OLIGOSIDE PRODUCTION BY HAIRY ROOT CULTURES TRANSFORMED BY Ri PLASMIDS[1)]

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Convenient methods have been developed to transform Panax ginseng with Ri plasmids of Agrobacterium rhizogenes. Crown galls were formed on the stems of ginseng plants by infecting them with A. rhizogenes. Hairy roots were generated from the crown galls when the stem segments bearing the crown galls were incubated on solid Murashige-Skoog's (MS) medium. The hairy roots were also generated on excised ginseng root segments which had been inoculated with A. rhizogenes. The ginseng hairy root cultures, established as liquid cultures in MS medium, produced 0.34-0.82% ginsenosides on a dry weight basis. The ginseng hairy roots were found to contain nucleosides, adenosine and guanosine, at signifivantly higher levels than normal ginseng plants. The transformed hairy roots of Glycyrrhiza uralensis were obtained by the infection of A. rhizogenes either in asceptically grown plantlets or in excised plant stems. One of the established liquid culture lines produced glycyrrhizin at an yield of 4.7 %.

KEYWORDS hairy root; Panax ginseng; Glycyrrhiza uralensis; Agrobacterium rhizogenes; Ri plasmid; ginsenoside; glycyrrhizin; nucleoside; saponin; oligoside

Plant tissue cultures have been developed for the large scale production of secondary metabolites.[2)] The culture lines for such production were established by the investigations of phytohormone composition and culture media. The production of secondary metabolites is often closely associated with morphological differentiation. Successful results have been reported in the production of alkaloids and ginseng saponins by root cultures which were induced by the control of phytohormone.[3)] Agrobacterium rhizogenes provides another approach to the production of secondary metabolites by plant tissue cultures. Hairy roots generated by transformation with Ri plasmids are differentiated organs, so the hairy root cultures produce the secondary metabolites occurring in the roots of the original plants.[4)] The best examples are the hairy root cultures of Solanaceous plants such as Scopolia japonica and Atropa belladonna, which produced alkaloids at significantly higher levels than the parent plant roots.[5)] It was difficult to produce these alkaloids by callus or cell suspension cultures. Recently, Yoshikawa and Furuya reported the production of ginseng saponins by the transformed hairy root cultures of Panax ginseng.[6)] This prompted us to report the results of our studies on the production of oligosides by the hairy root cultures of Panax ginseng (ginseng) and Glycyrrhiza uralensis (licorice), important drugs in Asian traditional medicines.

Yoshikawa and Furuya used the coculture method in obtaining the ginseng hairy roots.[6)] Cultured cells of ginseng were partially digested with cellulase and pectinase and incubated with A. rhizogenes. They obtained the transformed ginseng hairy roots after two months. One of the

successful approaches in our studies was the direct infection of ginseng plants with A. rhizogenes. When A. rhizogenes ATCC 15834 was inoculated at the several wounded sites on the stems of two ginseng plants (three years old), which were cultured in pots with soil, small crown galls were formed around the wouded sites of the stems after several weeks. This is well in accord with the observation that crown gall is formed instead of hairy roots when infection with A. rhizogenes occurs under dry conditions.[7] The stems were excised from the plants and cut into the segments that bore crown galls. The segments were sealed at the both ends with instant chemical glue (Alon-alpha), sterilized with ethanol (70%) and hypochlorite (1%), then incubated on solid Murashige-Skoog's (MS) medium containing the antibiotic claforan (300 mg/l). Seven hairy roots were generated from one crown gall after several weeks. Ginseng hairy roots were also obtained by a modified root disk method: excised root segments 2-5 cm long and 5-10 mm wide, which contained cambium layer, were cut out with a scalpel from previously sterilized roots. Both ends of the excised root segments were immediately sealed with chemical glue and sterilized with ethanol and hypochlorite. Then, the sealed ends of root segments were cut off with a scalpel. One of the mewly formed cut ends was immersed in solid MS medium containing claforan.[8] A. rhizogenes A4 was inoculated on the ginseng root segments. Of the 15 root segments tested, several ginseng hairy roots were generated from one root segment after a month. Thus obtained ginseng hairy roots were rather thick and lacked fine lateral branchings. Several culture lines were established as liquid cultures in MS medium with 3% sucrose and the production of opines, agropine and mannopine, was detected by paper electrophoresis.[9] Unidentified white spots migrating along with opines were observed on the electrophoresis paper.[6] These compounds were isolated with silica gel column chromatography and identified as nucleosides by spectroscopy (NMR, Mass and IR). The ginseng hairy root culture (No. 6) contained 0.05-0.22% adenosine and 0.01-0.05% guanosine.[10] The presence of nucleosides in ordinary ginseng has been reported. However, the contents of the nucleosides in the ginseng hairy root cultures were one order higher than those in the roots of cultivated ginseng plants.[11]

Table I. Contents of Ginsenosides in Ginseng Hairy Root Cultures (30 Days)

Sample	Total content[a]	Rg1	Re	Rd	Rc	Rb2	Rb1
No. 6	0.49	0.11	0.15	0.04	0.02	0.02	0.15
No. 7	0.82	0.08	0.25	0.09	0.04	0.06	0.32
No. 8	0.47	0.14	0.09	0.02	0.01	0.01	0.07
Main roots[b]	0.50	0.13	0.05	0.05	0.05	0.06	0.16
Lateral roots[b]	0.98	0.09	0.15	0.18	0.17	0.19	0.20

a) The sum of the ginsenoside contents of Rg1, Re, Rd, Rc, Rb2 and Rb1.
b) Ginseng plants cultivated at the Kemigawa Experimental Station for Medicinal Plant Studies, the University of Tokyo.

