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The *rolC* gene induces expression of a pathogenesis-related β-1,3-glucanase in transformed ginseng cells

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Abstract

The *Agrobacterium rhizogenes rolC* oncogene is capable of stimulating production of secondary metabolites in transformed plant cells that suggest its possible involvement in plant defense reactions. We tested whether the gene could also affect production of pathogenesis-related proteins. Using a well-known group of PR-proteins, such as β-1,3-glucanases, we observed a 10-fold increase in total β-1,3-glucanase activity in *rolC*-transformed *Panax ginseng* cells compared with normal cells. The increase was due to the production of a salicylic acid-activated β-1,3-glucanase isoform. We isolated cDNA of the corresponding β-1,3-glucanase gene (*Pg-glu1*), which shared 38–60% sequence identity with previously reported sequences of plant β-1,3-glucanases at the protein level. Levels of *Pg-glu1* mRNA transcripts were tightly correlated with expression of the *rolC* gene. Our data, together with previously reported information, indicate that *A. rhizogenes* can activate plant defense reactions via expression of T-DNA oncogenes.

Keywords: *Panax ginseng*; *rolC* gene; Pg-glu1; β-1,3-Glucanase; PR-proteins

1. Introduction

β-1,3-Glucanases (EC 3.2.1.39) comprise a PR-2 family of plant defense proteins. In many plants, β-1,3-glucanases are rapidly accumulated following pathogen attack, after elicitor treatment, and in response to stress hormones (Leubner-Metzger and Meins, 1999). The enzyme is involved not only in the inducible defense response of higher plants, but also participates in normal physiological processes, such as pollen development, embryogenesis, and seed germination (Leubner-Metzger and Meins, 1999).

The *rolC* gene of plant pathogen *Agrobacterium rhizogenes* plays an essential role in the development of hairy root disease and its expression in plants causes substantial morphological and biochemical alterations (Spena et al., 1987; Faiss et al., 1996). In addition to known properties of the *rolC* gene, a function of the *rolC* gene in formation of shoot meristems has been recently discovered (Gorpenchenko et al., 2006), thus suggesting its important role in formation of pluripotent stem cells. The *rolC* gene also affects normal animal (sea urchin) embryo development, causing formation of teratoma-like structures in transformed embryos (Bulgakov et al., 2006). Despite many efforts, the biochemical function of the RolC protein is not determined (Nilsson and Olsson, 1997).

A stimulatory effect of the gene on secondary metabolism was demonstrated by investigations with different groups of secondary metabolites, such as tropane alkaloids (Bonhomme et al., 2000), pyridine alkaloids (Palazón et al., 1998a), indole alkaloids (Palazón et al., 1998b), ginsenosides (Bulgakov et al., 1998), and anthraquinones (Bulgakov et al., 2002a, 2003). However, some examples exhibit

Abbreviations: SA, salicylic acid; MeJA, methyl jasmonate.

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decreased levels of antimicrobial polyphenols (rabdosiin and rosmarinic acid) and naphthoquinones (shikonin derivatives) in *rolC*-transformed *Eritrichium sericeum* and *Lithospermum erythrorhizon* cell cultures (Bulgakov et al., 2005). These data suggest that *A. rhizogenes* can perturb secondary metabolism of host plants by a T-DNA oncogene-mediated signaling mechanism.

In *Panax ginseng* root and callus cultures transformed with the *rolC* gene, increased β-1,3-glucanase activities were detected, whereas activities of other glucanases tested, such as 1,6-β-D-glucanase, 1,4-β-D-glucanase, and 1,4-α-D-glucanase, which are known to be unrelated to defense proteins, were unchanged (Bulgakov et al., 2002b).

Since understanding which genes or biological processes define and distinguish associative and infective interactions between plants and pathogenic microorganisms is a major problem (Pühler et al., 2004), we were interested in obtaining more detailed information about the possible involvement of the *rolC* gene in induction of marker PR-proteins such as β-1,3-glucanases. In the present investigation, we show that expression of the *rolC* gene in transformed ginseng cells is tightly correlated with the expression of a β-1,3-glucanase gene and a corresponding increase in β-1,3-glucanase activity.

