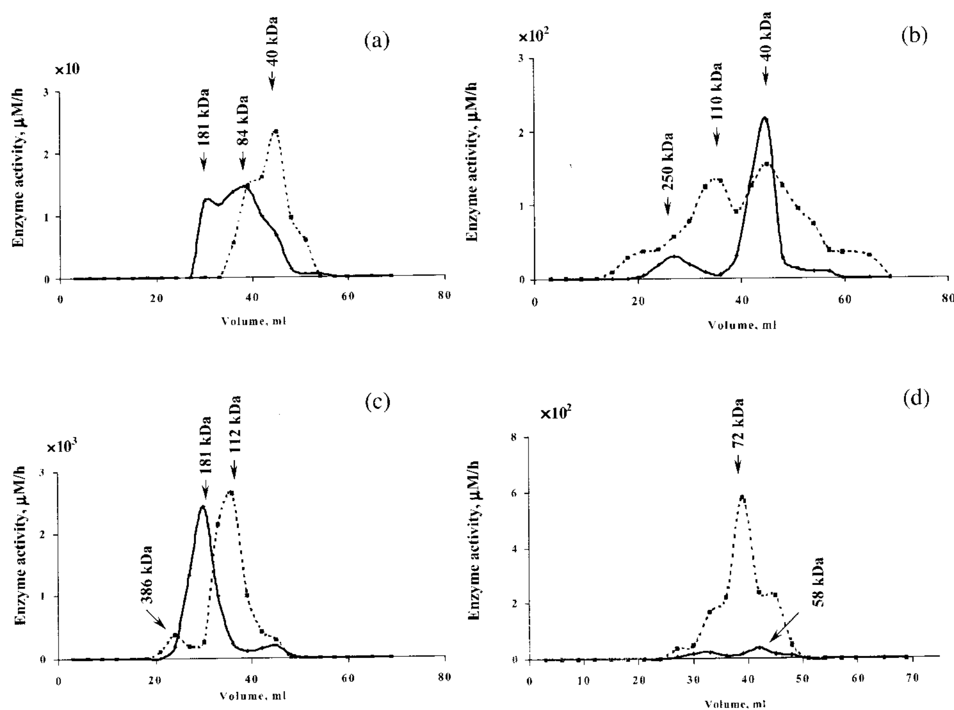

Fig. 1. Elution profiles of β-D-glucosidase (a), 1,3-β-D-glucanase (b), α-D-mannosidase (c) and β-D-galactosidase (d) activities fractionated from crude extracts of 1c culture (____) and 1c-*rolC*-II culture (-------) by use of a Toyopearl HW 65 column.

root lines and 2c3 callus culture in their carbohydrase activities, but we have concluded that the changes of carbohydrase activities, in general, cannot be explained by changes in the level of cell differentiation, e.g. root formation (Table 1).

Molecular forms of the enzymes were studied by gel-permeation chromatography of extracts from 1c calluses and 1c-*rolC*-II roots. The experiment was performed using a column calibrated with standard proteins in the range of 16–300 kDa. The molecular masses of 1c-callus β-D-glucosidases were estimated to be 84 and 181 kDa, while the molecular mass of β-D-glucosidase from the 1c-*rolC*-II roots was 40 kDa (Fig. 1a). The levels of β-D-glucosidase activities recovered from the columns were low for both transformed and non-transformed tissues.

The 1,3-β-D-glucanase activity was recovered from the column as a main peak of molecular mass 40 kDa for the 1c culture and two peaks of 40 and 110 kDa proteins for the 1c-*rolC*-II culture. (Fig. 1b) Apparently, a two-fold increase in 1,3-β-D-glucanase activity in 1c-*rolC*-II culture, compared with 1c culture (Table 1), can be explained by expression of the 110-kDa protein. Since β-D-glucosidase activity coeluted with β-1,3-glucanase activity, we could not exclude a possibility that the 40-kDa β-1,3-D-glucanase possessed both glucanase and glucosidase

activities because it was well established that some 1,3-β-D-glucanases could have a marked specificity for β-D-glucosides [10,11]. Therefore, true β-D-glucosidase activities of ginseng tissues could be less than shown in Table 1.

Mannosidase-related activity of the 1c-*rolC*-II root extract was localized in the fraction of molecular mass 112 kDa (Fig. 1c). α-Mannosidase of the transgenic roots appears instead 181-kDa protein, which originally was present in the 1c culture.

Two peaks of β-D-galactosidase activity were resolved when the 1c-callus extract was subjected to column chromatography. As in the case of mannosidase activity, the transgenic tissue showed quite different profiles of β-D-galactosidase activity, which have been revealed as a group of proteins with similar molecular masses. Among them, the most abundant 72-kDa protein was recovered (Fig. 1d). Thus, gel-permeation experiments seem to show that changes of carbohydrase activities in transgenic cells are the result of expression of new enzyme isoforms rather than of the induction of existing enzymes.

This work provides the evidence that activity of some carbohydrases could be increased in ginseng cells transformed by the *rolC* gene. Among them, the level of α-D-galactosidase activity was two–three-fold higher than that of the respective control. This enzyme may be useful for modification of human blood cells. The mechanism(s) of the *rolC* gene action on the level of carbohydrases in ginseng cells should now be investigated.

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