TOWARDS AGROBACTERIUM – MEDIATED TRANSFORMATION OF THE ENDANGERED MEDICINAL PLANT GOLDEN ROOT

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Abstract

Medicinal plants preparations are still the most popular life saving remedies for a larger part of the world population. However, exhaustion of natural resources demands new approaches in utilization and protection of these species. Biotechnology may be a powerful tool for enhancing the productivity of novel secondary metabolites. In recent years Agrobacterium mediated plant transformation, due to its simplicity and efficiency, has become quite used method for the introduction of foreign genes into plant cells followed by regeneration of genetically improved plants. The approach was successful in a number of technical and food crops but it is still limited in medicinal plants. One of the most appropriate methods for medicinal plants engineering is genetic transformation leading to increased synthesis of biological active substances in root cultures or in regenerated plantlets.

Rhodiola rosea is a medicinal plant with limited area of distribution. Roots and rhizomes contain biological active substances used in prevention and treatment of socially important diseases.

This article illustrates the possibilities of biotechnology for regeneration of Golden root and for genetic transformation aiming at stimulation of secondary metabolite production in vitro. Efficient system for propagation in vitro was developed using leaf nodes explants and was used for the genetic transformation experiments. Agrobacterium rhizogenes ATCC 15834 was used for hairy roots induction. Various factors (like density of bacterial suspension, co-cultivation time duration, concentration of the selective antibiotic) with impact on the efficiency of the transformation procedures were studied. Three transformation methods were compared: (i) spilling drops of bacterial suspension on the explants; (ii) injuring explants and dipping them into the bacterial suspension; (iii) infecting plantlets. Favorable and frustrating conditions were observed and discussed.

Key words: endangered plant, genetic transformation, golden root, in vitro propagation

INTRODUCTION

Genetic transformation is one the most popular method for increasing production of the secondary metabolites. Some economically important species were transformed by Agrobacterium rhizogenes to obtain transformed hairy roots. If the intact plant synthesizes a large quantity of biologically active substances, then their production could be increased significantly by transformed roots cultures as well as by transformed plantlets regenerants [12,16, 34]. The manipulations and optimization of the procedures for root transformation are usually the same like the other systems for in vitro cultivation and depend of the plant, the variety or cell line, nutrient media, the precursors, etc. [17]. The transformed “hairy root” culture obtained by Agrobacterium rhizogenes infected cells often maintain stable and high productivity on nutrient media free of phytoregulators [31]. Several species of the family Lamiaceae were transformed by A. rhizogenes for production of polyphenolic antioxidants. In „hairy roots” culture of Salvia officinalis (Salvia) the antioxidant activity and the level of rosmarc acid were higher compared to the untransformed tissue and organs [14, 15]. The efficient systems for production of “hairy roots” with similar action were established for some important medicinal species such as Atropa belladonna (Atropa, Solanaceae) [29], Artemisia spp. [5], species Duboisia hybrid and Datura metel (Solanaceae) [21], Papaver somniferum (Papaver, Papaveraceae) [26], Panax quinquefolius (Panax, Araliaceae) [28] and Taraxacum platycarpum (genus Taraxacum) [20], Hyssopus officinalis (Hyssopus, Lamiaceae) [10], Salvia
militorrhiza (Salvia, Lamiaceae) [38, 39], Ocimum basilicum (Ocimum, Lamiaceae) [1], Coleus blumei (Solenostemon) [2]. However, the plant regeneration was possible for some species but not for other important, medicinal plants as G. biloba (Ginkgo, Ginkgoaceae) [31] for example. The systems for micropropagation and transformation of Solanum laciniatum (Solanum, Solanaceae) by A. rhizogenes strain ATCC 15834 was established using leaf explant from in vitro propagated plants [23]. The levels of biological active substances in the hairy root culture were 0.3% to 1% higher than in vitro and in vivo developed shoots and plants. Using plant buds Wysokinska [37] obtained transformation of Catalpa ovata (Catalpa, Bignoniaceae) by A. rhizogenes (A4, LBA 9402, ATCC 15834, TR 105) and increased some glycosides levels in the „hairy roots”.

