식물조직배양학회지 제24권 제6호
Korean J. Plant Tissue Culture
Vol. 24, No. 6, 329~334 (1997)

Regeneration of Adventitious Shoots from *Populus deltoides* Bartr.

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미류나무 엽육조직에서 식물체 재분화

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Adventitious organs (shoots and roots) from mature leaves of *Populus deltoides* (Eastern cottonwood) clones were induced on WPM (Woody Plant Medium) containing fructose as a carbon source and different combinations of either BA (Benzyl aminopurin) or TDZ (Thidiazuron) with NAA (Naphthaleneacetic acid). The morphologies of adventitious shoots were different depending on the kinds of growth regulators and the length of shoots induced on the medium containing BA were generally longer than that of TDZ. BA 0.5 mg/L and TDZ 0.01 mg/L combined with either 0.05 or 0.1 mg/L NAA showed the highest values in the frequency of regeneration (63% to 100%) the number of shoots per explant and in the organogenic sites per explant among tested three clones (A, B and C). Although there was a minor clonal difference in the development of adventitious shoots from leaf segments, their responses to growth regulators were very similar.

Key words: adventitious shoots, BA, organogenic sites, TDZ,

The genus *Populus*, in the family *Salicaceae*, comprises a number of commercially important species and occupies a broad range of habitats throughout the world, especially planted in recreational areas, and harvested for pulps, timbers and biomass (Anderson and Krinard, 1985: Kennedy, 1985). Eastern cottonwood (*P. deltoides* Bartr.), in the *Aigeiros* section of this genus, regards as one of the most important species among forest trees due to having a considerable genetic variability, easy propagation by stem cuttings, and interspecific hybridization which can accelerate the breeding programs. Moreover, this species has a small genome size (1.1 pg/diploid nucleus) which can facilitate gene manipulation procedures for plant genetic engineering area (Dhillon, 1987).

Cottonwood has been attempted to establish in vitro systems (Kouider et al., 1984: Prakash and Thielges 1989: Savka et al., 1987: Uddin et al., 1988). However, this species showed the extreme cytokinin toxicity (especially to BA) which caused the necrosis of developing shoot tips in vitro (Coleman and Ernst, 1989: Sellmer et al., 1989). Prakash and Thielges (1989) reported in vitro regeneration system, but their system required a prolonged time for establishing callus before regenerating procedure and, moreover, most adventitious shoots derived through the callus phase which may increase the possibility of somaclonal variation. Coleman and Ernst (1989) were able to induce adventitious shoots directly from internodal segments of *P. deltoides*. According to their results, the regeneration was different from clones: among 16 clones, 4 clones produced adventitious shoots but no shoots could be observed from 12 clones.

Once reliable and repeatable regeneration systems for this species is established, those will be valuable tools for breeding programs and for plant genetic engineering. In this report, high frequency and efficient induction of direct organogenesis from three clones (*P. deltoides*) were developed by using various growth regulators and their combinations.

MATERIALS and METHODS

Shoot induction

Fully expanded leaves (5th to 7th from the each branch) were harvested from the three clones (designated by A, B and C) grown in the green house. They were dipped into 70% (v/v) ethanol for few seconds followed by rinsing with sterilized water for three times (5 min/rinse). Leaves were then sterilized in the solution containing sodium hypochlorite (10% diluted commercial clorox) and a few drops of Tween-20 for 20 min. After rinsing with sterile water for three time (5 min/rinses), leaves were dissected out by removing 1 cm^2 segments but midvein was excluded. Each leaf segment from three clones was placed onto agar-solidified medium with its adaxial side in contact with the WPM containing 20 g/L fructose supplemented with 4 levels of NAA (none, 0.01, 0.05, and 0.1 mg/L) in combination with either 4 levels of BA (0.05, 0.1, 0.5, and 1.0 mg/L) or with 4 levels of TDZ (0.01, 0.05, 0.1 and 0.5 mg/L). Each petri plate included 10 leaf segments and each treatment was consisted of three replications. In addition to these combinations, a complete set of identical media lacking growth regulators as control was used. Media were solidified with 0.8% agar and pH was adjusted to 5.6 before autoclaving. Cultures after 2 weeks under the dark condition were transferred into the 16 hr photoperiod condition with cool white fluorescent light (60 μ $moles^{-1}m^{-2}$) at 25°C until data was collected. For the elongation of shoots, the same medium (WPM) supplemented with 10 g/L fructose, 0.05 mg/L BA and 1 mg/L kinetin were used. Shoots over 2 cm in their lengths were transferred to the rooting medium (WPM containing 10 g/L fructose and 1 mg/L IBA).