2. Results

2.1. Transformed P. ginseng tissues and parameters of rolC gene expression

Previously, we reported a detailed description of the cultures which have been used in the present investigation (Bulgakov et al., 1998; Gorpenchenko et al., 2006). The origin of these cultures is presented in Table 1. The *rolC* gene was introduced to a 1c callus culture and five independently transformed primary *rolC*-tumors (1c-*rolC*-I, 1c-*rolC*-II, etc.) were established. From each of these primary tumor cultures, root cultures were established and two of them (1c-*rolC*-II and 1c-*rolC*-III) were used in the present investigation (Table 1). One of the primary tumor cultures, the 1c-*rolC*-II culture, was used to select root-forming and non-root-forming calli. Two callus cultures (2c2 and 2c3) were established from the non-root-forming calli by selection of vigorously-growing cell clusters. The 2c2 is a non-differentiated callus culture while the 2c3 culture is capable of forming somatic embryos (Gorpenchenko et al., 2006). Additionally, the 2cR3 and 2cR33 callus lines (secondary tumors) of presumably clonal origin were established from tips of the 1c-*rolC*-II roots. This combination of cultures allowed us to test glucanase activities and glucanase gene expression in cultures with different expression of the *rolC* gene. For the purpose of the present investigation, it is important to note that all of the cultures used contain a full-length and non-mutated *rolC* gene (Gorpenchenko et al., 2006).

2.2. Molecular analysis of the ginseng β-1,3-glucanase gene

A search in GenBank revealed that there are no known *P. ginseng* β-1,3-glucanases characterized at the molecular level. We designed degenerative primers according to published sequences of PR-2 glucanases of five different plant species (see Section 4). The forward primer was designed to react with a conserved sequence encoding the first catalytic (nucleophilic) Glu residue in plant β-1,3-glucanases (Henrissat et al., 1995). The reverse primer reacted with another conserved part of plant glucanase genes containing bases for the acid catalyst Glu residue. The expected RT-PCR product of about 420 bp was barely detectable or not detectable (in some experiments) when we analyzed the 1c and 1c-vector cultures, whereas the *rolC*-transgenic cultures showed one clear transcript of 420 bp (Fig. 1).

Table 1
Origin of *rolC*-transformed *Panax ginseng* cultures, their β-1,3-glucanase activities, and relative strength of *rolC* and *Pg-glu1* expression

Culture	Origin of the culture	β-1,3-Glucanase activity, pkat/g fresh wt	*rolC* expression[a]	*Pg-glu1* expression[a]
1c	Initial callus line	5.3 ± 0.8	nd[b]	0.03 ± 0.01
1c-vector	The 1c calli transformed with pPCV002	5.4 ± 1.1	nd[b]	0.03 ± 0.02
1c-*rolC*-II	Independently established cultures of primary tumors	25.0 ± 6.4	2.9 ± 0.4	0.31 ± 0.06[c]
1c-*rolC*-III		15.7 ± 2.2	2.5 ± 0.3	0.20 ± 0.06
1c-*rolC*-IV		8.7 ± 1.1	1.5 ± 0.1	0.09 ± 0.03
1c-*rolC*-V		16.5 ± 3.1	2.7 ± 0.3	0.22 ± 0.07
2c2	1c-*rolC*-II selected non-root-forming callus lines	10.1 ± 3.2	1.6 ± 0.2	0.09 ± 0.04
2c3		63.8 ± 10.6[c]	2.6 ± 0.4	0.70 ± 0.06[c]
2cR33	1c-*rolC*-II derived callus lines of clonal origin	56.0 ± 6.2[c]	9.2 ± 1.1	0.80 ± 0.17[c]
2cR2		69.0 ± 3.9[c]	10.1 ± 1.5	0.82 ± 0.19[c]
1c-*rolC*-II root culture	Independently established hairy root cultures	72.0 ± 6.9[c]	12.1 ± 1.2	1.21 ± 0.21[c]
1c-*rolC*-III root culture		78.4 ± 8.1[c]	11.0 ± 1.5	1.13 ± 0.20[c]

The data are based on 3–5 separate experiments with 3 replicates each.
[a] mRNA quantification was made using DNA microchip technology.
[b] Not detectable.
[c] $P < 0.05$ *vs.* values of the control (1c-vector) culture, Student's *t*-test.