Rhodiola rosea (Golden root, Rose root) (Rhodiola genus, Crassulaceae family) is a succulent medicinal plant with limited area of distribution, grows in cool regions of the world. Its roots and rhizomes contain biological active substances with high antioxidant activity, which are used in prevention and treatment of socially important diseases. Genetic manipulations in vitro are on of the ways to increase the level of biological active components.

The objective of the present work was to study the possibilities for genetic transformation of Golden root aiming at stimulation of secondary metabolite production in vitro.

MATERIALS AND METHODS

Agrobacterium strain. Agrobacterium rhizogenes strain ATCC 15834 was used in the experiments. Agrobacterium rhizogenes is a soil born, Gram negative, aerobic bacterium. The bacterium has one, two or tree plasmids, one of them carrying genes for induction of roots type „hairy roots”. Agrobacterium rhizogenes 15834 strain has root induction Ri plasmid - pRi15834. Agrobacterium rhizogenes strain 15834, inducing „hairy roots” formation in plants contains three large plasmids: pAR15834a (107 x 10^6 daltons), pAR15834b (154 x 10^6 daltons) and pAR15834c (258 x 10^6 daltons) [30]. Agrobacterium rhizogenes strain 15834 (used in the present experiments) is not included in the List of biologically active agents and is not considered biological hazard, hence, obtained genetically modified organism is not harmful.

Initial plant material. The transformation was performed using five different types of plant material: stem segments with leaves, stem segments, leaf segments, callus and entire plants obtained in vitro.

Dose response curves (LD 50). Two different types of tissue - (i) leaf explants from in vitro propagated plants (ii) calli obtained from leave explants from in vitro regenerants were used to draw the dose curve and to define LD50 for survival against the antibiotic action. The explants were cultivated on MS basic medium with addition of cloran in different concentrations (100, 150, 200, 250, 300, 350, 400, 450, 500, 550 and 600 mg/l). The survival rate was recorded on the 7th, 14th and 20th day taking in consideration the survival of the explant and its growth.

Nutrient media. The following nutrient media were used in the experiments:

1) YEB media (for 1 liter) for cultivation of A. rhizogenes [6] containing 5.0 g/l pepton, 1 g/l yeast extract, 5.0 g/l casein of animal origin, 5.0 g/l sucrose, 0.5 g/l MgSO4, 8 g/l bacterial agar-agar, pH 7.0 – 7.2.

2) Murashige and Skoog nutrient medium (1962) [22] enriched with 30 g/l sucrose and 7 g/l agar-agar for cocultivation of the plant explants.

3) MSZ1 nutrient media for in vitro plant propagation containing basic Murashige and Skoog medium, 2.0 mg/l zeatin, 0.2 mg/l IAA, 30 g/l sucrose and 7 g/l agar-agar (pH 5.7 - 5.8).

The nutrient medium was sterilized by autoclaving at pressure of 1.1 kg/cm^2 and 121 °C for 15 or 20 minutes for the bacteria material and for the plant material, respectively.
Agrobacterium activation. The Agrobacterium was stored at low temperature (4°C) and was activated prior to DNA transfer. For this purpose, a single bacterial colony was plated on solid nutrient medium in a Petri dish, then (one week later) a single bacterial colony was inoculated into 15-20 ml YEB medium in flasks (100 ml volume). The flasks were kept on a shaking machine (velocity 200, amplitude 3.5, (16 rad/s) at temperature of 28°C for 18-20 h for bacteria activation and growth (‘overnight culture’). Bacterial density was determined spectrophotometrically by measuring the extinction (Spekol 10) at 600 nm wave length. The necessary bacterial density was reached by appropriate dilution with YEB medium. Overnight culture of Agrobacterium rhizogenes strain ATCC 15834 reaches mid-log phase (OD600 = 0.5) for 24 h at temperature of 28°C. The bacterial density of 5.10⁹ cells/ml is defined by extinction of 1 at λ of 600 nm.

