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Characterization of *Ajuga* Plants Regenerated from Hairy Roots

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20-Hydroxyecdysone (20-HE), one of the phytoecdysteroids in plants, has practical possibilities for pest control[1], chemotherapy[2], and silk production[3]. In particular, Kozakai *et al.*[3] have demonstrated the use of 20-HE as a facilitator for the maturation of silkworms just before spinning cocoons.

We have reported the establishment of a rapidly growing root clone producing a high amount of steroid in *Ajuga reptans* var. *atropurpurea*, one of the phytoecdysone-producing plants, by transformation with *Agrobacterium rhizogenes*[4].

Hairy roots have the ability to regenerate plants[5]. The plants regenerated from hairy roots also have some special characteristics, so-called 'hairy-root syndrome', such as active formation of adventitious root, high growth rate of roots in culture, reduced apical dominance in both stems and roots, altered leaf and flower morphology, plagiotropic root growth (*i. e.* with altered geotropism) and reduced pollen and seed production[5]. The dwarfism by shortened internodes is also recognized in regenerated plants.

In this report, we demonstrate *Ajuga* plants regenerated from hairy roots, and their various properties such as growth, morphology of leaf, root mass, opine biosynthesis, and 20-HE productivity.

Materials and Methods

1. *Regeneration and culture of plants from hairy roots*

Hairy roots of *Ajuga reptans* var. *atropurpurea* were induced by leaf-disc inoculation method with *A. rhizogenes* MAFF 03-01724 (mikimopine type strain)[6,7] as described previously[4]. A piece of root, 3 cm from the tip of a hairy root, was explanted onto hormone-free MS medium[8] containing 30 g/*l* sucrose and 2 g/*l* Gellungum (San-ei Chemical Industries, Ltd.) and cultured for 30-45 days at 25° C in the dark. Spontaneously regenerated shoots (1-3 cm) were separated carefully from hairy roots and transferred to 10 m*l* of the same medium as above in a 50 m*l* culture flask. A control plant was also regenerated from non-transformed root cultures in a similar manner. These plants were grown with a 12 hour-photoperiod (2, 000 lux) at 25°C. A plantlet (about 1 g) cut from a one month -old plant, with a terminal bud and some newly developed leaves, was transferred to 100 m*l* of MS medium as described above in a 500 m*l* culture flask. The growth rate was calculated as the ratio of the weights of inoculated and harvested masses on a fresh weight basis. The percentage of root to whole plant was calculated as follows: (fresh weight of root)/(fresh weight of whole plant) × 100.

2. *Opine assay*

Mikimopine was detected with the quick opine assay method as described previously[9].

3. *Determination of plant dry weight and quantitative analysis of 20-HE*

The harvested plant was dried at 50°C for 8 hours in an oven, and then its dry weight was determined gravimetrically. Fifty mg of dried sample was extracted in 5 m*l* of methanol (MeOH) for 3 hours. The MeOH extract was chromatographed directly by HPLC (column, Nucleosil RP-5 C18 ; mobile phase, CH_3CN : MeOH : H_2O=2 : 18 : 80 ; detection, absorbance at 242 nm).

Results and Discussion

1. *Regeneration of plants*

All transformed roots regenerated shoots spontaneously in the dark. Especially, the hairy roots which were sufficiently grown for over 30 days after transfer showed a high frequency of shoot regeneration in the vicinity of their bases (**Fig. 1-a**). A similar result was obtained through the hairy root-culture by the addition of antibiotics in MS medium for removal of *A. rhizogenes*. The control roots also regenerated shoots under the same conditions as above. These results indicate that, in the case of *Ajuga* plant, shoots are frequently regenerated from hairy roots or non-transformed roots in the state of suppressed growth by the addition of reagents such as antibiotics or stationary growth. The yellowish shoots turned to green within three days under light (**Fig. 1-b**).

2. *Detection of mikimopine*

Since no significant differences in mikimopine production between the leaves and the roots in the transformed plants were observed (**Fig. 2**), it was assumed that the gene for mikimopine synthesis was expressed equally in the leaves and roots, but not in a specific organ, of these plants grown aseptically on medium.