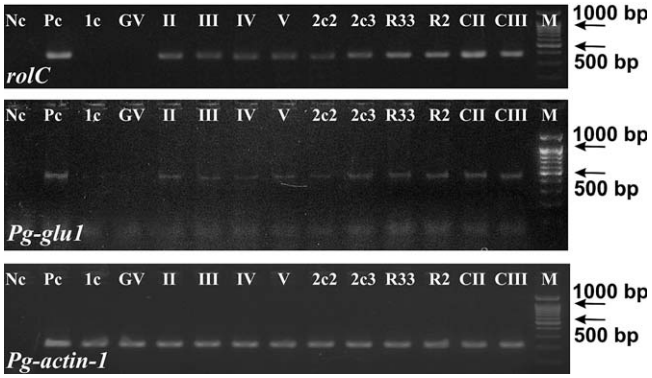

Fig. 1. RT-PCR analysis of the *rolC* and*Pg-glu1* transcripts in the transformed *Panax ginseng* cultures. *Nc* negative control (PCR mixture without plant DNA); *Pc* positive control (for *rolC* line: PCR product after amplification with pPCV002-*rolABC*; for *Pg-glu1* and *Pg-actin-1* lines: PCR products after amplification with 1c callus DNA); *GV* 1c-*rolC*-vector culture; *II*, *III*, *IV*, *V* cultures of 1c-*rolC* primary tumors; 2*c*2, 2*c*3 selected lines of 1c-*rolC*-II primary tumors; *CII*, *CIII* 1c-*rolC*-II and 1c-*rolC*-III root cultures; *R*33, *R*2 lines of secondary 1c-*rolC*-II (root-derived) tumors.

No amplification of mRNAs with the primers was observed when the RT step was omitted.

The isolated cDNA fragment was sequenced. Comparison of the deduced amino acid sequence of this fragment with those in GenBank indicates that the gene encodes β-1,3-glucanase. In order to recover the full-length transcript, RACE-PCR was used. 3′ RACE recovered the remaining 3′ part of the glucanase mRNA, whereas 5′ RACE was unsuccessful because of interference with abundant glucose phosphate isomerase transcripts (data not shown). Therefore, we performed another amplification reaction with a degenerative primer reactive with a sequence located near the 5′ end of the gene. As a result, we isolated and sequenced approximately 81% of the full-length transcript (Supplementary Fig. 1).

This β-1,3-glucanase gene, identified in *P. ginseng* cells for the first time and designated *Pg-glu1* (GenBank Accession No. DQ015705), shares 38–60% sequence identity with previously characterized plant β-1,3-glucanases at the protein level (Table 2). The deduced amino acid sequence of the *Pg-glu1* gene shows 38% identity with *Arabidopsis* β-1,3-glucanases BG4 and BG5 (Delp and Palva, 1999). These glucanases are thought to participate in normal plant development but are not activated by pathogens, stress, and SA, distinguishing them from the pathogenesis-related *Arabidopsis* β-1,3-glucanases, such as BG2. BG2 is more homologous to Pg-glu1 (50% identity) than BG4 and BG5 (Table 2). A higher level of identity, up to 60%, was found to the defense β-1,3-glucanases of tobacco, tomato, and potato (Table 2), as well as glucanases of nine other distantly related plant species which do not belong to Solanaceae (data not shown). The *Pg-glu1* also showed relatively high identity with *Cichorium* hybrid CG1 (60%) and tobacco stylar matrix β-1,3-glucanases SP41a (57%) and SP41b (57%), which do not appear to be involved in plant defense and have been referred as ''PR-like'' proteins (Leubner-Metzger and Meins, 1999). Thus, ginseng Pg-glu1 exhibits a clear amino acid identity to previously characterized plant β-1,3-glucanases.

