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Characterization of *In Vitro* Totipotency by *Armoracia rusticana*

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서양고추냉이의 기내 전형성능에 관한 특성

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Plantlets were regenerated from various explants (shoot tip, leaf blade, petiole and root segments) via organogenesis and/or somatic embryogenesis from *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb.. Shoot regeneration rate from callus was highest on the MS medium supplemented with 0.5 mg/L IAA, 5.0 mg/L BA and 10.0 mg/L spermine. A Low frequency of regeneration occurred on hormone-free MS medium. Multiple shoots were regenerated at a pH of 4.0 to 8.0 on MS medium supplemented with 1.0 mg/L BA and 0.1 mg/L NAA. Polyamines promoted shoot- and root-formation by 2 to 4 times normal, Specific proteins associated with organogenesis were identified. Somatic embryogenesis occurred directly from the leaf blade, petiole and root segments cultured on MS medium with 2.0 mg/L BA and 2.0 mg/L NAA. Three types of regeneration in *A. rusticana* were clearly established, which could be applied to the study of morphogenesis and genetics at cell, tissue and organ levels.

Key words : embryogenesis, growth regulator, organogenesis

Each living cell of a multicellular organism should be capable of independent development if provided with the proper external conditions (White, 1951). A totipotent cell is one that is capable of developing by regeneration into a whole organism, but there are some barriers to regeneration of the cells in culture. If there is a loss of certain functions, the cells are no longer totipotent. The loss of regeneration capacity during subcultures and the genetic instability of cultured cells were considered as the barriers to plant tissue culture (Evans and Sharp, 1986). Although many researchers have been focused on the development of efficient plant regeneration systems, regeneration *via* organogenesis and embryogenesis has been reported only in a few higher plant species. Efficient regeneration systems *via* organogenesis and embryogenesis have been observed in some higher plant species: embryogenesis from root explants of carrot (Steward et al., 1964), organogenesis to multiple shoots from leaf explant of tobacco (Horsch et al., 1985) and organogenesis to multiple shoots from root explant of *Arabidopsis* (Marton and Browse, 1991).

Plant regeneration of *Armoracia rusticana* has not been reported except from the leaf blade (Akira 1988: Meyer et al., 1984). The process of organogenesis begins with cell division, and biochemical and physiological changes occur during this process. The biochemical indicators associated with organogenesis have been reported (Kay and Basile, 1987). The changes in protein and DNA contents were studied in *Hydrangea macrophylla* by Molnar and LaCroix (1972), and recently specific proteins related with organogenesis were detected in the somatic embryos of spruce (Roberts et al., 1990).

The present work established the regeneration systems *via*

embryogenesis and organogenesis from various explants of *A. rusticana*. and examined the protein changes in relation to organogenesis. This information could be useful for the study of morphogenesis at cell, tissue and organ levels.

MATERIALS AND METHODS

Plant materials

Explants were excised from *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb. at 4 to 5 leaf stages, and were rinsed with 70% ethyl alcohol for 30 sec, and then placed in 2% a sodium hypochlorite solution for 15 min. The sterilized explants were then washed four times with sterile distilled water. Excised segments were placed on the MS (Murashige and Skoog, 1962) solidified medium in 100 mL flasks. The culture media were adjusted to pH 5.8 prior to autoclaving and were solidified with 0.8% agar. The cultures were kept at 25°C ± 2°, for an 18/6 hour day/night photoperiod at 4.5 W/m^2 light intensity with fluorescent light. After eight weeks in culture, plantlets were used for subsequent experiments.

Investigation of regeneration system

Shoot tip (1-2 mm long), leaf blade (disks of 0.5 cm in diameter), petiole and root (about 10 mm long) segments were cultured on the MS medium supplemented with 0.1 mg/L 2,4-D for callus induction. In order to investigate organogenesis, the calli induced from leaf blade segments were cut into blocks approximately 0.5 cm in size and transferred to MS medium supplemented with plant growth regulators and other compounds as shown in Table 1. At least 20 explants were cultured per treatment, and all the experiments were replicated more than three times.

To investigate the conditions of organogenesis and embryogenesis, the segments of leaf blade, petiole, and root were transferred to MS medium containing combinations of BA and NAA in concentrations of 0, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/L, respectively.