3. *Properties of regenerated plants*

The leaf size of the transformed plants was smaller than that of the control ones, while the number of leaves greatly increased in the former plants (**Fig. 3**). The roots initiated from the

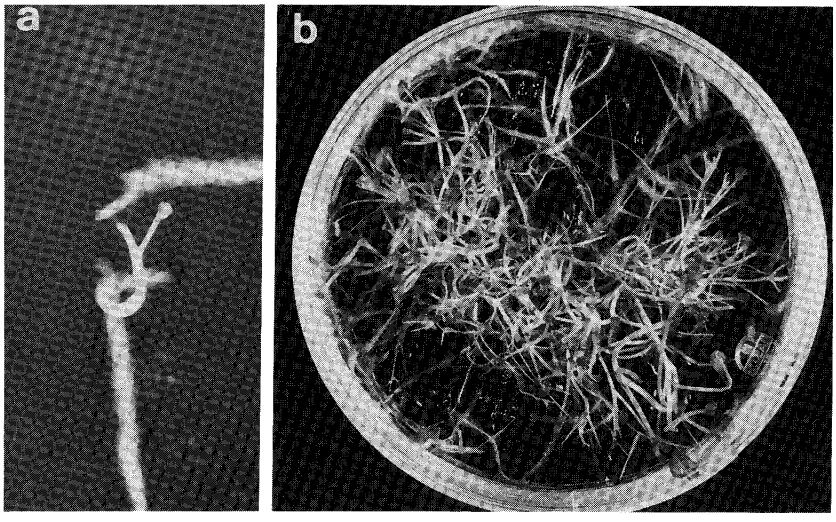

Fig. 1 Plants regenerated from hairy roots of *A. reptans*.
a : Shoots regenerated in the vicinity of hairy root basis. b : Immature plants regenerated from hairy roots cultured for over one month in the dark.

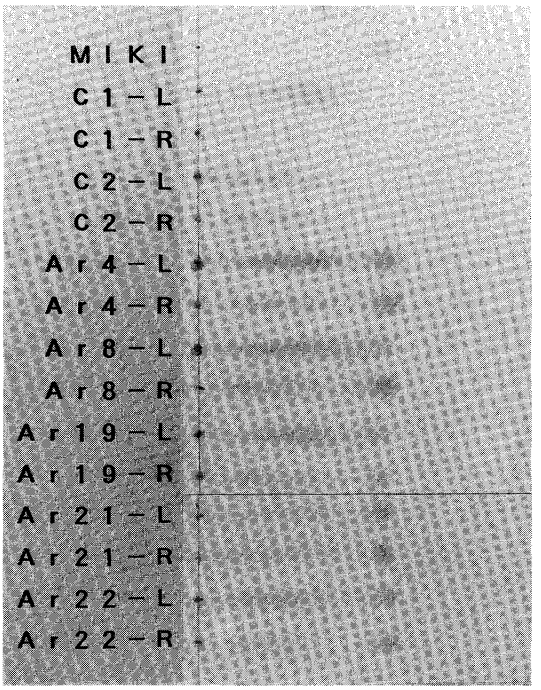

Fig. 2 Detection of mikimopine in leaf and root of cultured plants regenerated from hairy roots of *A. reptans* by paper electrophoresis.
Each sample was applied directly by squeezing about 1 mg of the plant tissue on a filter paper. Electrophoresis was performed in 5 g/*l* ammonium carbonate solution (pH. 9. 8) for 30 min at 500 V. L and R represent leaf and root, respectively. Lane Miki contains authentic mikimopine. Lane C1 and C2 contain an extract from independent control plants. Lanes Ar4, Ar8, Ar19, Ar21, and Ar22 contain an extract from independent trasformed plants.

Fig. 3 A control plant (left) and a transformed plant (right) regenerated from a control root and a hairy root clone Ar-4, respectively.

Table 1. Growth rates, percentages of root fresh mass against total plant fresh mass, and 20-hydroxyecdysone (20-HE) content of *Ajuga* plants regenerated from hairy roots.

Clone No.	Growth Rate*1 (fold)	% of Root*2	20-HE Content (% per dry weight)	
			Stem and Leaf	Root
Original plant*3	—	—	ND	0.030
Control*4	3	29	0.014	0.074
Ar- 4	17	45	0.017	0.070
Ar- 8	8	45	0.017	0.067
Ar-19	16	52	0.019	0.077
Ar-21	19	36	0.026	0.074
Ar-22	20	42	0.009	0.088

Data represent the average values obtained from triplicate samples cultured for a month.
*1 (fresh weight of harvested plant)/(fresh weight of inoculated plant).
*2 (fresh weight of root)/(fresh weight of whole plant) × 100.
*3 Original plants were cultivated over a year in a greenhouse.
*4 Control indicates a plant regenerated from cultured roots of non-transformed plants.

transformed plants exhibited active growth, and they elongated not only into the solid medium but also into air because of the altered geotropism. With the increase of the leaf number and the root mass, the growth rates of the transformed plants increased 8-to 20-fold a month, while those of the control plants increased about 3-fold (**Table 1**). The percentage of root fresh mass against total plant fresh mass ranged from 40 to 50% in the transformed plants as compared with 25 to 30% in the control plants (**Table 1**), and this increase of the percentage of root mass remarkably indicates one of the properties of the plant regenerated from hairy roots. On the other hand, most leaves of the transformed plants were smaller than those of the control though both of them were morphologically similar (**Fig. 4**). The leaf morphology of clone 19, however, became more circular and