2.3. Expression of rolC in transformed cells coincides with Pg-glu1 expression and a corresponding increase in β-1,3-glucanase activity

Using laminaran as a substrate, we determined the β-1,3-glucanase activity in samples of different callus and root cultures of *P. ginseng* transformed with the *rolC* gene. Determination of the pH-dependence of β-1,3-glucanase activities in these cultures revealed an optimal pH at values between 4.5 and 6.5. Therefore, the measurements were performed at pH 5.4. In the tested samples, the *rolC* and *Pg-glu1* transcript levels were also determined. Both the 1c and 1c-vector cultures showed low glucanase activities (Table 1). Slightly increased β-1,3-glucanase activities were detected in those *rolC*-transformed cultures, where the *rolC* gene was transcribed at low levels. In contrast, the actively *rolC*-expressing cultures showed about 10-fold higher β-1,3-glucanase activities compared with the vector culture. This elevation well correlated with expression of the *Pg-*

Table 2
Identities of the *Pg-glu1* amino acid sequence with plant glucanases

β-1,3-Glucanase gene	Accession number in GenBank	Identity (%)	Characteristics	Reference
Panax ginseng Pg-glu1	DQ015705	–	SA-inducible	This work
Cichorium hybrid *CG1*	AJ249292.1	60	Induced during embryogenesis	Helleboid et al. (2000)
Tomato *E13A*	Q01412	60	Pathogen-inducible	van Kan et al. (1992)
Tobacco *GL9*	M60460	59	Pathogen and SA-inducible	Linthorst et al. (1990), Van de Rhee et al. (1993)
Tobacco *cI101*	C38257	59	Pathogen and SA-inducible	Linthorst et al. (1990)
Tobacco *Sp41a*	X54430	57	Stylar-specific, not inducible by pathogen	Ori et al. (1990)
Tobacco *Sp41b*	X54431	57	Stylar-specific, not inducible by pathogen	Ori et al. (1990)
Potato *gluB*	AJ009932	57	Pathogen-inducible	Mac et al. (2004)
Tobacco *GL153*	M60463	56	Pathogen-inducible	Ward et al. (1991)
Tobacco *PR-2d* (*PR-Q′*)	X54456	55	Pathogen and SA-inducible	Hennig et al. (1993)
Nicotiana plumbaginifolia gn1	M38281	55	Pathogen and SA-inducible	Castresana et al. (1990)
Arabidopsis *BG2*	M58463	50	Pathogen and SA-inducible	Dong et al. (1991)
Arabidopsis *BG5*	X79694	38	Not inducible by pathogens and SA	Delp and Palva (1999)
Arabidopsis *BG4*	X79694	38	Not inducible by pathogens and SA	Delp and Palva (1999)

Table 3
Effect of salicylic acid and methyl jasmonate on growth and β-1,3-glucanase activity of *Panax ginseng* 1c culture

Treatment	Fresh callus wt.	β-1,3-Glucanase activity, pkat/g fresh wt	*Pg-glu1* expression[a]
Non-treated cells	1.50 ± 0.12	5.6 ± 0.9	0.02 ± 0.01
SA (50 μM)	0.80 ± 0.06	22.4 ± 3.8[b]	0.14 ± 0.02[b]
MeJA (10 μM)	0.75 ± 0.08	8.4 ± 0.8	0.04 ± 0.01

The 1c culture was grown for 30 days before harvesting and analysis.
[a] mRNA quantification was made using DNA microchip technology.
[b] $P < 0.05$, Student's *t*-test.

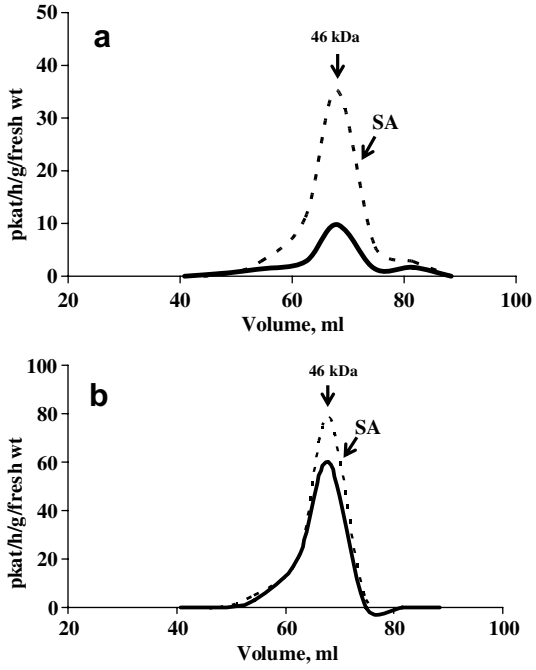

Fig. 2. Effect of salicylic acid (SA) on β-1,3-glucanase activity (*x*-axis) of the 1c culture (a) and the *rolC*-transformed 2c3 culture (B) of *Panax ginseng*. Crude protein extracts from the calluses, which were grown for 30 days in the presence of SA (50 μM), were analyzed by gel-permeation chromatography as indicated in Section 4.