Factors affecting organogenesis

To examine the effects of pH and polyamines on the organogenesis, the pH of the medium was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0, and the concentrations of polyamines: Putrescine, spermidine or spermine to 0.0, 5.0, 10.0, 25.0, or 50.0 mg/L. The MS medium containing 1.0 mg/L BA and 0.1 mg/L NAA was used. The cultures were placed under a continuous light condition of 4.5 W/m^2 with fluorescent light for 4 weeks.

Protein electrophoresis

In order to observe the changes of the protein pattern in relation to different types of organogenesis, callus, callus with roots, callus with shoots and normal shoots were harvested separately. Five hundred milligrams of harvested sample was ground into a fine powder with liquid nitrogen. Samples were centrifuged (12,000 rpm) at room temperature for 10 min.

Table 1. Effects of growth regulators and spermine (mg/L) supplemented to MS medium for the organogenesis from the callus of *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb. cultured for 6 weeks.

Medium code[z]	IAA[y]	NAA[x]	BA[w]	Kinetin[v]	Spermine	No. of roots per explant	No. of shoots per explant
M 1	-	-	-	-	-	40.3[bu]	3.3[e]
M 2	-	-	2.0	-	-	5.3[de]	22.7[b]
M 3	0.5	-	2.0	-	-	4.0[e]	22.0[bc]
M 4	0.5	-	2.0	-	10.0	7.7[cd]	25.7[ab]
M 5	0.5	-	2.0	-	50.0	2.0[ef]	21.3[bc]
M 6	-	-	5.0	-	-	0.0[f]	19.6[c]
M 7	0.5	-	5.0	-	-	0.0[f]	18.4[cd]
M 8	0.5	-	5.0	-	10.0	1.0[f]	36.0[a]
M 9	0.5	-	5.0	-	50.0	0.0[f]	25.5[a]
M 10	0.5	-	5.0	-	-	0.0[f]	23.7[b]
M 11	-	0.1	1.0	-	-	10.3[c]	15.3[d]
M 12	1.0	-	-	2.0	10.0	45.3[a]	2.1[e]

[z]Medium code indicates Murashige and Skoog's basal medium (Murashige and Skoog, 1962) supplemented with the respective growth regulators and other compounds. [y]Indol-3-acetic acid. [x]α-naphthalene acetic acid. [w]6-benzyl aminopurine. [v]6-furfurylaminopurine. [u]Duncan's multiple range test at 5% level

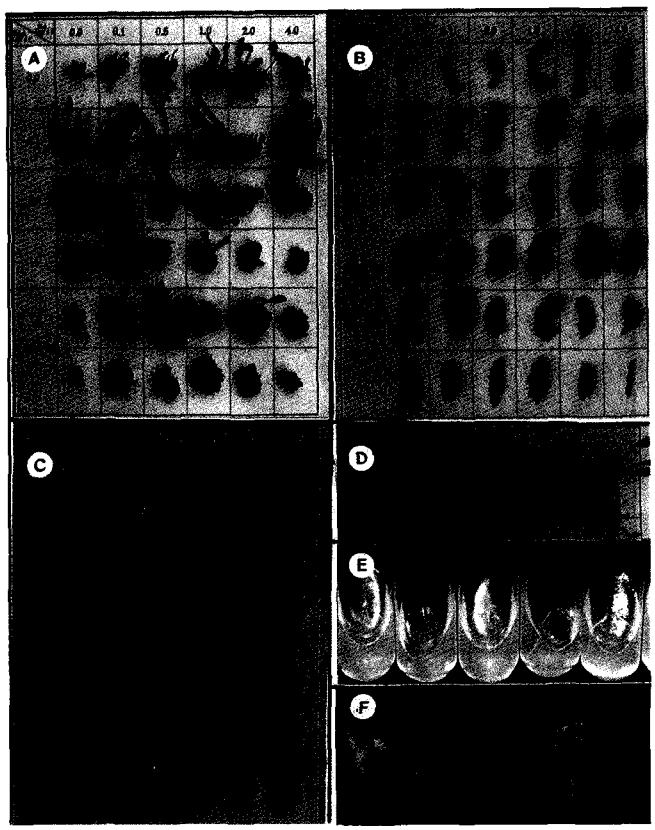

Figure 1. Organogenesis from *in vitro* culture of *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb. A, B and C: Organogenesis from the leaf blade, petiole and root segments, respectively, cultured on the MS media containing various combinations of NAA (0.0, 0.1, 0.5, 1.0, 2.0 or 4.0 mg/L) and BA(0.0, 0.1, 0.5, 1.0, 2.0 or 4.0 mg/L). D: Organogenesis from the petiole segments cultured on the MS media containing 0.0, 0.1, 0.5, 1.0, 2.0 or 4.0 mg/L of BA. E: Plant regeneration from the shoot tip segments cultured on the MS medium free of growth regulators. F: Multiple shoot development from the leaf blade segments cultured on the MS medium containing 4.0mg/L of zeatin.

