Salidroside Production by Hairy Roots of *Rhodiola sachalinensis* Obtained after Transformation with *Agrobacterium rhizogenes*

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Hairy roots induced by *Agrobacterium rhizogenes* grow faster, and are considered as genetically stable. These hairy roots can be used as an interesting material for the production of secondary metabolites of pharmaceutical value. Salidroside has been identified as the major compounds from the roots of *Rhodiola sachalinensis* A. BOR. Here, we provide an update that adds new perspectives on the prospects and challenges of producing Salidroside from hairy roots induced by *Agrobacterium rhizogene* in *Rhodiola sachalinensis* A. BOR. For high salidroside production, the optimal concentration for precursor (Tyrosol, Tyrosine, and Phenylalanine) and elicitor (*Aspergillus niger*, *Coriolus versicolor*, and *Ganoderma lucidum*) was added in the LB liquid medium, respectively. The addition of elicitor in the liquid MS medium and the utilization of precursor from chemical feeding enhanced biomass accumulation and salidroside production. The optimal concentration for elicitor and precursor in the liquid medium was 0.05 mg/l and 1 mmol/l, respectively.

Key words *Agrobacterium rhizogenes*; hairy root; salidroside; *Rhodiola sachalinensis*

Agrobacterium rhizogenes is responsible for the formation of adventitious roots known as “hairy roots”. Hairy roots result from the transfer and integration of the genes located on the root-inducing plasmid Ri of *Agrobacterium rhizogenes* into the plant genome and their expression therein.¹⁾ These roots are characterized by an extensive growth, associated with lateral branching, leading to an important mass of adventitious roots exhibiting a typical phenotype. Furthermore, these roots are found to produce a high yield of secondary metabolites as compared to that of undifferentiated plant cell suspensions.^{2–4)} The greatest advantage of hairy roots is that hairy root cultures often exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants. Hairy root cultures have been shown to accumulate many valuable secondary metabolites. For example, several laboratories have shown that hairy roots can produce artemisinin, tropane alkaloid, and so on.^{5–8)} Another advantage of hairy roots is that hairy root system is genetic stable compared to the roots of a whole plant.⁹⁾ Recently, many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, which are potentially important pharmaceutical products.^{10–13)} *Rhodiola sachalinensis* A. BOR, a perennial herb, belonging to the family Crassulaceae, is mainly distributed in mountains at the altitudes of 1700–2500 m. It is a typical alpine plant and a very important medicinal plant with high activities of anti-fatigue, anti-senescence, and anti-radiation, due to the secondary metabolite salidroside, one of the major compounds from the roots of *Rhodiola* species (Crassulaceae).¹⁴⁾ Since the natural supply of this herb is rapidly decreasing, plant cell suspension culture has been considered an alternative of producing salidroside. Although there are some production of salidroside in the plant cell suspension culture of *Rhodiola sachalinensis*,¹⁵⁾ the biggest challenge of producing secondary metabolites from the suspension cultures is that secondary metabolites are usually produced by specialized cells differentiated at distinct developmental stages.^{16,17)} To

produce Salidroside more efficiently and stably, we established the hairy root system of *Rhodiola sachalinensis* for high yield salidroside production with *A. rhizogenes*. The results have demonstrated that salidroside production in most cases of the system is higher than in normal field-grown plants.

This paper firstly reports the production of transformed roots using *A. rhizogenes* strain A4 and regeneration hairy roots from such transformants. The stable transformation of transgenes has been shown by polymerase chain reaction (PCR) and PCR-Southern blotting. As far as it is known, this is the first time that salidroside production by hairy roots of *Rhodiola sachalinensis* obtained after transformation with *Agrobacterium rhizogenes*.

