Technical Note

Evaluation of somatic embryogenesis from immature cotyledons of Japanese soybean cultivars

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Received December 5, 2006; accepted January 26, 2007 (Edited by S. Ogita)

Abstract To examine the capacity for plant regeneration through somatic embryogenesis, Japanese soybean cultivars were investigated by 3-step-somatic embryogenesis: 1) induction of somatic embryos (SEs) from immature cotyledons, 2) proliferation of SEs in liquid medium and 3) differentiation of SEs into cotyledon-stage embryos. At each step, properties of the cultures varied among cultivars. Notably, significant differences were observed on 1) efficiencies of SE induction, 2) compactness of individual SEs during proliferation, and 3) yields of differentiated embryos (numbers of cotyledon-stage embryos differentiated from 100 mg globular SEs). We found that the compactness of SEs in liquid medium was very important factor for the recovery of well differentiated embryos. In fact, Yuuzuru and Yumeyutaka which indicated high ratios of compact SEs at proliferation step showed high yields of well differentiated embryos. The abilities of these Japanese cultivars to yield cotyledon-stage embryos were superior or comparable to North American cultivar 'Jack', which is used as one of the most preferable genotypes for somatic embryogenesis, indicating their high potentials for plant regeneration.

Key words: Immature cotyledon, somatic embryo, soybean.

Soybean [*Glycine max* (L.) Merrill] is a traditional food crop in several Asian countries such as Japan, Korea and China. It is also an important crop for oil and protein production in North and South America. Since genetically modified soybeans are now widely cultivated in many countries, this crop appears to be one of the most popular plant species as targets of biotechnological improvement. However, molecular biological studies of soybean have been limited by genotype-dependent suitability for transformation.

Two plant regeneration systems, which are mediated by somatic embryogenesis or shoot organogenesis, are exploited in soybean transformation (Somers et al. 2003). Since the first report on plant regeneration from soybean somatic embryos (SEs) (Christianson et al. 1983), culture conditions have been repeatedly modified for efficient and stable regeneration of soybeans (Finer and Nagasawa 1988; Samoylov et al. 1998; Santarem et al. 1997). Currently, soybean SEs are efficiently induced from immature cotyledons on solid culture medium supplemented with high concentrations (for example 40 mg l^{-1}) of 2,4-dichlorophenoxyacetic acid (2,4-D), proliferated in liquid or solid media containing 2,4-D at lower concentrations, and differentiate into cotyledon-stage embryos in the absence of exogenous auxin (http://www.cropsoil.uga.edu/soy-engineering/index.html). Indeed, this culture protocol has been employed for transformation of North American soybean cultivars such as 'Jack' (Chiera et al. 2004; Stewart et al. 1996), 'Williams' (Ko et al. 2003) and 'Fayette' (Finer and McMullen 1991).

Previously, Komatsuda and Ohyama (1988) reported that some Japanese soybean cultivars have high competence for somatic embryogenesis on solid media containing 2 mg l^{-1} 2,4-D or 10 mg l^{-1} naphthaleneacetic acid. Using soybean varieties including Japanese cultivars, they also showed that the efficiency of somatic embryogenesis is affected by genotype and/or sucrose concentration in the culture media (Komatsuda et al. 1991). However, there is no report on SE-mediated transformation of Japanese soybean cultivars so far. A recent study demonstrated that Japanese soybean cultivars are genetically distant from North American cultivars (Ude et al. 2003), suggesting different

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; FNL, Finer and Nagasawa Lite; MS, Murashige and Skoog; SE, somatic embryo