One-dimensional SDS-PAGE was carried out according to Laemmli (1970) using 10% acrylamide resolving gel and 4% stacking gel 1 mm thick. The supernatant was mixed with Laemmli buffer (1:1 v/v, 62.5 mM Tris-HCl pH 6.8 : 1% sodium dodecyl sulfate (SDS), and 5% 2-mercapto ethanol), and boiled for 3 min (Laemmli, 1970). The resolving gel contained 10% acrylamide, 80 mM Tris-HCl (pH 8.8), 1% SDS (w/v), 1.5% ammonium persulfate (AMPS), and N,N,N′-tetramethylene diamine (TEMED). The Stacking gel was made of 4% acrylamide, 62.5 mM Tris-HCl (pH 6.8), 1% SDS, 1.5% AMPS and TEMED. The electrophoresis (10 μg of protein lane^{-1}) was done at a constant 30 mA for 4h at room temperature. After running, the SDS polyacrylamide gels were stained by the silver staining method (Daiichi Chemicals Co., Ltd. Japan). Protein quantification was carried out by the method of Lowry et al. (1951).

RESULTS

Regeneration system

Calli were induced with high frequency (95 to 100%) from shoot tip, leaf blade, petiole or root explants cultured on MS medium supplemented with 2,4-D 0.1 mg/L. Shoots regenerated with high frequency when the calli were transferred to the differentiation media except on the hormone-free MS basic medium (Table 1 M1) or on the MS medium supplemented with 1.0 mg/L IAA, 2.0 mg/L kinetin, and 10.0 mg/L spemine (Table 1 M12). The root formation was optimized to more than 40 roots per explants on M1

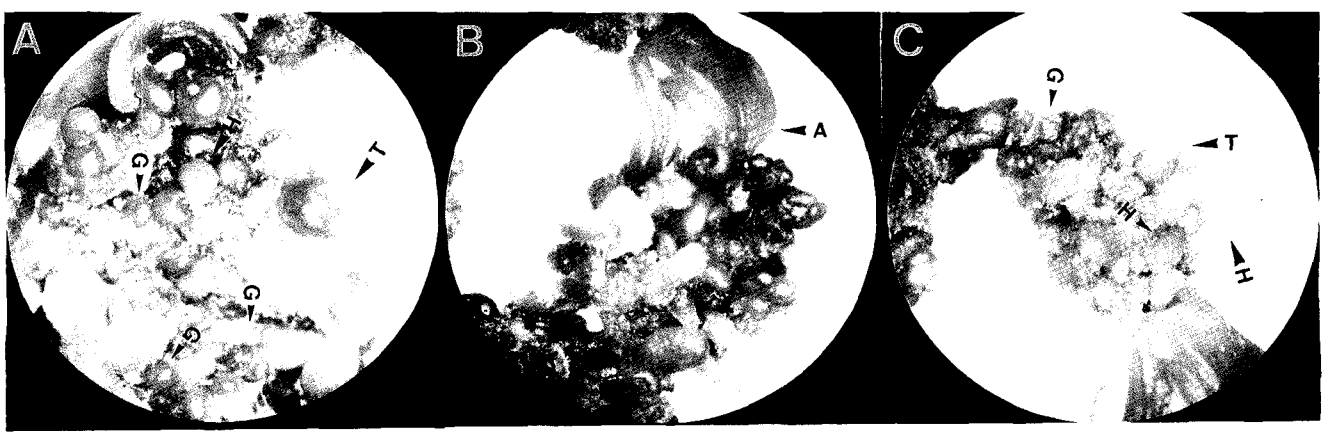

Figure 2. Somatic embryos at various developmental stages on the leaf (A), petiole (B) and root (C) segments of *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb. cultured on the MS medium containing NAA 2 mg/L and BA 2 mg/L. The arrows indicate G: Globular stage, H: Heart stage, T: Torpedo stage and A: Abnormal embryos.

