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Difference in the Ability of Initiation and Maintenance of Embryogenic Cultures among Sugi (*Cryptomeria japonica* D. Don) Seed Families

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Abstract

Zygotic embryos of *Cryptomeria japonica* were collected from 20 clones and cultured on half-concentration of Murashige and Skoog medium containing 10 μM 2, 4-dichlorophenoxyacetic acid to examine the differences in embryogenic tissue (ET) initiation among seed families. ETs were obtained in all the seed families tested, with an overall initiation rate of 45.6 % (689 from 1512 explants). However, initiation rates and maintenance rates differed considerably among seed families. They varied from 7.5 % to 78.5 % and from 1.2 % to 27.8 %, respectively.

Sugi (*Cryptomeria japonica* D. Don), the family Taxodiaceae, is the most important commercial forest tree species in Japan, and sugi plus trees have been selected from 1950's, mainly based on growth characteristics. Somatic embryogenesis has great potential for the mass propagation of sugi trees with desirable characteristics (e.g. rapid growth and commercially attractive wood qualities). Embryogenic tissues (ETs) could also be used as a target for transformation. Plantlet regeneration through somatic embryogenesis of coniferous species was first induced in *Picea abies* (Hakman and von Arnold, 1985) and *Larix decidua* (Nagmani and Bonga, 1985). Recently, ET induction in *C. japonica* was reported by Ogita *et al.* (1999), from both immature and mature zygotic embryos collected from a single tree. However, genetic variation in somatic embryogenesis ability in *C. japonica* has been known but the reason for this is unknown. In order to use the somatic embryogenesis in *C. japonica* breeding, it is very important to investigate the differences in capacity for somatic embryogenesis among plus tree families. In this study, therefore, we report differences in initiation rate of ETs and maintenance rate of embryogenic cell lines in twenty seed families of *C. japonica* plus trees.

Open pollinated cones of *C. japonica* plus trees were collected on July 14 and 23, 1998, in the crossing garden of the Forest Tree Breeding Center located in Juo (Ibaraki, Japan). We decided on these collection dates according to the results of a preliminary study, in which we collected immature cones from early June to late July at weekly intervals and cotyledonary embryos collected at mid July were found to be the best explants for ET initiation (data not shown). Collected cones were stored in polyethylene bags at 5 ℃ until dissection, for a maximum of five days after collection. They were then surface disinfected for 1 min in 70 % (vol/vol) ethanol, followed by 15 min treatment in 7.5 % (vol/vol) hydrogen peroxide, and rinsed three times in sterile deionized water. The immature seeds were then extracted from the cones, surface disinfected for 1 min in 7.5 % (vol/vol) hydrogen peroxide and rinsed three times in sterile deionized water. The immature seeds were dissected, and explants (excised zygotic embryos) were placed onto initiation medium: half-concentration of Murashige and Skoog (MS) (1962) components supplemented with 2,4-D (10 μM), sucrose (30 g l^{-1}), and agar (8 g l^{-1}). After three to five weeks of culture on the initiation media, all explants were transferred onto maintenance medium. The maintenance medium was the same as the initiation medium, except that it contained 1 g l^{-1} casein hydrolysate and 0.5 g l^{-1} L-glutamine (Jain *et al.*, 1995) and its concentrations of inorganic nitrate salts and 2,4-D were reduced to 50 % and 20 % of that of initiation medium. The pH of the all media was adjusted to 5.8 with KOH and HCl prior to autoclaving at 120 ℃ for 15 min. Approximately 50 m*l* of medium was poured into 90 x 20 mm petri dishes. All cultures were incubated in the dark at 25 ℃. Two months after monthly subculture, the number of explants forming ETs was

counted. All the ETs were then subcultured monthly on maintenance medium solidified with 4.5 g l^{-1} gellan gum.

Within one month after inoculation of explants, two types of tissue were induced. One was ET, which was white to translucent, moist and mucilaginous, and consisting of small dense cells (embryonic region) and elongated and vacuolated cells (suspensor region) (**Fig. 1A**). These morphologies were typical for ETs of conifer (Jain *et al.*, 1995). ET was mainly formed on hypocotyl, and sometimes on cotyledons when cotyledons had been very small. Another type was non-embryogenic tissue, which was yellowish, less moist than ET, and consisting of small cells. This type was formed on cotyledons. ET could be easily distinguished from non-embryogenic tissue by the naked eye. The ET initiation rates of 20 seed families examined are shown in **Table 1**. ETs were formed from explants in all seed families collected on both sampling dates. Initiation rates varied from 9.1 to 78.0 %, with a mean rate of 50.3% in explants collected on July 14, and from 3.4 to 82.5 % with a mean rate of 40.5 % in explants collected on July 23. The overall mean rate was 45.6% (689/1512). This is higher than that found for immature zygotic embryos of *Pinus* species (for example, 1.4% for 10 seed families of *P. taeda*, Becwar *et al.*, 1990, and 15.3% for 13 seed families of *P. strobus*, Garin *et al.*,