Data collection and Statistical analysis

The number of leaf segments with organogenic sites (root, shoots or root/shoot), the total number of organogenic sites per the leaf segments and the total number of shoots were counted after 5 weeks in culture. Each organogenic site was characterized by a local swelling site where one or more adventitious organs be emerged. Number of distinctive organs from each organogenic site were counted and recorded separately.

To analyze the effects of the growth regulators on the frequency of leaf segments developing shoots, average number of shoots per segments, and average number of organogenic sites per segment, analysis of variance (ANOVA) tests with a 3-way, fixed-effects model (clone, BA, TDZ and NAA as main-effects and their interactions) using PROC ANOVA of the Statistical Analysis System (SAS) package (SAS institute, 1985) were employed. Percentage data were transformed into arcsine for normalizing their distribution prior to ANOVA analysis.

RESULTS

Two weeks after culturing leaf segments under the dark condition, leaf segments began to swell and small white calli appeared on the surface of them. The swollen leaf segments turned into red and began to produce adventitious shoots after transfer to the light condition. Most shoots formed at or near the red, compact calli, while some developed directly from the leaf tissues (Fig. 1). In addition, some organogenic sites developed into structures resembling somatic embryos (Fig. 1).

Adventitious shoots were induced at higher concentrations of BA (0.5 and 1 mg/L) in combination with all concentration of NAA tested (Table 1), whereas either shoots, roots, or both were induced at lower concentrations of BA (0.05 and

Table 1. *In vitro induction of shoots* (S), *roots* (R), *or shoots and roots* (S/R) *from P. deltoides leaf segments originating from fully expanded, greenhouse-grown leaves. Data are collected separately from leaves of three clones.*

Clone	BA × NAA[a]	NAA 0	NAA 0.01	NAA 0.05	NAA 0.1	TDZ × NAA	NAA 0	NAA 0.01	NAA 0.05	NAA 0.1
A	BA 0.05	-	-	R	R	TDZ 0.01	S	S	S	S
	BA 0.1	S	S	S/R	S/R	TDZ 0.05	-	S	S	S
	BA 0.5	S	S	S	S	TDZ 0.1	S	S	S	S
	BA 1	S	S	S	S	TDZ 0.5	S	-	-	-
B	BA 0.05	-	-	-	R	TDZ 0.01	S	S	S	S
	BA 0.1	-	-	-	-	TDZ 0.05	S	S	S	S
	BA 0.5	-	S	S	S	TDZ 0.1	S	S	S	S
	BA 1	-	S	S	S	TDZ 0.5	-	-	-	-
C	BA 0.05	-	-	R	R	TDZ 0.01	S	S	S	S
	BA 0.1	-	-	S	-	TDZ 0.05	S	S	S	S
	BA 0.5	S	S	S	S	TDZ 0.1	S	S	S	S
	BA 1	S	S	S	S	TDZ 0.5	S	S	S	S

[a]Unit of concentration: mg/L

Figure 1. Development of adventitious shoots directly from an unwounded area of a leaf explants from a greenhouse-grown *P. deltoides* (clone: C). WPM containing 0.01 mg/L TDZ and 0.05 mg/L NAA was used.

0.1 mg/L) depending on clones tested (Table 1). Only roots were induced only from three clones cultured on the media containing 0.05 or 1 mg/L BA in combination with 0.05 or 0.1 mg/L NAA. In contrast to BA, no root was observed on the leaf segments grown on the medium containing TDZ (Table 1).