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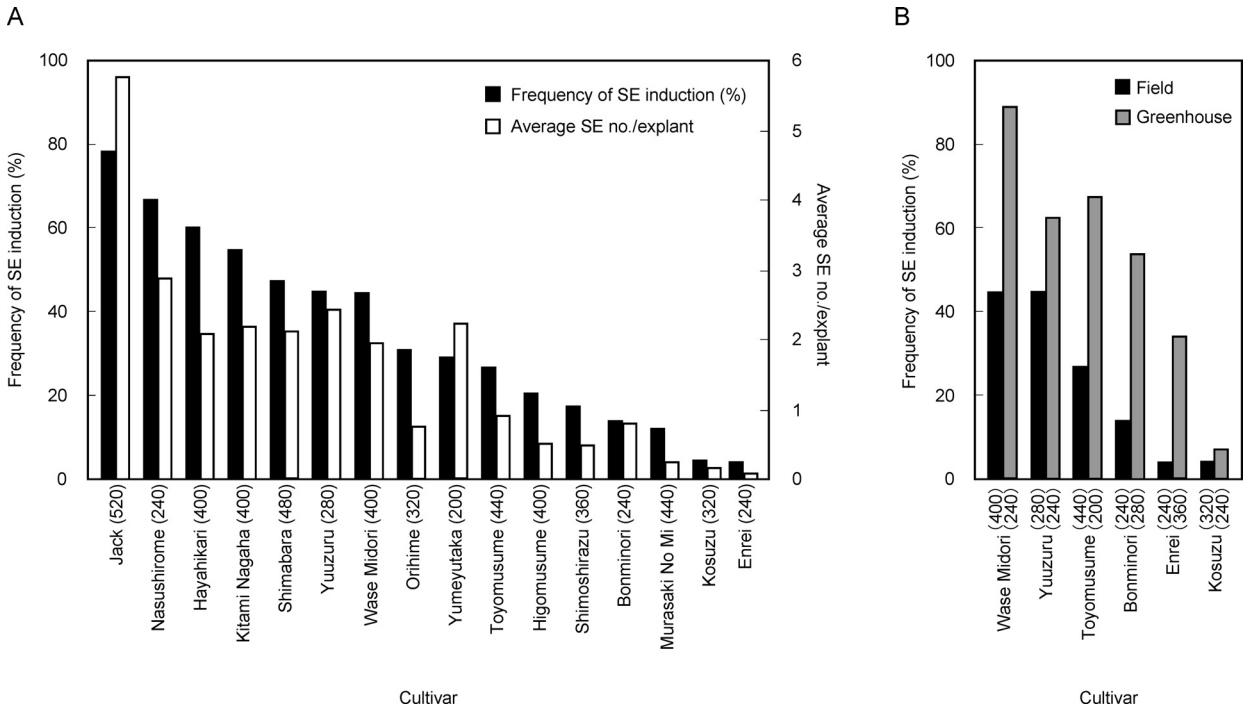

Figure 1. Efficiencies of SE induction from immature cotyledons. Immature cotyledons were extruded from developing seeds which have been excised from surface-sterilized young pods of soybean plants. Immature cotyledons were placed onto solid MSD40 medium, with abaxial side facing the medium (flat side up), and incubated in a growth chamber adjusted at 25 °C and 23 h photoperiod (5–10 μmol m^{-2} s^{-1} from cool white fluorescent lamp). After 1 month, numbers of SEs on every explant were counted under a stereoscopic microscope. Efficiencies of SE induction were evaluated by frequency of SE induction (% of explants which formed at least one SE) and average SE number (total number of induced SEs per total number of used explants). (A) Evaluation of SE induction efficiencies using 16 field-grown soybean cultivars. (B) Frequencies of SE induction evaluated using both greenhouse- and field-grown plant materials. The numbers of tested explants are indicated in parentheses.

adaptability of Japanese and North American soybean cultivars to tissue culture. Therefore, further basic information on the somatic embryogenesis is necessary for the biotechnological improvements of Japanese soybeans.

To establish the efficient soybean transformation system using Japanese cultivars, we evaluated the efficiencies of somatic embryogenesis from immature cotyledons. Cultivar 'Jack', which is one of the most suitable genotypes for somatic embryogenesis, was also used as a reference cultivar for our experimental conditions. Since the efficiencies were shown to be different among cultivars at the steps of SE induction, proliferation and differentiation (Bailey et al. 1993), we separately evaluated the efficiencies at each steps of somatic embryogenesis.