glu1 gene (Table 1 and Fig. 1). These results indicate that expression of the *rolC* gene is sufficient to activate expression of the β-1,3-glucanase gene and elevates β-1,3-glucanase activity.

2.4. Pg-glu1 encodes a SA-dependent but MeJA-independent glucanase

Since SA induces genes of pathogenesis-inducible β-1,3-glucanases, this plant defense hormone serves as a marker for glucanases involved in defense reactions (Castresana et al., 1990; Jung et al., 1993; Hennig et al., 1993). Jasmonates are known to induce genes encoding proteinase inhibitors, defensin, thionin, and some basic PR-proteins (Zhou, 1999). We determined whether salicylic acid and methyl jasmonate could affect β-1,3-glucanase activity in control and *rolC*-transformed ginseng cultures. SA caused a 4-fold

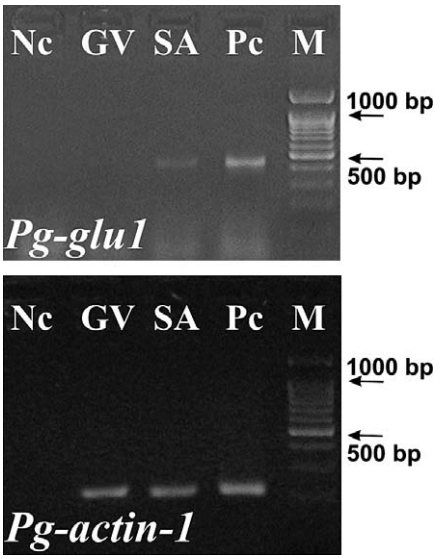

Fig. 3. Expression of *Pg-glu1* in the SA-treated 1c-vector culture (*GV*). Lane designations are the same as in Fig. 1.

induction of the total β-1,3-glucanase activity in the 1c culture (Table 3). The increase was exclusively due to enhanced production of β-1,3-glucanase with an apparent molecular mass of 46 kDa (Fig. 2). MeJA appears to be ineffective in this system (Table 3).

To determine whether the SA-induced increase of the β-1,3-glucanase activity was caused by enhanced expression of the *Pg-glu1* gene, we extracted mRNA from the SA-treated 1c calli and performed RT-PCR analysis. The analysis yielded the same 420 bp fragment as found in *rolC*-transformed cells (Fig. 3). The identity of this fragment as the *Pg-glu1* gene was confirmed by cDNA sequencing. We interpreted these results as an indication that *Pg-glu1* belongs to pathogenesis-related β-1,3-glucanases.

3. Discussion

Bacterium–plant interactions can be subdivided into three classes: pathogenic, associative, and symbiotic (Pühler et al., 2004). The current data on plant–microbe interaction suggest that pathogens often develop strategies aimed to suppress plant defense responses (Pühler et al., 2004; Nomura et al., 2005). *A. tumefaciens*, which are closely related to *A. rhizogenes*, can detoxify hydrogen peroxide, a primary component of plant oxidative burst, and suppress induction of the hypersensitive response in plants (Escobar and Dandekar, 2003). The effect of single *A. tumefaciens* oncogenes on plant defense reactions is largely unknown. Gális et al. (2004) reported that the *A. tumefaciens* AK-6*b* oncogene was capable of modulating the synthesis of phenolic secondary metabolites in tobacco. The authors did not reveal a correlation between mRNA levels of the AK-6*b* gene and key genes of phenylpropanoid

metabolism and proposed that the oncogene perturbs reactions generating CoA-derivatives of phenolic metabolites.