The percentage of explants producing shoots varied considerably depending on the growth regulators and on the clones. The regeneration percent was the highest when leaf segments cultured on the media containing 0.5 mg/L BA and either 0.05 or 0.1 mg/L NAA (Fig. 2). No shoot induction was observed at 0.05 mg/L BA, and shoot was considerably less at 1.0 mg/L BA than it was at 0.5 mg/L BA. The organogenic responses to growth regulators was similar among the clones, even though regeneration frequency of clones A and C was higher than that of B. The results of ANOVA showed that the main effects of clone, BA and NAA were all significant (Table 2), as well as all the interaction terms except clone × NAA. This suggested that the specific combinations of BA and NAA for shoot development are necessary depending on clones. In contrast to BA, shoot development was the highest at 0.01 mg/L TDZ, and steadily decreased at higher concentrations of TDZ. Shoot development increased as higher concentrations of NAA increase (0.05 and 0.1 mg/L). Main effects of clone, TDZ and NAA were significant (Table 2). Among interaction effects, only the interaction between clone × NAA was significant.

Most of the explants from clone C developed shoots in response to either cytokinins (95% of the explants developed shoots in 6 of the 32 combinations of growth regulators) (Fig. 2). However, only a small part of the explants from the

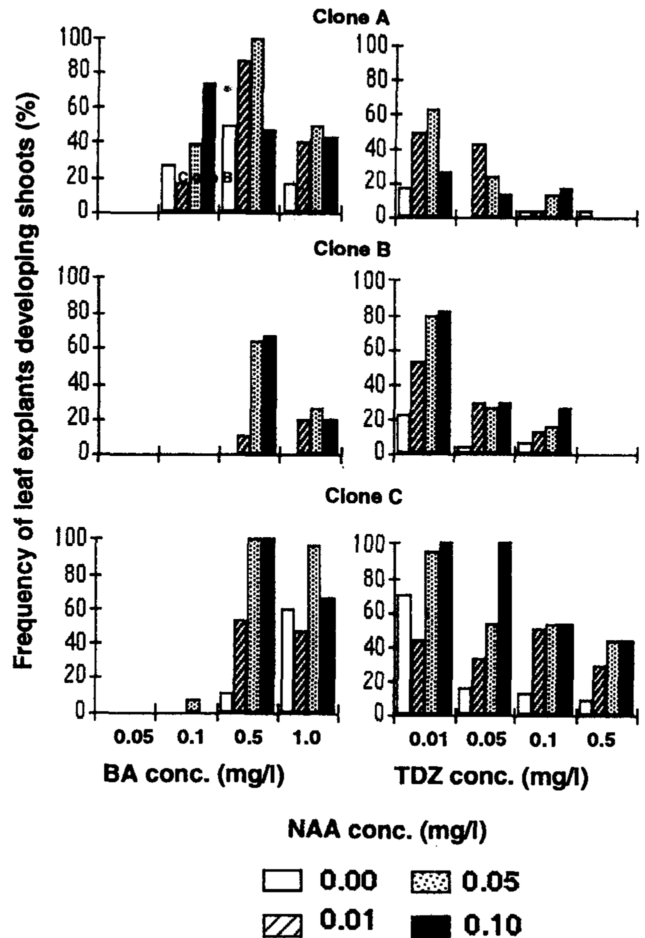

Figure 2. Frequency of leaf explants(% of total) developing one or more shoots on leaf explants from three *P. deltoides* clones cultured on the WPM containing NAA in comination with either BA or TDZ.

other two clones developed shoots. The number of explants developing shoots was more at higher concentrations of NAA (i.e, 0.05 to 0.1 mg/L) than at lower concentrations of NAA regardless of cytokinin concentrations.

Another measurement of organogenesis is the number of shoots induced per explant. More shoots developed at higher levels of BA (0.5 or 1.0 mg/L, Fig. 3, Table 2) than at lower levels of BA. However, there was significant variation among clones: the clone C showed the highest shoots per explant (15.8-16.8) at 0.1 mg/L NAA combined with either 0.5 or 1.0 mg/L BA (Fig. 3) but clones A and B showed 6.5 and 2.5, respectively at 0.5 mg/L BA combined with 0.05 mg/L NAA. The number of shoots was more at lower levels of TDZ than at higher levels (Fig. 3, Table 2). As in BA, clone C developed more shoots per explant than the other two clones, but organogenic responses of clones to TDZ were more consistent than to BA. The average number of shoots induced

Table 2. ANOVA tables for frequency of explants developing shoots (A), number of shoots (B), and number of organogenic sites (C). Percentage were transformed to arcsin prior to analysis.