SEs were induced from immature cotyledons prepared from soybean plants which were grown in fields. To obtain sections of immature cotyledons, developing seeds were excised from surface-sterilized young pods. We used seeds with major axis length between 4.0 and 5.5 mm, because SE induction efficiencies of 7 different cultivars peaked around this size. To collect a large number of explants at once, excised seeds were stored overnight at 7 °C, without reduction in the ability to induce SEs. Efficiency of SE induction was evaluated 1 month after incubation of the immature cotyledons on solid MSD40 medium which consists of Murashige and Skoog (MS) salts (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), 3% sucrose, 40 mg l^{-1} 2,4-D and 0.2% Gellan Gum (pH 7.0). As shown in Figure 1A, the efficiency of SE induction was greatly different among cultivars. Three Japanese cultivars (Nasushirome, Hayahikari and Kitami Nagaha) showed SE induction frequencies more than 50%. The SE induction frequencies of 'Yuuzuru', 'Shimabara' and 'Wase Midori' were 40 to 50%, and those of other Japanese cultivars were lower than 30%. Under the same conditions, 78% of 'Jack' explants induced SEs. Generally, the cultivar with high SE induction frequency also showed high average number of SEs per explant. We also evaluated efficiencies of SE induction using greenhouse-grown plant materials of several cultivars. The frequencies of SE induction were again different among cultivars, and higher than those of field-grown plants (Figure 1B). As our preliminary experiments showed no significant difference in the SE induction efficiencies among individual plants of identical cultivar (using 100 to 180 explants per plant), these results indicate that observed difference in the SE induction efficiency is genotype-dependent.

Next, induced SEs were excised from explant/callus

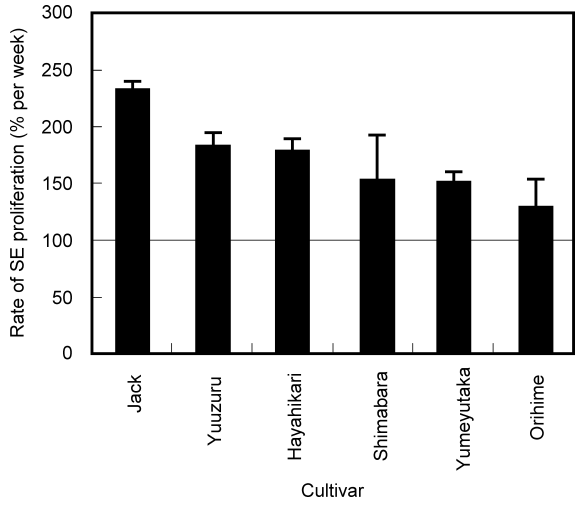

Figure 2. Rates of SE proliferation in liquid medium. Induced SEs were excised from explant/callus complexes on MSD40 plates, and transferred into 100 ml Erlenmeyer flasks containing 25 ml FNL medium. Flasks were shaken on gyratory shakers (100 rpm) in a growth chamber adjusted to the same temperature and light settings as SE induction. Embryogenic suspension cultures were maintained by transferring SE clusters (<100 mg) to fresh medium every week. Rate of SE proliferation was evaluated by increase in their fresh weight (%) per week, using 4- to 8-week-old suspension cultures. Data are means and SD from 4 or 5 flasks.

complexes and proliferated in liquid Finer and Nagasawa Lite (FNL) medium (FNL macro salts, MS micro salts, B5 vitamins, 1% sucrose, 1 g l^{-1} asparagine and 5 mg l^{-1} 2,4-D, pH 5.8), where FNL macro salts consist of 2830 mg l^{-1} KNO_3, 463 mg l^{-1} $(NH_4)_2SO_4$, 370 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 185 mg l^{-1} KH_2PO_4 and 300 mg l^{-1} $CaCl_2 \cdot 2H_2O$ (Samoylov et al. 1998). SEs were repetitively induced from preexisting SEs, and proliferated as SE clusters. In contrast to SE induction, difference in the rates of SE proliferation was small among cultivars (Figure 2). SE clusters of 5 Japanese cultivars proliferated in FNL medium at average rates from 130 to 180% per week, while those of 'Jack' proliferated at 230% per week. In most cases, a small fraction of SEs showed negative characters such as bleaching, browning and surface dehiscence, and were discarded at each transfer. SEs of 3 Japanese cultivars, 'Nasushirome', 'Kitami Nagaha' and 'Wase Midori', did not proliferate at all due to these negative characters. Although SE induction efficiencies of these 3 cultivars were relatively high among examined cultivars (Figure 1A), induced SEs could not survive in the liquid medium, suggesting the genotype-dependent adaptability to our liquid culture conditions.