Profiles of ginsenoside production in the transformed hairy roots were analyzed by HPLC.[12] The ginseng hairy roots were cultured in 500 ml Erlenmeyer's flasks with 100 ml MS medium containing 3% sucrose on a rotary shaker at 50 rpm. The culture lines were significantly different from each other (Table I). The total saponin contents were 0.82-0.47%. The best growth was observed in the No.6 culture line which was originally derived from a ginseng stem. Its dry weight increased 20.1-fold in 30 days culture. Studies of the time course production of saponins in the ginseng hairy roots culture (No 6) revealed that the production patterns of ginsenosides could be classified into three types (Fig. 1): ginsenoside Rb1 reached maximum content in an early stage of culture and then fell to a half of the maximum. The contents of Rg1 and Re reached their maxima in a later stage of culture. Ginsenoside Rb2, Rc and Rd maintained the same levels throughout the culture period.

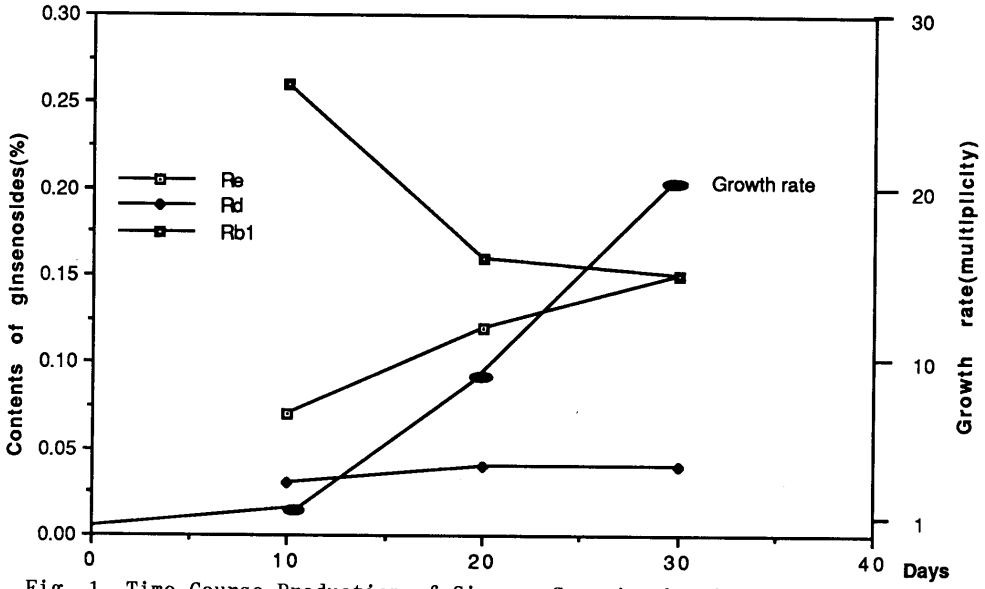

Fig. 1. Time Course Production of Ginseng Saponins by the Hairy Root Cultures

The hairy roots of Glycyrrhiza uralensis were obtained by conventional methods, infection of A. rhizogenes ATCC 15834 to asceptically grown plantlets and to excised plant stems of asceptically grown plantlets. Three culture lines were obtained from the seeds of two different origins.[13] They showed significantly different morphologies and growth rates. The hairy roots of culture line (No. 1-4), derived from the seeds of Japanese origin, were rather thin and tended to become dark, except for newly developing yellow parts. The other culture lines (No. 2-2 and 2-5), derived from the seeds of Chinese origin grew rapidly and active branchings occurred at the points where callus-like tissues were formed. Glycyrrhyzin was isolated by HPLC, and identified by negative FAB mass spectra as glycyrrhizin $(M-1)^-$ = 821 and as its methyl ester $(M-1)^-$ = 863. The identification was also made with 3D chromatography.[14] Glycyrrhizin contents were quantified by HPLC. One line (No.2-5) had a 4.7% content on a dry weight basis.[15] Preliminary investigations of the culture media have revealed that Nitsch and Nitsch (NN) medium is the most suitable for the production of glycyrrhizin (Table II). Further studies of hairy roots on differnt kinds of Glycyrrhiza species are in progress in our laboratory.

Table II. Glycyrrhizin Contents in Licorice Hairy Root Cultures (30 Day)

Sample	Origin of seeds	Medium[a)]	Content(% dry weight)
No.1-4	Japan	MS(30)	2.96
No.1-4	Japan	MS/2(30)	1.18
No.2-5	China	MS(30)	2.18
No.2-5	China	MS/2(30)	0.97
No.2-5	China	NN(30)	4.70
No.2-2	China	NN(30)	1.89

a) Figures in the parantheses indicate the g/l concentration of sucrose in the medium.

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