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; Faisst 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...
decreased levels of antimicrobial polyphenols (rabdosin 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 onco-gene-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>
<thead>
<tr>
<th>Culture</th>
<th>Origin of the culture</th>
<th>β-1,3-Glucanase activity, pkat/g fresh wt</th>
<th>rolC expressiona</th>
<th>Pg-glu1 expressiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>Initial callus line</td>
<td>5.3 ± 0.8</td>
<td>ndb</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>1c-vector</td>
<td>The 1c calli transformed with pPCV002</td>
<td>5.4 ± 1.1</td>
<td>ndb</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>1c-rolC-II</td>
<td>Independently established cultures of primary tumors</td>
<td>25.0 ± 6.4</td>
<td>2.9 ± 0.4</td>
<td>0.31 ± 0.06c</td>
</tr>
<tr>
<td>1c-rolC-III</td>
<td>15.7 ± 2.2</td>
<td>2.5 ± 0.3</td>
<td>0.20 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>1c-rolC-IV</td>
<td>8.7 ± 1.1</td>
<td>1.5 ± 0.1</td>
<td>0.09 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>1c-rolC-V</td>
<td>16.5 ± 3.1</td>
<td>2.7 ± 0.3</td>
<td>0.22 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>2c2</td>
<td>1c-rolC-II selected non-root-forming callus lines</td>
<td>10.1 ± 3.2</td>
<td>1.6 ± 0.2</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>2c3</td>
<td>1c-rolC-II derived callus lines of clonal origin</td>
<td>63.8 ± 10.6c</td>
<td>2.6 ± 0.4</td>
<td>0.70 ± 0.06c</td>
</tr>
<tr>
<td>2cR2</td>
<td>69.0 ± 3.9c</td>
<td>9.2 ± 1.1</td>
<td>0.80 ± 0.17c</td>
<td></td>
</tr>
<tr>
<td>1c-rolC-II root culture</td>
<td>Independently established hairy root cultures</td>
<td>72.0 ± 6.9c</td>
<td>12.1 ± 1.2</td>
<td>1.21 ± 0.21c</td>
</tr>
<tr>
<td>1c-rolC-III root culture</td>
<td>78.4 ± 8.1c</td>
<td>11.0 ± 1.5</td>
<td>1.13 ± 0.20c</td>
<td></td>
</tr>
</tbody>
</table>

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. Ne 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); GY1 – rolC-vector culture; H, III, IV, V cultures of Ic-rolC primary tumors; CI, CII i-rolC-II and Ic-rolC-III root cultures; R33, R2 lines of secondary Ic-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.

### Table 2

<table>
<thead>
<tr>
<th>β-1,3-Glucanase gene</th>
<th>Accession number in GenBank</th>
<th>Identity (%)</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panax ginseng Pg-glu1</td>
<td>DQ015705</td>
<td>–</td>
<td>SA-inducible</td>
<td>This work</td>
</tr>
<tr>
<td>Cichorium hybrid CG1</td>
<td>AJ249292.1</td>
<td>60</td>
<td>Induced during embryogenesis</td>
<td>Helleboid et al. (2000)</td>
</tr>
<tr>
<td>Tomato E134</td>
<td>Q01412</td>
<td>60</td>
<td>Pathogen-inducible</td>
<td>van Kan et al. (1992)</td>
</tr>
<tr>
<td>Tobacco GL9</td>
<td>M60460</td>
<td>59</td>
<td>Pathogen and SA-inducible</td>
<td>Linthorst et al. (1990), Van de Rhee et al. (1993)</td>
</tr>
<tr>
<td>Tobacco ci101</td>
<td>C38257</td>
<td>59</td>
<td>Pathogen and SA-inducible</td>
<td>Linthorst et al. (1990)</td>
</tr>
<tr>
<td>Tobacco Sp41a</td>
<td>X54430</td>
<td>57</td>
<td>Stylar-specific, not inducible by pathogen</td>
<td>Ori et al. (1990)</td>
</tr>
<tr>
<td>Tobacco Sp41b</td>
<td>X54431</td>
<td>57</td>
<td>Stylar-specific, not inducible by pathogen</td>
<td>Ori et al. (1990)</td>
</tr>
<tr>
<td>Potato gluB</td>
<td>AJ009932</td>
<td>57</td>
<td>Pathogen-inducible</td>
<td>Mac et al. (2004)</td>
</tr>
<tr>
<td>Tobacco GL153</td>
<td>M60463</td>
<td>56</td>
<td>Pathogen-inducible</td>
<td>Ward et al. (1991)</td>
</tr>
<tr>
<td>Tobacco PR-2d (PR-Q)</td>
<td>X54456</td>
<td>55</td>
<td>Pathogen and SA-inducible</td>
<td>Hennig et al. (1993)</td>
</tr>
<tr>
<td>Nicotiana plumagonifolia gn1</td>
<td>M38281</td>
<td>55</td>
<td>Pathogen and SA-inducible</td>
<td>Castresana et al. (1990)</td>
</tr>
<tr>
<td>Arabidopsis BG2</td>
<td>M58463</td>
<td>50</td>
<td>Pathogen and SA-inducible</td>
<td>Dong et al. (1991)</td>
</tr>
<tr>
<td>Arabidopsis BG5</td>
<td>X79694</td>
<td>38</td>
<td>Not inducible by pathogens and SA</td>
<td>Delp and Palva (1999)</td>
</tr>
<tr>
<td>Arabidopsis BG4</td>
<td>X79694</td>
<td>38</td>
<td>Not inducible by pathogens and SA</td>
<td>Delp and Palva (1999)</td>
</tr>
</tbody>
</table>