As described previously (Hazel et al. 1998), SE clusters of some cell lines were rich in compact SEs characterized by tightly packed globular structure, while those of another embryogenic cultures were more lobed in appearance (Figure 3A). To examine whether the difference in the appearance of proliferating SEs are

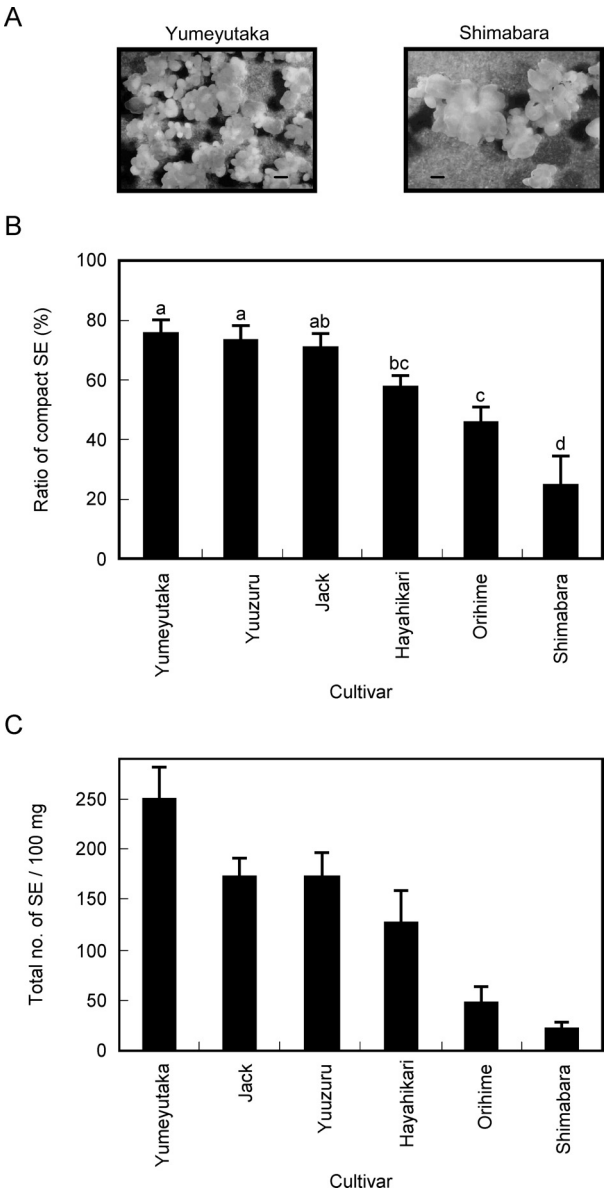

Figure 3. Different ratios of compact SEs among cultivars. SE clusters (<20 mg) were transferred from FNL to MSM6AC media (100 mg per plate). Immediately after the transfer, numbers of compact and non-compact SEs were separately counted under a stereoscopic microscope. Individual SEs were conveniently distinguished by apparent major axis length, as compact (less than 1 mm) or non-compact (above 1 mm) SEs. (A) Examples of the clusters consisting of compact (Yumeyutaka) and non-compact (Shimabara) SEs. Bar=1 mm. (B) Ratios of compact SEs in 100 mg SE clusters. (C) Total numbers of SEs per 100 mg clusters. Data are means and SD from 4 or 5 replicates. Mean values with different letters are significantly different by Scheffe's test (α=0.05).

genotype-specific, we compared compactness of SEs in suspension cultures. The ratios of compact SEs (less than 1 mm) were indeed different among cultivars (Figure 3B). Reflecting the size of SEs, compact-SE-rich cultivars such as 'Yumeyutaka', 'Yuuzuru' and 'Jack' showed higher numbers of SEs in 100 mg SE clusters compared to compact-SE-less cultivars such as 'Orihime' and 'Shimabara' (Figure 3C).