A. Frequency of leaf explants developing shoots *

Clone × BA × NAA interactions

Source	DF	ANOVA SS	F	PR>F
Clone	2	11376.6725	21.85	0.0001
BA	3	59599.7766	76.31	0.0001
NAA	3	11238.1240	14.39	0.0001
Clone * BA	6	13308.5905	8.52	0.0001
Clone * NAA	6	1111.2805	0.71	0.6421
BA * NAA	9	11509.2160	4.91	0.0001
Clone * BA * NAA	18	9357.4205	2.00	0.0168
Error	96	24991.3384		
Total	143	142492.4184		

Clone × TDZ × NAA interactions

Source	DF	ANOVA SS	F	PR>F
Clone	2	21266.5158	38.52	0.0001
TDZ	3	33779.9145	40.79	0.0001
NAA	3	10964.4625	13.24	0.0001
Clone * TDZ	6	856.5632	0.52	0.7931
Clone * NAA	6	4285.6982	2.59	0.2281
TDZ * NAA	9	3889.6914	1.57	0.1366
Clone * TDZ * NAA	18	6855.4651	1.38	0.1595
Error	96	26499.1253		
Total	143	108399.4361		

B. Average number of shoots

A. Clone × BA × NAA interactions

Source	DF	ANOVA SS	F	PR>F
Clone	2	262.9839	42.31	0.0001
BA	3	488.6863	48.12	0.0001
NAA	3	191.8108	20.57	0.0001
Clone * BA	6	359.2355	19.26	0.0001
Clone * NAA	6	232.9978	12.49	0.0001
BA * NAA	9	178.9495	6.04	0.0001
Clone * BA * NAA	18	348.9361	6.24	0.0001
Error	96	298.3600		
Total	143	2321.9600		

Clone × TDZ × NAA interactions

Source	DF	ANOVA SS	F	PR>F
Clone	2	1124.4904	45.05	0.0001
TDZ	3	814.2125	21.75	0.0001
NAA	3	276.9525	7.4	0.0002
Clone * TDZ	6	412.1446	5.5	0.0001
Clone * NAA	6	289.2295	3.86	0.0017
TDZ * NAA	9	216.4014	1.93	0.0570
Clone * TDZ * NAA	18	337.0999	1.5	0.1065
Error	96	1198.0867		
Total	143	4668.6175		

C. Average number of organogenic sites

Clone × BA × NAA interactions

Source	DF	ANOVA SS	F	PR>F
Clone	2	11.8606	16.32	0.0001
BA	3	61.7014	56.61	0.0001
NAA	3	22.2425	20.41	0.0001
Clone * BA	6	15.5628	7.14	0.0001
Clone * NAA	6	13.2633	6.08	0.0001
BA * NAA	9	21.4225	6.55	0.0001
Clone * BA * NAA	18	27.3133	4.18	0.0001
Error	96	43.8800		
Total	143	208.2426		

Clone × TDZ × NAA interactions

Source	DF	ANOVA SS	F	PR>F
Clone	2	20.6493	27.26	0.0001
TDZ	3	51.3369	45.18	0.0001
NAA	3	18.9669	16.69	0.0001
Clone * TDZ	6	6.5913	2.90	0.0121
Clone * NAA	6	8.3913	3.69	0.0024
TDZ * NAA	9	18.4362	5.41	0.0001
Clone * TDZ * NAA	18	11.4949	1.69	0.0551
Error	96	36.3600		
Total	143	172.2266		

from explants was the highest at 0.01 mg/L TDZ in combination with 0.05 mg/L NAA in all three clones.

The number of organogenic sites per explant (Fig. 4) was smaller than the number of shoots per explant (Fig. 3). Clone C developed 15-20 shoots per explant whereas the other clones developed 2-7 shoots per explant. The number of organogenic sites was the highest on explants cultured on medium containing 0.5 mg/L BA or 0.01 mg/L TDZ in combination with 0.1 mg/L NAA (Fig. 4). The optimal combinations of growth regulators for the development of organogenic sites and adventitious shoots were similar. The results of ANOVA showed that all three main effects as well as their interaction were significant (Table 2).

Adventitious shoots elongated rapidly when transferred to fresh WPM containing 10 g/L fructose, 0.01 mg/L BA and 1 mg/L kinetin. Within three week, the shoots elongated about 2 to 3 cm and those were rooted easily on WPM containing 10 g/L fructose supplemented with 1 mg/L IBA.