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-
These results indicate that expression of the rolC gene is sufficient to activate expression of the \( \beta-1,3 \)-glucanase gene and elevates \( \beta-1,3 \)-glucanase activity.

2.4. \( \text{Pg-glu1} \) encodes a SA-dependent but MeJA-independent glucanase

Since SA induces genes of pathogenesis-inducible \( \beta-1,3 \)-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 \( \beta-1,3 \)-glucanase activity in control and rolC-transformed ginseng cultures. SA caused a 4-fold induction of the total \( \beta-1,3 \)-glucanase activity in the 1c culture (Table 3). The increase was exclusively due to enhanced production of \( \beta-1,3 \)-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 \( \beta-1,3 \)-glucanase activity was caused by enhanced expression of the \( \text{Pg-glu1} \) 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 \( \text{Pg-glu1} \) gene was confirmed by cDNA sequencing. We interpreted these results as an indication that \( \text{Pg-glu1} \) belongs to pathogenesis-related \( \beta-1,3 \)-glucanases.

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh Callus Wt.</th>
<th>( \beta-1,3 )-Glucanase Activity, pkat/g Fresh Wt.</th>
<th>( \text{Pg-glu1} ) Expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated cells</td>
<td>1.50 ± 0.12</td>
<td>5.6 ± 0.9</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>SA (50 ( \mu )M)</td>
<td>0.80 ± 0.06</td>
<td>22.4 ± 3.8b</td>
<td>0.14 ± 0.02b</td>
</tr>
<tr>
<td>MeJA (10 ( \mu )M)</td>
<td>0.75 ± 0.08</td>
<td>8.4 ± 0.8</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

The 1c culture was grown for 30 days before harvesting and analysis.

* mRNA quantification was made using DNA microchip technology.

b \( P < 0.05 \), Student’s t-test.

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### Fig. 2

**Fig. 2.** Effect of salicylic acid (SA) on \( \beta-1,3 \)-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 \( \mu \)M), were analyzed by gel-permeation chromatography as indicated in Section 4.

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### Fig. 3

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

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### 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-6b 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-6b 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 encourage 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 WACP medium in the dark at 25 °C for 30 days (Bulgakov et al., 1998). SA and MeIA 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-sucinate 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’TACATAGCTGTG GVAATGARGT and 5’GGCCAGCCACTTTGCAGAYACA 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 CTTGAGAAAGCTGGTG and the anchoring primer were used to perform the first step of 3′ RACE. Then the nested primer 5’CATGTTAGAGGA GGGGAATGGGE 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’CCAATGACCCGGA ATATGTTGCGCCG and the nested primer 5’CCGGA AGGACAAATGGCAGCTG) were used for 5′ RACE.

RT-PCR products were subcloned into a pTZ57R/T plasmid using InsT/Aclone PCR Product Cloning Kit (FERMENTAS, Vilnus, 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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