An example of associative bacteria is plant-growth-promoting rhizobacteria (such as *Pseudomonas fluorescens*), which are characterized by their ability to colonize plant roots without causing disease and to confer benefit to the plant. This benefit generally comes through the suppression of pathogens (Pühler et al., 2004). One of the biocontrol traits is the production of anti-fungal metabolites, including secondary metabolites.

Rhizobia are symbiotic soil bacteria of the family Rhizobiaceae. Rhizobial stimulation of transient or localized plant-defense responses has been reported for various legumes, especially during ineffective symbioses (e.g. Vasse et al., 1993). However, recent evidence indicates that single genes of *Rhizobium* could repress plant defense reactions. The *nopL* gene of *Rhizobium* species NGR234 blocks inducible plant defense responses (Bartsev et al., 2004). In particular, expression of the *nopL* gene in tobacco caused a 10-fold inhibition of β-1,3-glucanase activity.

Taking these data into account, one could expect that *A. rhizogenes*, a typical plant pathogen, would have a lifestyle strategy aimed at inhibiting the defense reactions of host cells. However, in our experiments, one of the important oncogenes of *A. rhizogenes*, the *rolC* gene, caused an opposite effect. In the *rolC*-transformed *P. ginseng* calli, expression of the *Pg-glu1* gene was specifically activated (Fig. 1 and Table 1). *Pg-glu1* most probably belongs to the PR-glucanase family, because the deduced amino acid sequence of *Pg-glu1* shares high identity with plant PR-glucanases (Table 2) and expression of the *Pg-glu1* gene is activated by SA in non-transformed calli (Fig. 3). The enhanced expression of the *rolC* gene in transformed ginseng cells correlated with the enhanced expression of the ginseng *Pg-glu1* β-1,3-glucanase gene and a corresponding increase in β-1,3-glucanase activity (Table 1).

These results are in agreement, with one exception (Bulgakov et al., 2005), with the observation that the *rolC* gene could ensure a stimulatory effect on secondary metabolism (Palazón et al., 1998a,b; Bulgakov et al., 1998, 2002a,b, 2003; Bonhomme et al., 2000). Our results indicate that a list of *rolC*-stimulated secondary metabolites can be expanded by including such important phytoalexins as stilbenes (in *rolC*-transformed cultures of *Vitis amurensis*), isoflavones, and prerocarpans (in *rolC*-transformed cultures of *Maackia amurensis*) (data not shown). In ginseng calli, expression of the *rolC* gene caused a significant increase in ginsenoside production (Bulgakov et al., 1998). Moreover, numerous investigations with wild-type *A. rhizogenes*-transformed hairy roots have conclusively shown increased levels of secondary metabolites in transformed roots (Sevon and Oksman-Caldentey, 2002). Another *A. rhizogenes* oncogene, the *rolD* gene, was also shown to be involved in activation of plant defense responses (Bettini et al., 2003). Thus, it is evident that *A. rhizogenes* can manipulate defense pathways in transformed plant cells via expression of T-DNA oncogenes. Regarding the physiological importance for such a function of the *rolC* gene, one can hypothesize that activation of defense reactions in transformed plants could provide advantages for *A. rhizogenes* by inhibiting concurrent soil-borne microorganisms, such as fungi. An example is known where expression of the *rolC* gene in genetically modified hybrid aspen plants could suppress mycorrhizal colonization of roots by four fungal species (Kadorf et al., 2002).

Although a general rule postulates that plant pathogenic bacteria inhibit defense reactions in host plants while associative and symbiotic bacteria activate defense reactions, more and more evidences indicate exceptions from the rule, providing a basis for the hypothesis (Pühler et al., 2004) that there is a continuum from pathogenic to associative interactions. Our results provide corroborative evidence for this hypothesis indicating that the plant pathogen can manipulate host defense reactions in a manner which shares some similarity with associative and symbiotic interactions.

4. Experimental

4.1. Chemicals

Laminaran was obtained from the brown seaweeds *Laminaria cichorioides* as described (Zvyagintseva et al., 1999). Plant tissue culture reagents and standard molecular weight proteins were obtained from Sigma.