For differentiation from globular- to cotyledon-stage embryos, SEs were incubated on hormone-free MSM6AC medium (MS salts, B5 vitamins, 6% maltose, 0.5% activated charcoal and 0.2% Gellan Gum, pH 5.8). Efficiency of SE differentiation was evaluated by counting differentiated embryos 1 month after transfer to MSM6AC plates. When differentiated embryos were counted, 15 to 40% of embryos showed abnormal morphologies as fused cotyledons, long hypocotyls and vestigial cotyledons and cup-shaped cotyledons (Figure 4A). These abnormal embryos were not counted, because such morphologies were reported to be detrimental to the germination process for plant regeneration (Buchheim et al. 1988). As shown in Figure 4B, 'Yumeyutaka' showed the highest yield of well differentiated embryos (approx. 75 embryos per 100 mg SE clusters) which was 15-fold higher than the lowest yield of 'Shimabara' (approx. 5 embryos per 100 mg SE clusters). Compact-SE-rich cultivars such as 'Yumeyutaka', 'Jack' and 'Yuuzuru' yielded more differentiated embryos than compact-SE-less cultivars such as 'Orihime' and 'Shimabara'. In contrast to the yield of differentiated embryos, there was a small variation in the frequency of differentiation among cultivars (Figure 4C), ranging from 25% (Shimabara) to 35% (Jack).

In this study, we evaluated efficiencies of, 1) SE induction from immature cotyledon in the presence of 40 mg l^{-1} 2,4-D, 2) SE proliferation at globular-stage in the liquid medium containing 5 mg l^{-1} 2,4-D, and 3) SE differentiation into cotyledon-stage embryos on the hormone-free medium. Examined soybean cultivars were greatly different in, 1) efficiencies of SE induction, 2) ratios of compact SEs at proliferation step, and 3) yields of well-differentiated embryos. As for soybean cultivars examined in this study, yield of differentiated embryos considerably depends on the compactness of globular-stage SEs, since there was a small variation in the frequencies of SE differentiation among cultivars. This idea is supported by a positive correlation between the ratios of compact SEs and the yields of differentiated embryos of 12 soybean cultivars including those described in this study ($R=0.88$, $\alpha=0.01$, data not shown). Thus, ability of globular SE induction and compactness of globular SEs are likely to be the major factors affecting overall efficiency of soybean somatic embryogenesis.

It has been shown that the levels of exogenous auxin determine the efficiency of SE induction. For example, SEs were induced from cultured carrot cells by transfer