Tree methods for infection were tested:
(1) Leaf and stem segments from in vitro propagated plants were used as explants. They were placed in Petri dishes with nutrient media and dropped with Agrobacterium suspension allowing infection of some of the plant cells. The explants were cultivated on MS medium without phytohormones and antibiotics.

(2) Leaf and stem segments, and calli from in vitro plants were used. They were injured and dipped into the Agrobacterium suspension for different time duration (10 and 15 min) and were transferred onto MS medium without phytohormones and antibiotics.

(3) The in vitro obtained Rh. rosea plants in the test tube were punched by a thin needle attached to a syringe containing suspension of A. rhizogenes strain 15834. A tiny drop was injected into the tissue avoiding leakage on the media. Treated plants were cultivated on MSZ1 medium for organogenesis.

2). Co-cultivation was in dark at temperature of 21-22°C till bacterial growth around the infected site was observed.

3). Post cultivation procedure consisted of rinsing the explants with dH₂O, dried up with filter paper and transferred to selective medium. Co-cultivation with the bacterium lasted 3 days when the first and the second methods were applied. Infected explants were transferred to basic MS medium without phytohormones but containing 3 % sucrose, 0.7 % agar and 0.50 g/l antibiotic Claforan (500 mg/l) preventing bacterial growth. Claforan’s concentration was decreased after 10 days to 400, 300 and 250 mg/l till the complete extinction of the bacteria.

4). Cultivation of the explants was in dark at 24°C. The selective medium was changed each 10 days with reduction of the antibiotic.

Two types of leaf disks and callus served as control:
K1 – noninjured leaves/calli from in vitro obtained uninfected with Agrobacterium plants
K2 – injured leaves/calli from in vitro obtained uninfected with Agrobacterium plants.

Experiments from 88 to 140 explants were tested in the transformation, and from 30 to 50 explants were used in the control. Experiments were repeated from 4 to 6 times. Cultivation was under two regimes – in dark and in dim light.

RESULTS AND DISCUSSIONS

The basic approach in the experiments to develop a system for gene transfer in golden root was the modification of the existing methods for transformation used for other plants. Important factors for successful genetic transformation are a reliable system for in vitro propagation and cultivation, easy maintenance and high efficiency organogenesis and estimation of the follow parameters:

(1) Determination of the influence of the selective factor – drawing of the dose response curve to specify the sublethal and lethal doses of the antibiotic;

(2) Determination of the optimal bacteria suspension density.

It is important for genetic transformation to achieve a previous development of a system for the in vitro cultivation in order to give possibilities for effective organogenesis and to obtain large number of plant for the short period. This system was used in these experiments [32]
Dose response relationship (LD50). The dose curves were created using leaf explants and callus cultivated on MS nutrient medium with addition of different concentrations of the antibiotic Cloran. Cloran sublethal concentration LD50 was 400 mg/l both for the leaf explants and for the callii (Figures 1A and B).

Transformation. Application of the first method when the explants were placed on MS medium in Petri dishes and dropped with suspension of A. rhizogenes ATCC 15834 usually lead to the development of severe infection. The subsequent procedures for elimination of the bacteria were accompanied with tissue death.

The presented results showed that most of the leaf explants were dead and/or reinfected followed by the necrosis of the tissue. About 6% of the leaf explants initiated callus formation on the 12th day from cultivation at extinction 0.42 of the bacterial suspension for 15 minutes (Table 1).

In case of the infection of a whole in vitro propagated plant (in test tube), bacterial infection usually did not develop and plants survived and grew, however, without formation of roots.