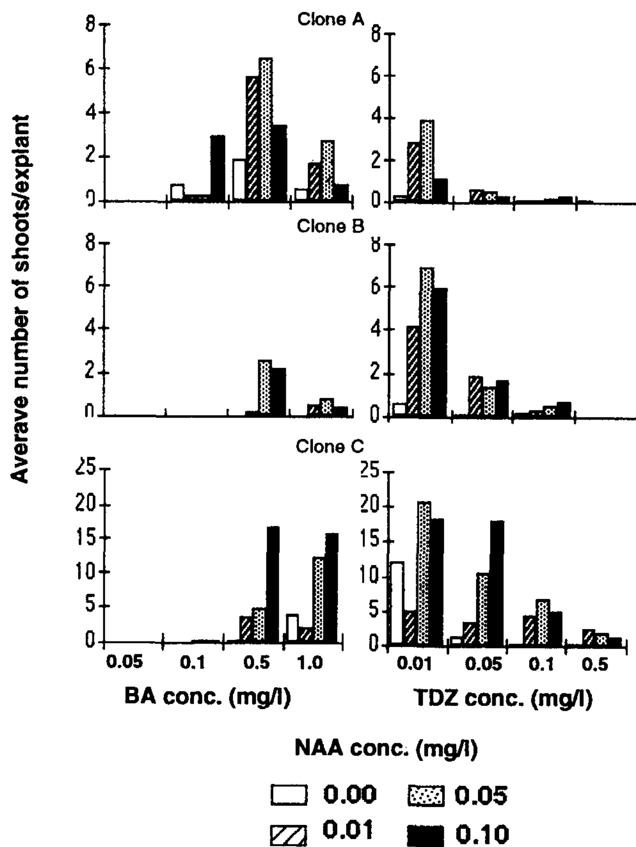

Figure 3. Average number of developing shoots per leaf segment from three *P. deltoides* clones cultured on the WPM containing NAA in comination with either BA or TDZ.

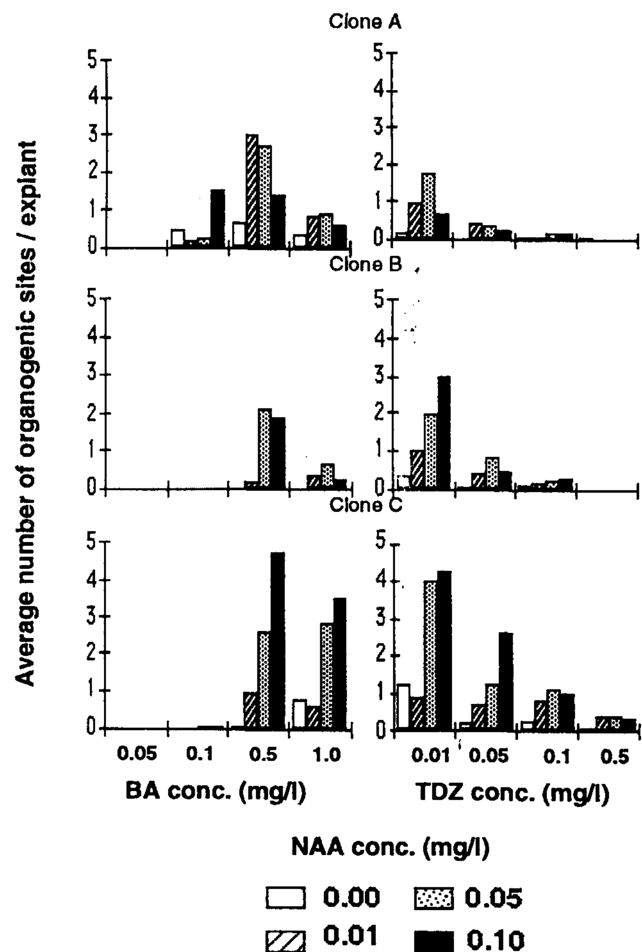

Figure 4. Average number of organogenic sites per leaf segment from three *P. deltoides* clones cultured on the WPM containing NAA in comination with either BA or TDZ.