Table 2. Effects of polyamines on the organogenesis from the whole leaves of *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb.

Polyamines mg/L		No. of shoots per explant	No. of roots per explant
Control		5.4^{dz}	2.3^{ef}
Putrescine	5	12.6^{bc}	3.1^{de}
	10	13.3^{bc}	4.4^{cd}
	25	15.4^{b}	10.3^{a}
	50	15.5^{b}	10.5^{a}
Spermidine	5	13.3^{bc}	5.2^{c}
	10	14.0^{b}	3.3
	25	15.1^{b}	6.7^{c}
	50	20.4^{a}	8.4^{b}
Spermine	5	10.5^{c}	2.3^{ef}
	10	14.7^{b}	2.4^{ef}
	25	20.4^{a}	5.3^{c}
	50	5.4^{d}	1.6^{f}

Cultured for 5 weeks on a modified MS medium containing 0.1 mg/L NAA and 1.0 mg/L BA

^{z}Duncan's multiple range test at 5% level

and M12 media. Shoot regeneration was optimized to 36 shoots per explants on the MS medium supplemented with 0.5 mg/L IAA, 5.0 mg/L BA, and 10.0 mg/L spermine (M8). Furthermore, five years-old subcultured callus also has plant regeneration ability. Adventitious root formation was suppressed when the concentration of BA exceeded 5 mg/L. Multiple shoots from each segment of leaf blade, petiole, and root were observed after 3 to 4 weeks of culture on the medium containing 0.5 to 4.0 mg/L BA, or 0.1 to 2.0 mg/L BA combined with 0.1 to 0.5 mg/L NAA, whereas no shoot formation was observed on medium containing more than 4.0 mg/L of NAA (Fig. 1-A,B, and C). The effect of zeatin on multiple shoot formation was similar to that of BA (Fig. 1-F). The effect of kinetin on multiple shoot formation was not noticed (data not shown). Low frequency of shoot formation was observed from all different explants cultured on hormone free medium (Fig. 1-A, C, D, and E). Somatic embryogenesis was also observed from the cultures of leaf, petiole and root segments (Fig. 2). Direct somatic embryogenesis from the explants cultured on MS medium was induced by 2.0 mg/L NAA and 2.0 mg/L BA. Frequency of abnormal embryo development in the cultures was about 10%.

Factors affecting multiple shooting

After four to five weeks cultures of leaf blade, petiole, root, and whole leaf segments, shoot development were observed in the media whose pHs ranged from 4.0 to 8.0, and optimun pH was between 6.0 to 7.0. Addition of putrescine, spermidine, and spermine stimulated shoot and root formation by 2 to 4 times except for 50 mg/L of spermine, compared with the control (Table 2). When regenerated plants were planted into the soil, 95 percent of the regenerants easily adapted to the soil (Fig. 3). Any morphological variation of regenerants compared to the control plants was not observed .

Figure 3. Regenerated plants from the whole leaves of *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb.. The plants grown in soil were cultivated for 3 months.

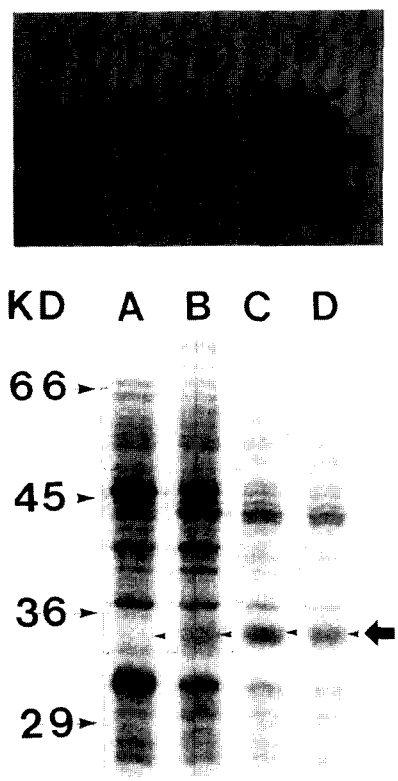

Figure 4. SDS-PAGE of the leaf blade segments of *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb. A: Callus cultured on the MS medium supplemented with 2,4-D 0.1 mg/L, B: Callus and roots subcultured on the MS medium containing 0.1 mg/L 2,4-D, C: Callus and shoots cultured on the MS medium supplemented with NAA 0.1 mg/L and BA 1.0 mg/L, D: Shoots obtained from C. The arrows indicate major specific proteins of 34 kD