MATERIALS AND METHODS

Bacterial Strain and Cultivation *Agrobacterium rhizogenes* strain A4 was used for inoculation of the explants originated from root, stem, leaf and cotyledon of *Rhodiola sachalinensis* A. BOR. Strain A4 was obtained from Professor D. Tepfer, Cedex, France. The bacteria were grown overnight at 28 °C on Luria–Bertani medium (LB: contains 10 g/l Bacto-trypton, 5 g/l Bacto-yeast extract, and 10 g/l NaCl, adjusted to pH 7.0) with shaking at 4000 rpm in darkness. The bacteria were then collected by centrifugation and resuspended in LB medium supplemented with 75 mM acetosyringone and 0.1 M galactose.

Transformation and Root Cultures The explants from leaf of sterile seedlings of the wild-type *Rhodiola sachalinensis* were shaken for 20 min in a suspension of *A. rhizogenes*, which contained the Ri plasmid for transformation. The inoculated explants were cultured on solid MS-medium,¹⁸⁾ containing 1.5% agar, Gamborg’s B5 vitamins¹⁹⁾ at 22 °C. After 3 d of co-cultivation, the explants were washed in sterile distilled water three times and transferred to the fresh medium containing cefotaxime (500 mg/l) and carbenicillin (250 mg/l) in order to eliminate residual bacte-

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ria. The tissue was maintained on the same medium for at least 3 weekly subculture periods for hairy root induction. Roots developed at the infection sites and in order to obtain the root lines, single roots were picked off and placed onto new media together with cefotaxime and carbenicillin, until the bacteria were eliminated. Hairy roots with on bacterial contamination were cultured on hormone-free MS solid medium in the dark at 24 °C. After 1—2 months of subculturing the roots every two weeks on fresh solid medium, the root lines were transferred to 1/2 MS liquid medium and kept in a rotary shaker at 100 rpm, 24 °C in the dark. Subcultures were performed every one week in fresh medium. The root cultures grew with different morphology. Some lines grew as short, thick, and slightly translucent roots; most other lines had typical hairy roots appearance with thin long roots and many side branches. Root culture lines were cultivated in 100 ml conical flasks in 35 ml 1/2 MS liquid medium on a gyratory shaker (100 rpm, 24 °C) in the dark. For each measurement three flasks were harvested. Roots were blotted dry for the determination of fresh mass and dried to constant mass before dry mass measurement.

DNA Extraction and PCR Performance Total DNA was isolated from single root, whose weight ranged about 0.25 g. The bacteria were removed from the roots by washing in sterile distilled water three times and the roots were rinsed in sterile deionized water afterwards. The roots, dried on sterile filter paper, were quickly frozen in liquid nitrogen and ground to a fine powder.¹⁶⁾ Each PCR reaction was carried out on 25 ng of total DNA with 1.2 U *Taq* DNA polymerase (TaKaRa) in a total volume of 25 μ l. Primer I 5'-GATATATGCCAAATTACACTAG-3' and primer 5'-GTAA-CAAAGTAGGAAACAGG-3'. Primers used for the amplification reactions came from internal sequences of the *rolC* gene of the TL-DNA of pRi of *A. rhizogenes* A4, produced a DNA fragment of 500 bp. The amplification temperatures for *rolC* gene were 94 °C for denaturation (45 s), 45 °C for annealing (30 s), 72 °C for extension (45 s) for 30 cycles. Then, another 10 min at 72 °C for extension. The amplified fragments were separated in a 1.5% agarose gel and the gels were scanned in a UV image instrument (UVIPhotoMW, BTS-20.M, EEC).

PCR-Southern Blot Hybridization For PCR-Southern blot hybridization, the PCR products on agarose gel were transferred to nylon membrane. The filters were then pre-hybridized for 15 min at 55 °C. The probes (from PCR product of *A. rhizogenes* strains as template) were prepared using the Gene ImagesTM labeling kit (AlkPhos, RPN 3680/1) according to the manufacturer's instruction. Filters were hybridized overnight at 55 with gentle agitation. The excess probes were washed off with primary washing buffer and secondary washing buffer. Filters were then soaked in CDP-StarTM detection reagent for 2 min at room temperature, wrapped in a protective film and exposed to a photographic film. The details of the procedure are described in the gene image.