4.2. Cell cultures and media

In the present investigation, we used 8-year-old cultures of ginseng (*Panax ginseng* C.A. Meyer) obtained by transformation of the 1c callus culture with the pPCV002-CaMVC construct (Bulgakov et al., 1998). The origin of the initial non-transformed culture 1c of *P. ginseng*, the transformation of the 1c culture, and the establishment of the 1c-*rolC* and 1c-vector callus cultures were described in previous papers (Bulgakov et al., 1998; Gorpenchenko et al., 2006). Culture conditions were identical for both transgenic and control cultures; they were grown on solid W_{4CPA} medium in the dark at 25 °C for 30 days (Bulgakov et al., 1998). SA and MeJA were added to autoclaved media aseptically at desired concentrations.

4.3. Activity and gel-permeation chromatography of β-1,3-glucanases

Ginseng tissues (0.5 g) were frozen at −20 °C, crushed in a mortar, and extracted with 1 ml 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.

The activities of β-1,3-glucanases were measured by determination of reducing sugars released from laminaran (1 μg/ml in 0.025 M acetate buffer, pH 5.4) (Bulgakov et al., 2002b).

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 5.4), was incubated for 18 h at 37 °C. Molecular masses of β-1,3-glucanases were evaluated by gel-filtration chromatography using a Sephacryl S 300 column (Bulgakov et al., 2002b). All experiments were performed with 30-day cultures, since a maximum peak of β-1,3-glucanase activity was shown at this time.

4.4. Semiquantitative RT-PCR

RNA isolation and semiquantitative RT-PCR analysis were performed as described previously (Bulgakov et al., 2005). RNA expression profiles were normalized with expression of the *P. ginseng* actin gene (GenBank Accession No. AY907207). The analysis of the *rolC* gene was performed as described (Bulgakov et al., 2005). To amplify sequences corresponding to plant β-1,3-glucanase genes, we used the degenerative primers 5′TACATAGCTGTTG GVAATGARGT and 5′GGCCAGCCACTTTCAGAYA-CHA designed according to GenBank sequences of *Sambucus nigra* (AF434173), *Vitis riparia* (AY353062), tobacco (A16120), *Populus* hybr. (AF230109), and *Arabidopsis thaliana* (M90509) β-1,3-glucanases. In the semiquantitative RT-PCR reactions, PCR products were collected after 25, 30, 35, 40, and 45 cycles to determine the linearity of the PCR. The linearity of the PCR was determined for the *rolC* gene between 30 and 35 cycles and the *Pg-glu1* gene between 40 and 45 cycles.

4.5. mRNA quantification

Quantitative analysis of mRNAs was performed using a microchip technology with a DNA 1000 LabChip® kit (Agilent 2100 Bioanalyzer, Agilent Technologies, Germany) following the manufacturer's protocol and recommendations. The data is presented as relative fluorescent units normalized to expression of the *P. ginseng* actin gene.

4.6. Isolation and sequence analysis of Pg-glu1

The 423 bp cDNA fragment of *Pg-glu1* obtained by RT-PCR was used as a template. 5′ and 3′ RACE amplifications were performed using 5′/3′ RACE Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol and recommendations. A gene-specific primer 5′ACTCAGCA CTTGAGAAAGCTGGTGG and the anchoring primer were used to perform the first step of 3′ RACE. Then the nested primer 5′CATGTTAGAGGA GGGAATGGGA-CACC and the anchoring primer were used to perform the second step of 3′ RACE and the resulting 250 bp fragment was subcloned into a pTZ57R/T plasmid and sequenced. Specific primers (5′CCAATGAGACCGGA-ATATGTGGCCG and the nested primer 5′CCGGA-AGGACAAAGTTAACGTACTG) were used for 5′ RACE.

RT-PCR products were subcloned into a pTZ57R/T plasmid using InsT/Aclone PCR Product Cloning Kit (FERMENTAS, Vilnius, Lithuania) and sequenced using a Big Dye Terminator Cycle Sequencing Kit (Perkin–Elmer Biosystems, Forster City, CA) following the manufacturer's protocol and recommendations. After purification with ethanol, the sequences were identified on an ABI 310 Genetic Analyser (Perkin–Elmer Biosystems). The sequences of plant glucanase genes were accessed from GenBank. The BLAST search program was used for sequence analysis. Multiple sequence alignments were done with ClustalX program (Altschul et al., 1990).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.07.019.

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