Protein patterns in different types of organogenesis

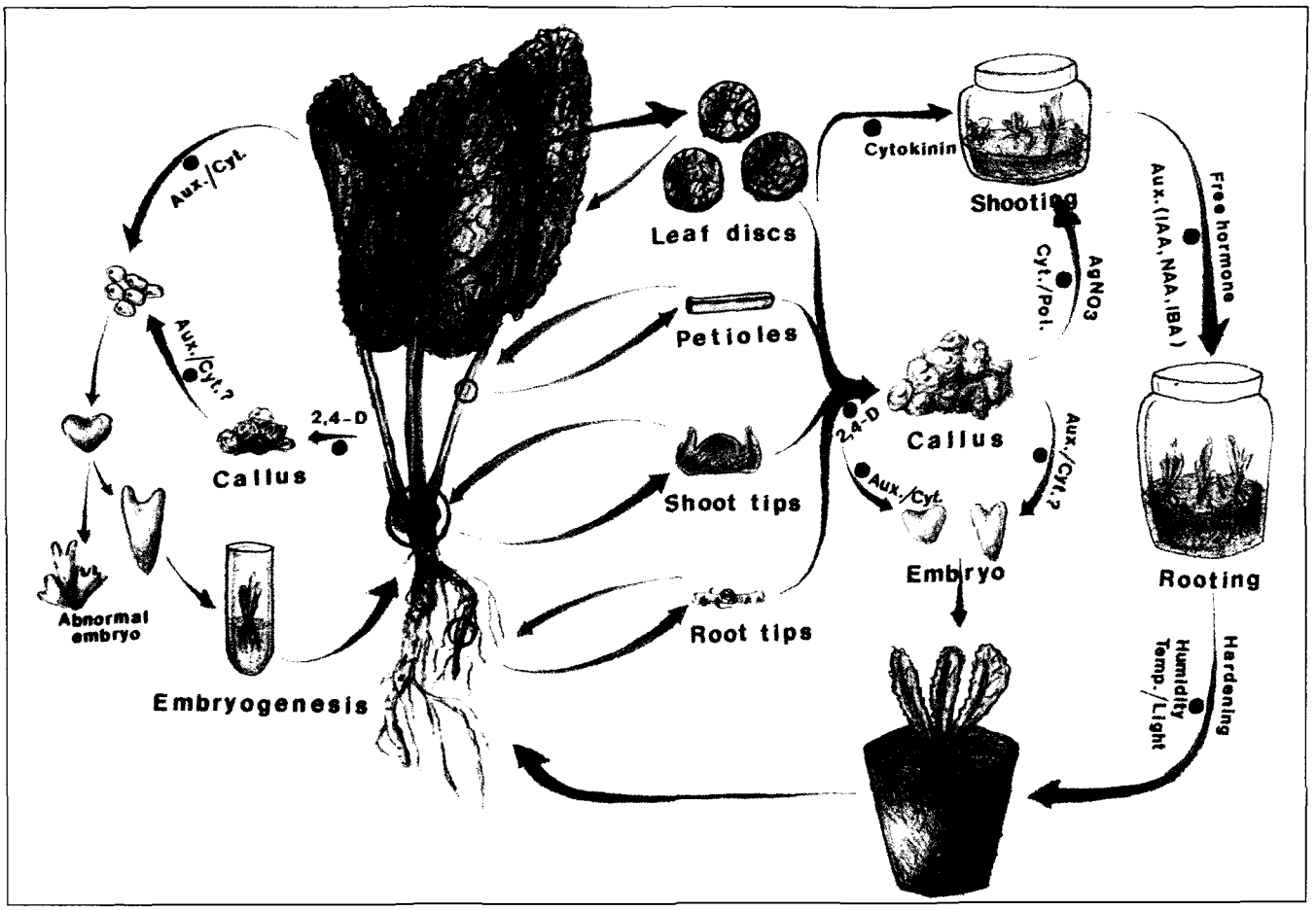

Figure 5. A diagram of *in vitro* regeneration systems from all kinds of explants in *Armoracia rusticana* (Lam.) Gaertn., Mey. et Scherb.. Solid circle(●) indicates the main factor(s) involved in the regeneration step. Aux.: Auxin, Cyt.: Cytokinin, Pol.: Polyamines and Tem.: Temperature.