Determination of Salidroside To prepare the assay samples of salidroside in hairy roots of *Rhodiola sachalinensis* and roots of wild *Rhodiola sachalinensis*, an ultrasonic extraction method was used. The optimal extracted conditions were distilled water as the solvent, 60 °C as the extracted temperature and 40 min as the extracted time. Salidroside concentrations of the samples were determined

with HPLC analysis. The HPLC system (LC-8A; Shimadzu Japan) consisted of an Agilent Hypersil BDS-C18 column (5 μ m, 4.6 $\times$ 150 mm), waters 600 pump and waters 2487 Dual λ Absorbance Detector. The optimal conditions were shown below. The mobile phase was a gradient mixture of acetonitrile and water (1:9). The flow rate was 1.0 ml/min and the detection wavelength was 280 nm.

Elicitor (Precursor) Dosage Response Hairy roots were subcultured with an inoculum of 0.2 g fresh root segments in 100 ml conical flasks in 35 ml 1/2 MS liquid medium on a gyratory shaker (100 rpm, 24 °C) in the dark. Twenty-five days after cultivation, hairy roots were transferred to fresh 1/2 MS liquid medium containing different volumes of elicitor solution or 1 ml of distilled water (control). On 10-d of elicitation, hairy root cultures were harvested and the biomass and contents of salidroside were determined. The experiment was performed in six independent tests. The similar procedure was performed in precursor dosage response.

RESULTS AND DISCUSSION

Optimization of the Agrobacterium Infection Process

A system of hairy roots of *Rhodiola sachalinensis* was obtained after transformation with *Agrobacterium rhizogenes* (Fig. 1). Several parameters of the agro-infection method

Fig. 1. Hairy Root Induction of *Rhodiola sachalinensis*

(A) *Rhodiola sachalinensis* A. Bor., a perennial herb, belonging to the family Crassulaceae. (B) Sterile seedlings of *Rhodiola sachalinensis*. (C) Induction of hairy roots by co-culture method. Infected segments were cultured on MS solid medium containing cefotaxime (500 mg/l) and carbenicillin (250 mg/l) at 24 °C in the dark for 20 d. (D) Infected leaves were cultured on MS solid medium at 24 °C in the dark for 35 d. (E) The selected hairy root clones were cultured on MS solid medium at 24 °C in the dark for 35 d. (F) The typical hairy roots were cultured in MS liquid medium at 24 °C in the dark for 60 d.

Fig. 2. Infection Rate of Hairy Roots in *A. rhizogenes*-Induced System of *Rhodiola sachalinensis*

(A) *Agrobacterium rhizogenes* versus infective effect. (B) The time of incubation explants in the bacterial suspension and infection rate. (C) Duration of co-culture and infection rate.

were adjusted such as the bacteria concentration, the time of submerged leaf disks in the bacterial suspension, pH value in media, and duration of co-culture on hairy root induction using leaf explants and *A. rhizogenes* A4. For the best *Agrobacterium* concentration test, the various concentrations (measured as OD value 0.15, 0.25, 0.35, 0.42, 0.51, 0.75, and 1.25) of *A. rhizogenes* A4 were used to treat the leaf explants. The results indicated that the best rate of infection (69.4%) was achieved at OD₆₀₀=0.51 (Fig. 2A). When the *Agrobacterium* concentration was lower than 0.25 the efficiency of infected plant was also low. Whereas when the *Agrobacterium* concentration was greater than 1, there was much severe harm to the explants after infection. The time of submerged leaf disks in the bacterial suspension were: 3 min, 8 min, 20 min, 1 h, 2 h, and 4 h. In all cases the best results were obtained when the sections were incubated for 20 min in the bacterial suspension (Fig. 2B). This time has been greatly reduced as compared to Horsch *et al.*²⁰⁾ who submerged leaf disks overnight in the bacterial culture. This time has been greatly increased as compared to Bonhomme *et al.*²¹⁾ who submerged leaf disks 5 min in the bacterial suspension. The co-culture times were 0.5 d, 1 d, 2 d, 3 d, 5 d, and 7 d. The optimal time of co-culture on solid medium was 3 d of the A4 strain (Fig. 2C). The remainders were discarded as