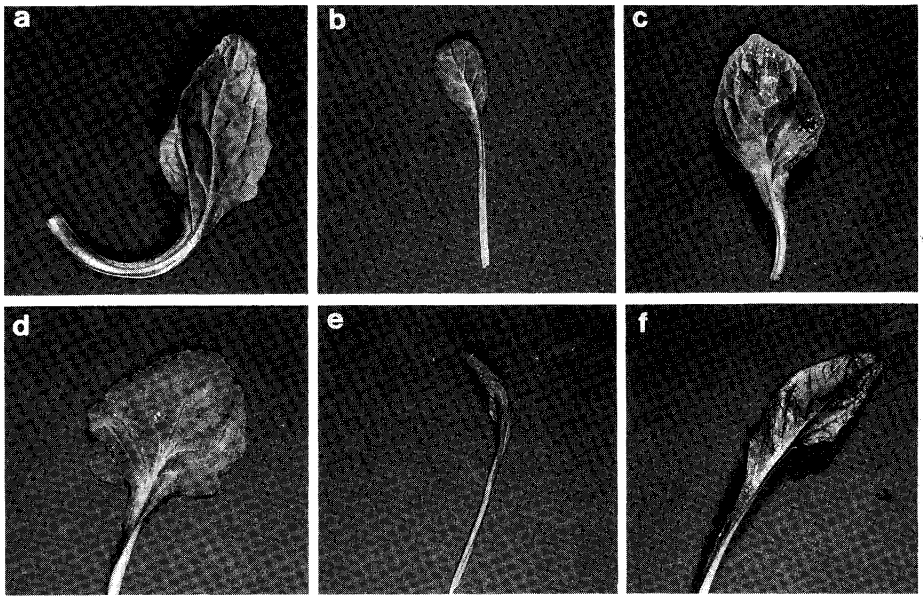

Fig. 4 Morphology of leaves of a control plant and transformed plants.
a : Control plant. b-f : Transfomed plants ; b : Ar-4, c : Ar-8, d : Ar-19, e : Ar-21, and f : Ar-22.

wavier than that of the control (**Fig. 4-a** and **-d**). Since the characteristics of the plants regenerated from hairy roots, such as the enhacement of root growth rate, the increase of root mass, and the alteration of leaf morphology as described above, seem to be influenced by the expression of rooting locus (*rol*) genes[10], the introduction of these genes into useful plants may be utilized as a means for plant improvement.

4. *Contents of 20-HE in regenerated plants*

20-HE was detected at about 0.07% and 0.01-0.02% per dry weight in the roots and in the leaves of the transformed plants, respectively (**Table 1**). Similar contents were observed in the control plants regenerated from cultured roots of non-transformed plants (**Table 1**), and thus, no remarkable differences in the contents between these plants were obtained. However, these plants demonstrated more than twice as high 20-HE contents as that of the original *A. reptans* var. *atropurpurea* plant continuously grown in a greenhouse, which was about 0.03% per dry weight in their roots (**Table 1**). However, the contents in roots of transformed plants were lower than that in hairy roots. We already observed that the ecdysteroid content decreased to one-third to -forth in the hairy roots cultured under light irradiation[4]. Most likely the decreasing contents in the transformed plants was caused by the cultivation with 12 hour-photoperiod. On the other hand, 20-HE was also detected in the leaves and stems of the regenerated plants, irrespective of the transformation with Ri plasmids, but not in those of the original plant (**Table 1**). Though the culture conditions such as the composition of medium, the illumination, and/or the temperature must have affected the production of 20-HE in the regenerated plants, we have not yet obtain a precise explanation of what causes of the existence of 20-HE in the leaves and stems.

Investigations of the properties and the content of 20-HE of the transformed *Ajuga* plants cultivated in a greenhouse are currently in progress.

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《和文要約》

毛状根から再生したアジュガ植物体の特徴

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20-hydroxyecdysone 生産植物 *Ajuga reptans* var. *atropurpurea* の毛状根を 30 日間以上培養すると多数の植物体が再生した．これらの植物体は，培養時では根の生育が旺盛な上，小型化した葉が多数展開し，増殖が著しく活発で，非形質転換対照植物の 3～7 倍の増殖率を示した．一方，根における 20-hydroxyecdysone 含量は 0.07% 前後で，栽培時の起源植物の約 2 倍となったが，非形質転換対照植物のそれと大差無かった．