Figure 4 shows the one-dimensional electrophoregram of the proteins synthesized during various types of organogenesis from the leaf segments culture of *A. rusticana*. Differences between major and minor protein bands were not clearly noticed, but a major polypeptide with a molecular weight of 34 kD was detected in cases of redifferentiated tissues: callus with roots, callus with shoots, and shoots.

DISCUSSION

Three types of plant regeneration have been reported in higher plants: direct regeneration from organs, regeneration *via* callus and somatic embryogenesis (Horsch et al., 1985: Steward et al., 1964). However, there are few reports on higher plant species which can be regenerated from all kinds of explants through these three types of regeneration. Regeneration of shoots from all kinds of explants cultured *in vitro* was reported only in *Arabidopsis* (Marton and Browse, 1991). In the present study, we established three types of efficient plant regeneration from explants (leaf blade, petiole, root) of *A. rusticana*. IAA as well as NAA promoted root formation while 2,4-D was effective only for callus induction. A higher concentration of BA was required for shoot formation from calli rather than organs. Despite the low frequency, shoot induction occurred on MS medium free of growth regulators. This indicates that the endogenous hormones required for regeneration are present in the plant organs. The ratio of auxin to cytokinin determines the type of segments: that is a low auxin level combined with high cytokinin produced multiple shoots with fewer roots, while a high auxin level combined with low cytokinins produced fewer shoots and more roots. Somatic embryogenesis occurs directly from the explants or indirectly *via* callus (Sharp et al., 1980). In this study, direct somatic embryogenesis was observed on the surface of the leaf blade, petiole, and root segments, and indirectly *via* callus in a low frequency. The promotion of somatic embryogenesis was observed in the presence of 2.0

mg/L BA and 2.0 mg/L NAA. pH and polyamines were investigated to evaluate their effects on organogenesis. The optimal pH in the culture media for most plant species ranged from pH 5.5 to 6.0 (Murashige and Skoog, 1962). However, multiple shoots on *A. rusticana* occurred at a relatively wide range of pH, from 4.0 to 8.0. Polyamines promoting the shoot formation as well as the root formation (Jarvis et al., 1985), were found to have the same effect in the present study. The specific protein (34 kD) appearing during organogenesis could provide valuable information on redifferentiation and on the genes controlling organogenesis in *A. rusticana*, if identified

In conclusion, three types of plant regeneration were certified using various explants from *A. rusticana* (Fig. 5). These results indicated that *A. rusticana* can be used as a model plant species for the study of morphogenesis and for transformation systems (Bea et al., 1994).

적 요

십자화과 향신료 작물인 서양고추냉이 (*Armoracia rusticana* (Lam.) Gaertn., May. et Scherbs)의 재분화에 미치는 제 요인을 조사하였다. 서양고추냉이의 callus로부터 식물체 재분화는 IAA 0.5 mg/L, BA 5.0 mg/L, spermine 10 mg/L이 첨가된 MS배지에서 가장 높았다. BA 2.0 mg/L과 NAA 2.0 mg/L가 첨가된 MS배지에 엽, 엽병, 뿌리의 조직을 치상한 결과 각각의 조직으로부터 체세포배가 발생하였으며, 기관발생 경로를 거쳐서 식물체가 재분화 되었으며, 빈도는 낮았지만 생장조절물질이 포함되지 않은 MS배지에서도 각 조직으로부터 식물체가 분화되었다. 수소이온 농도는 pH 4.0부터 8.0의 비교적 넓은 범위에서 multiple shoot가 형성되었으며, polyamine류의 첨가는 shoot와 뿌리의 형성을 촉진시켰다. Callus로부터 뿌리 또는 잎이 분화 중인 조직에서 특이적인 단백질이 생성되는 것이 확인되었다.

이상의 결과, 서양고추냉이는 생장점 배양, 체세포배발생, 기관발생과정을 각기 거쳐 식물체가 재생될 수 있는 식물체로 확인되었다. 서양고추냉이의 이러한 특성은 세포, 조직, 기관의 형태형성 연구 및 형질전환 연구에도 좋은 재료로 이용되리라 생각된다.

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