Fig. 3. PCR (A) and PCR-Southern (B) Analysis of Hairy Root Cultures in *Rhodiola sachalinensis*

(A) Agarose gel electrophoresis of PCR-amplified products from genomic DNA of transformed hairy root culture and untransformed roots. PCR was performed with *rolC* primers. (B) Hybridization was carried out with a labeled *rolC* fragment as probe. For PCR-Southern blot hybridization, the PCR products on agarose gel were transferred to nylon membrane. 1–5, Hairy root samples transformed by Ri plasmid; 7, Ri plasmid positive control; 6 and 8, seminal seedling roots as the negative control; M, marker (DL2000). PCR-amplified products are indicated by the arrow.

non-growers or too slow growers to produce enough biomass, salidroside contents, and hairy roots. Roots never appeared on control leaf disks. Therefore, for further experiments, some better hairy root lines were kept from the infection with *A. rhizogenes* A4.

DNA Analysis PCR analysis (Fig. 3A) showed that amplification with the *rolC* primers showed a 500 bp band for the transformed roots (lanes 1–5) and for the strain *A. rhizogenes* A4 (lane 7). No band was observed for the untransformed roots (lanes 6, 8). To confirm whether the detection of the *rolC* gene by PCR in hairy root-derived plants was the result of true transformation or from contamination of *A. rhizogenes* A4, the following procedure was performed. Bacterial cells were eliminated from the co-cultures by incubation of hairy root cultures on MS medium with antibiotics, then hairy root cultures were grown on a medium without antibiotics. Later on hairy root tissues were homogenized for several days, but the growth of *A. rhizogenes* was never observed. The PCR-Southern blot hybridization had further confirmed integration of *rolC* gene to the plant genome. The *rolC* gene was detected in transformed plants, but not in untransformed plant (Fig. 3B).

Effect of Elicitor Dosage The quantitative determination was restricted to salidroside by HPLC method. As shown in Fig. 4A, the use of the elicitors (*Aspergillus niger*, *Coriolus versicolor*, and *Ganoderma lucidum*) at all four concentration resulted in a great increase in biomass accumulation (from 178 g/l in the control to as much as 258.86 g/l) (Fig. 4A), salidroside content (from 0.42% of dry weight in the control to as much as 0.71%) (Fig. 4B). Among the known data, the better results appeared at medial elicitor dosages (0.05 mg/l).

Precursor of Elicitor Dosage After we have checked that no salidroside could be detected in the culture media, the salidroside content of the transformed roots (g g⁻¹ DW) was evaluated 5. The use of the precursor (Tyrosol, Tyrosine, and Phenylalanine) at all five concentration (0, 0.5, 1, 1.5, 2 mmol/l) resulted in a great increase in biomass accumulation (Fig. 5A). A rapid increase in salidroside content was seen in Fig. 5B, (from 0.42% of dry weight in the control to as much as 0.66%) (Fig. 5B). The results showed that the

Fig. 4. Effects of the Elicitor Concentration on Biomass Accumulation (A), Salidroside Content (% Dry Weight) (B)

0, 0.025, 0.05, and 0.1 mg/l of elicitor (*Aspergillus niger*, *Coriolus versicolor*, and *Ganoderma lucidum*) was added per flask, respectively. Values are means of six results and error bars show standard deviation.

growth of hairy roots was greatly improved due to the addition of elicitor, or precursor, a further increase in the contents of salidroside was observed in treated hairy roots with elicitor or precursor. The optimal concentration for elicitor and precursor in the liquid medium was 0.05 mg/l and 1 mmol/l, respectively.

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Fig. 5. Effects of the Precursor Concentration on Biomass Accumulation (A), Salidroside Content (% Dry Weight) (B)

0, 0.5, 1, 1.5, and 2 mmol/l of precursor (Tyrosol, Tyrosine, and Phenylalanine) was added per flask, respectively. Values are means of six results and error bars show standard deviation.

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