DISCUSSION

Coleman and Ernst (1989) and Sellmer et al. (1989) observed browning and necrosis on explants exposed to medium with sucrose and cytokinins, suggesting that cytokinins, especially BA, might be toxic *P. deltoides* or its hybrids (*P. deltoides* × *P. nigra*) when cultured them in vitro. To minimize cytokinin toxicity, Coleman and Ernst (1989) used natural plant growth regulator (zeatin) in their medium. In contrast to their attempts (Coleman and Ernst, 1989), BA and TDZ both sucessfully applied and stimulated the development of adventitious shoots from three clones of *P. deltoides* in our research. This is probably the reason from application of fructose instead of sucrose as a carbon source. Our earlier report (Sul and Shin, 1997) indicated that in vitro cultures of *P. deltoides* leaves especially on the WPM with fructose and BA were induced adventitious shoots. Furthermore, adventitious shoots were readily elongated on media containing the same carbon source (fructose) and low concentrations of BA.

An important consideration in tissue culture is to minimize handling times by reducing serial transfers in vitro, especially for woody plants, because they respond slowly in vitro (Pierik, 1987). Although Prakash and Thielges (1989) reported that adventitious shoots were induced from *P. deltoides* but required long term processes for callus induction, subculture and regeneration. The advantages of our system is that only 3 months are enough to produce rooted plants in vitro. Moreover, direct organogenesis was induced using various growth regulators' combination while minimizing the possibility of somaclonal variation. Therefore, shoots from this system can be used as clonal propagation.

Genotypic dependence in inducing adventitious shoots from various clones of *P. deltoides* have been reported (Coleman and Ernst, 1989) in which adventitious shoots were produced in only 2 clones among 16 clones tested and the number of shoots produced per explant was only 3.6 to 4.3. All three

clones tested in our system, they all produced adventitious shoots on the WPM supplemented with 0.5 mg/L BA or 0.01 mg/L TDZ combined with either 0.05 or 0.1 mg/L NAA. While different levels and types of cytokinins induced different types of organs: specific combinations of BA and NAA induced roots but not on all combinations of TDZ with NAA. Thus, our system can be applied to other *P. deltoides* clones with above specific plant growth regulators' combinations.

Adventitious shoots induced by TDZ, in comparison to those induced by BA, were often numerous, small and very compact. Counts of organogenic sites can offer other advantages for selecting somaclonal variants or transgenic plants because of selecting regenerants from independent origins. Prakash and Thielges (1989) reported that 0.02 mg/L TDZ induced adventitious shoots on calli from *P. deltoides*. Other reports (apples) showed that 2 mg/L or 0.6 mg/L TDZ combined with specific concentrations of auxin produced the best results for inducing adventitious shoots from leaf segments (Abu-Qaoud et al., 1990: Chevreau et al., 1989: Sriskandarajah et al., 1990). Our results showed that for *P. deltoides*, a low concentration (0.01 mg/L) of TDZ combined with a higher concentration (0.05 mg/L) of NAA produced the best results.

Most adventitious shoots were induced at or near wound sites and organogenic sites had structures resembling somatic embryoids (Fig. 1), which failed to germinate on new medium. Additional research will be required whether or not these structures truly represent the somatic embryogenesis. Our direct regeneration system based on mature leaves may be suitable for *Agrobacterium* and biolistic transformation of *P. deltoides*.

적 요

미류나무의 잎절편들을 fructose를 첨가한 WPM에서 배양하였으나, TDZ와 BA 그리고 NAA의 여러 농도와의 조합에서 다른 형태의 기관들(뿌리나 줄기)이 관찰되었으며, 체세포 배와 유사한 기관들의 형태도 관찰되었다. 3계통 미류나무들의 잎절편들을 사이토카이닌(BA 또는 TDZ)와 오옥신(NAA)를 첨가한 WPM 배지에서 접종시켜, 재분화를 유도한 결과, BA (0.5 mg/L)나 TDZ (0.01 mg/L)와 NAA (0.05 mg/L 또는 0.1 mg/L)의 조합에서 재분화 율은 각각 63%에서 100%이고, 재분화 개체 수는 잎절편당 평균 2.5부터 20개, 그리고 부정줄기 형성체군(organogenic site) 들은 평균 1.8에서 4.5개로 높게 나타났다. 또한 세 계통의 미류나무들은 각기 유사한 처리 조합에서 높은 재분화율을 나타내었다.

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(1997년 2월 6일 접수)