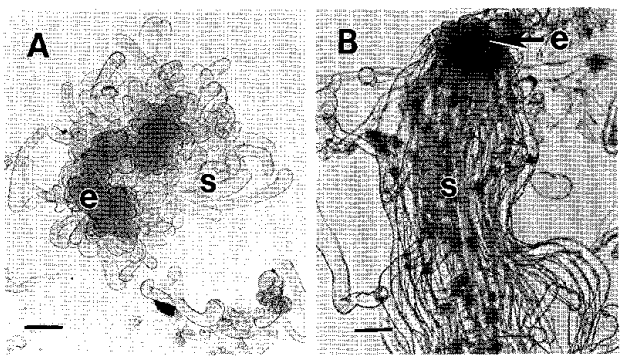

Fig. 1 Embryogenic cultures of *C. japonica*. (A) Microscopy of embryogenic tissue, consisting of embryonic region and suspensor cells. Embryonic regions were intensely stained red by acetocarmine. Bar = 100 μm. (B) Somatic embryo, developed on maintenance medium solidified with gellan gum. Embryonic region was subtended by the suspensor cells. Bar = 100 μm.
e, embryonic region. s, suspensor cells.

Table 1. Embryogenic response of 20 open-pollinated seed families using zygotic immature embryo explants of *C. japonica*. The number of explants forming embryogenic tissue (ET) three months after initial culture, and the number of embryogenic cell lines (ECLs) proliferating nine months after initial culture are shown.

Seed families	Seeds collected on July 14		Seeds collected on July 23	
	Number of explants forming ET	Number of ECLs with proliferating ET after 9 months	Number of explants forming ET	Number of ECLs with proliferating ET after 9 months
Abe 1	8/ 41 (19.5)	1/ 41 (2.4)	6/ 27 (22.2)	2/ 29 (6.9)
Ashigarashimo 8	4/ 35 (11.4)	2/ 35 (5.7)	1/ 29 (3.4)	1/ 31 (3.2)
Gujo 4	28/ 40 (70.0)	0/ 40 (0.0)	25/ 40 (62.5)	1/ 40 (2.5)
Ishikawa 3	31/ 42 (73.8)	1/ 42 (2.4)	17/ 40 (42.5)	0/ 40 (0.0)
Ishiki 4	20/ 40 (50.0)	1/ 40 (2.5)	9/ 40 (22.5)	1/ 40 (2.5)
Kofu-sho 2	29/ 45 (64.4)	3/ 45 (6.7)	21/ 40 (52.5)	3/ 40 (7.5)
Kuji 28	15/ 40 (37.5)	1/ 40 (2.5)	8/ 28 (28.6)	1/ 26 (3.8)
Kuno 1	29/ 39 (74.4)	13/ 39 (33.3)	33/ 40 (82.5)	9/ 40 (22.5)
Minaminasu 5	6/ 33 (18.2)	0/ 33 (0.0)	10/ 41 (24.4)	1/ 41 (2.4)
Minamitama 5	30/ 40 (75.0)	4/ 40 (10.0)	27/ 41 (65.9)	5/ 41 (12.2)
Nakanojo 7	13/ 40 (32.5)	1/ 40 (2.5)	9/ 38 (23.7)	1/ 38 (2.6)
Nishitama 12	13/ 40 (32.5)	1/ 40 (2.5)	16/ 35 (45.7)	2/ 35 (5.7)
Shimotakai 14	32/ 41 (78.0)	7/ 41 (17.1)	18/ 40 (45.0)	4/ 40 (10.0)
Sunami 1	20/ 35 (57.1)	7/ 35 (20.0)	13/ 39 (33.3)	7/ 39 (17.9)
Takahagi 13	24/ 40 (60.0)	6/ 40 (15.0)	22/ 41 (53.7)	10/ 41 (24.4)
Takahagi 16	12/ 38 (31.6)	1/ 38 (2.6)	17/ 40 (42.5)	0/ 40 (0.0)
Takasaki 5	22/ 41 (53.7)	0/ 41 (0.0)	16/ 40 (40.0)	1/ 41 (2.4)
Tanzawa 3	28/ 39 (71.8)	3/ 39 (7.7)	16/ 41 (39.0)	2/ 41 (4.9)
Yaita 5	3/ 33 (9.1)	0/ 33 (0.0)	1/ 20 (5.0)	1/ 20 (5.0)
Yoshida 103	26/ 39 (66.7)	5/ 39 (12.8)	11/ 31 (35.5)	6/ 31 (19.4)
Total	393/781 (50.3)	57/781 (7.3)	296/731 (40.5)	58/734 (7.9)

Note: Values in parentheses are percentages.