Table 1. Transformation effectiveness on the A. rhizogenes strain ATCC 15834 using leaf explants isolated from 15-20-days old in vitro propagated plants after cultivation on MS medium containing 400 mg/l cloran (co-cultivation with bacteria for 3 days in dark)

<table>
<thead>
<tr>
<th>№</th>
<th>Explant [number]</th>
<th>Extinction</th>
<th>Infection time [min]</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.713</td>
<td></td>
<td>Necrotic tissue</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>0.696</td>
<td>15</td>
<td>Reinf. and necrosis of the tissue</td>
</tr>
<tr>
<td>3</td>
<td>140</td>
<td>0.659</td>
<td>15</td>
<td>Necrotic tissue on the 7th day</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>0.500</td>
<td>15</td>
<td>Necrotic tissue</td>
</tr>
<tr>
<td>5</td>
<td>140</td>
<td>0.490</td>
<td>15</td>
<td>Necrotic tissue</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>0.458</td>
<td>15</td>
<td>Reinf. and necrosis of the tissue on the 2nd week</td>
</tr>
<tr>
<td>7</td>
<td>250</td>
<td>0.440</td>
<td>15</td>
<td>Necrotic tissue on the 15th day</td>
</tr>
<tr>
<td>8</td>
<td>300</td>
<td>0.420</td>
<td>15</td>
<td>6% callus like formations</td>
</tr>
<tr>
<td>9</td>
<td>220</td>
<td>0.600</td>
<td>10</td>
<td>Necrotic tissue on the 12th day</td>
</tr>
<tr>
<td>10</td>
<td>320</td>
<td>0.425</td>
<td>10</td>
<td>Necrotic tissue on the 18th day</td>
</tr>
</tbody>
</table>

Transformation of calli. The callus tissue was heavily infected, being very difficult to eliminate the bacteria. Calli underwent necrosis followed by death within a period of 2 to 4 weeks.

Infected explants were cultivated on MS medium containing antibiotic Cloran for elimination of the bacteria. The concentration of the antibiotic was not less than 400 mg/l. In our experiments, addition of 250 mg/l and 300 mg/l led to recurrence of the bacteria infection within 2-3 days (explant reinfection). Suppression of recurrent bacterial growth was suppressed by increasing the antibiotic concentration up to 400 and 500 mg/l. However, most often the largest part (more than 85%) of the cases led to lethality, making calli unsuitable object of transformation (Fig. 2, 3 A and B).
Stem segments proved to be unsuitable object, too, but due to another reason. In 10% of the cases, these explants formed roots, which were typical for the plant without being transformed. The methods for the transformation of the plant genome by Agrobacterium exploit the pathogenic infection processes [8]. Usually, the initial reaction of the plants to the pathogenic attack is oxidative stress with fast and short-term production of free radicals [35]. This reaction of the plants is the defensive mechanism as far as the reactive oxygen species (ROS) as active radicals can destroy the bacteria or suppress their growth [36]. The production of the free radicals usually results in super sensitivity towards the pathogen, leading to the fast cell death (necrosis) [8, 13], which was observed for the most of the explants used in our experiments.

The mechanisms of interaction between Agrobacterium and the plants are not fully understood yet. Some authors report about necrosis and poor survival rate of the target plant tissue after transformation by Agrobacterium [4, 7, 8, 9, 10, 11, 18, 24, 25, 27, 33, 40]. Another problem is the inability of the plant cell culture to respond in a desired way to achieve results [3]. Even so, under normal conditions in culture, without transformation many plants are unable to respond during manipulations in in vitro culture - "in vitro recalcitrance is attributed to several factors but the exact reasons remain unclear so far [3]. In the future, one of the ways for a successful genetic transformation might be the suppression of the high antioxidant activity which, in fact, is one of the most useful components of the defensive system of plants. In this respect, difficulties with Agrobacterium transformation of Rhodiola might be expected.

CONCLUSIONS

The article represents the possibilities of biotechnological methods to increase the
biological active substances in *Rhodiola rosea*. The obtained results could be used as a basis to determine the optimal parameters for effective genetic transformation. The latter could be an alternative way for secondary metabolites production under laboratory conditions for the protection and conservation of this endangered species.

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REFERENCES