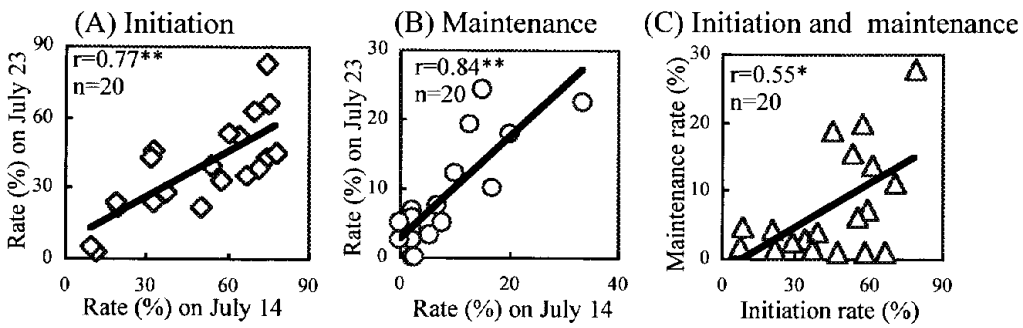

Fig. 2 Relations between (A) embryogenic tissue initiation rates, and (B) embryogenic cell line maintenance rates for explants collected from seed families on July 14 and 23. (C) Relations between rates of initiation and maintenance among 20 seed families. Plots in (C) were average data of two collection dates. r, correlation coefficient. **, significant at the p = 0.01 level. *, significant at the p = 0.05 level.

1998). However, it is lower than that reported for *Picea* species (for example, 53.1% for 25 seed families of *P. glauca*, Park *et al.*, 1993). The initiation rates among the 20 seed families collected on July 14 and July 23 were found to be positively correlated (correlation coefficient r=0.77, significant at the p=0.01 level) **(Fig. 2A)**. This suggests that ET initiation rates are genetically controlled. The maximum initiation rate was 78.5% (62/79) for seed family Kuno 1, and the minimum initiation rate was 7.5% (4/53) for seed family Yaita 5. The wide range of initiation rates obtained here indicates that considerable genetic variability in ET initiation capacity exists among plus tree populations of *C. japonica*. This range is much wider than that (2.6–23.3%) recorded for *Pinus strobus* (Garin *et al.*, 1998).

When ETs were maintained on the medium solidified with gellan gum, outgrowth of suspensors with small embryonal heads at their apices was often observed **(Fig. 1B)**. This outgrowth showed that ETs could develop more on gellan gum medium than agar medium. However, a dramatic decrease in the number of embryogenic cell lines on the maintenance medium was observed six months after the initial culture. This phenomenon was also reported in other conifers (Grain *et al.*, 1998). After that, their numbers decreased only slightly. Embryogenic cell lines were obtained from all the seed families nine months after initial culture **(Table 1)**. The rate of cell lines with proliferating ETs (*i.e.* the rate of maintenance) varied from 1.2% (1/82, for seed family Ishikawa 3) to 27.8% (22/79, for seed family Kuno 1) with an overall mean rate of 7.6% (115/1512). As in the case of ET initiation rates, a positive correlation was found between the rates of maintenance among the 20 seed families collected on July 14 and July 23 (r=0.84, significant at the p =0.01 level) **(Fig. 2B)**. Therefore, the differences in maintenance rates among the families might be caused by genetic differences. Moreover, there was a positive correlation between the rates of initiation and maintenance among the 20 seed families (r= 0.55, significant at the p=0.05 level) **(Fig. 2C)**, indicating that seed families which initiate ETs at high frequency tend to maintain at a high frequency during subculture.

In conclusion, we have shown that there are large differences in capacity for initiation and maintenance of embryogenic tissues among seed families in *C. japonica*. The results also showed that certain families can initiate and maintain embryogenic tissues at high frequencies. To our knowledge, maturation and germination of somatic embryos have not been reported in *C. japonica*. We must establish the methods of plant regeneration from somatic embryos to enable the somatic embryogenesis technique to be applied to a large number of genotypes.

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