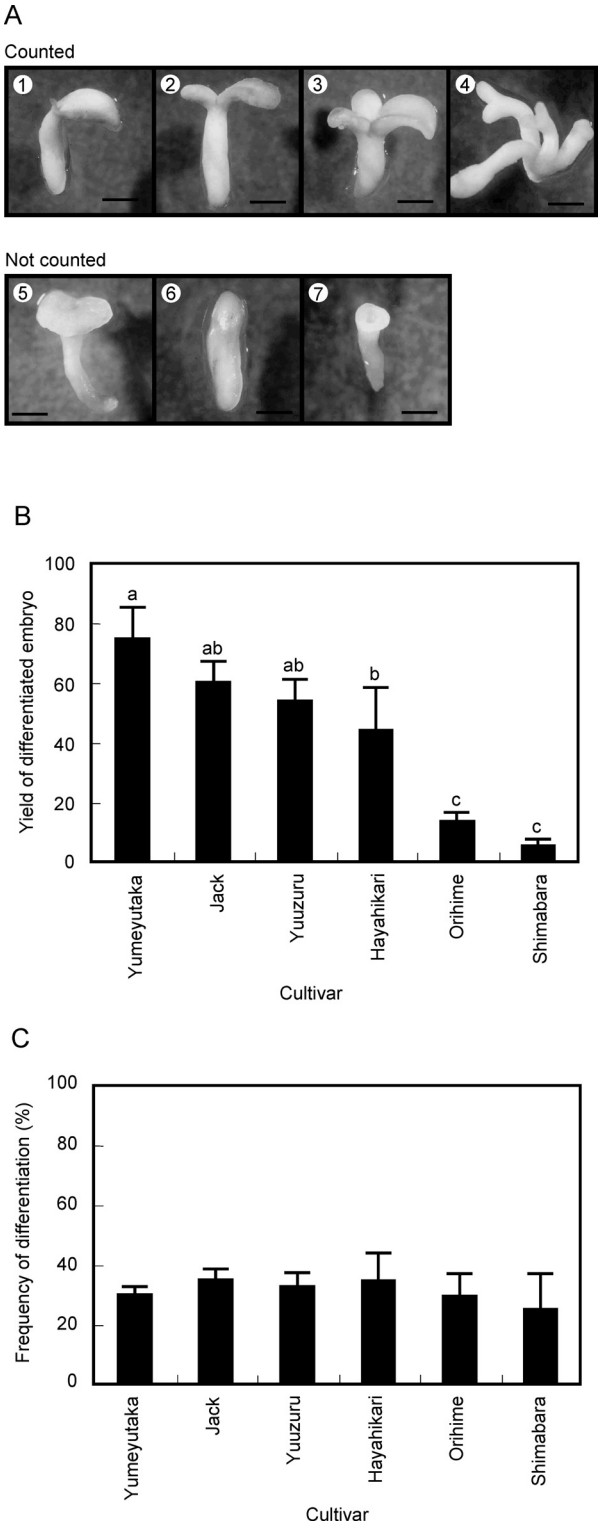

Figure 4. Different yields of differentiated embryos among cultivars. SE clusters were incubated on MSM6AC plates under the same temperature and light conditions as SE induction. After 1 month, numbers of well differentiated embryos were counted under a stereoscopic microscope. SE differentiation efficiencies were evaluated by yield of differentiated embryos (number of well differentiated embryos derived from 100 mg SE clusters), and frequency of differentiation (% of well differentiated SEs). Data are means and SD from 4 or 5 plates. Mean values with different letters are significantly different by Scheffe's test ($\alpha=0.05$). (A) Examples of embryo morphologies. Monocotyledonous (1), dicotyledonous (2), polycotyledonous (3) and moderately fasciated (4) embryos were counted as well differentiated embryos. Abnormal embryos with fused cotyledons (5), long hypocotyls and vestigial cotyledons (6) and cup-shaped cotyledons (7) were not counted. Bar=3 mm. (B) Yields of differentiated embryos. (C) Frequencies of differentiation.

from the medium containing 1 mg l^{-1} 2,4-D to hormone-free medium (Satoh et al. 1986). On the other hand, SE induction from shoot-apical-tip or floral-bud explants of *Arabidopsis* occurred after stress-treatment and subsequent administration of 1 mg l^{-1} 2,4-D (Ikeda-Iwai et al. 2003). Efficient induction of SEs from immature cotyledons of soybean requires relatively high concentration of 2,4-D (for example 40 mg l^{-1}), indicating characteristic response of soybean immature cotyledon to exogenous auxin. In our preliminary experiments, we observed a difference in the effective concentrations of 2,4-D for SE induction between 2 soybean cultivars. Efficiency of SE induction from 'Fayette' explants peaked at 40 mg l^{-1} 2,4-D, and declined to about 1/3 at 60 and 80 mg l^{-1}. In contrast, maximal SE induction efficiency in 'Suzuyutaka' was observed at 40 to 80 mg l^{-1}. These observations suggest that the differences in SE induction efficiencies among soybean cultivars are likely to be attributed to the differences in the sensitivities to auxin or the endogenous levels of auxin.

Among Japanese soybean cultivars tested in this study, 'Yuuzuru' and 'Yumeyutaka' are found to be genotypes with high potentials of plant regeneration through somatic embryogenesis. Although efficiencies of SE induction from immature cotyledons of these cultivars were intermediate levels among Japanese cultivars, the abundance of the compact SEs would compensate for the total numbers of proliferating SEs. Compactness of SEs also contributes to high yield of well differentiated embryos of these cultivars. It was reported that the genotypes of high yield of well differentiated embryos showed high frequencies of conversion from cotyledon-stage embryos to plantlets (Bailey et al. 1993). On the basis of reported frequencies of conversion from cotyledon-stage embryo which were differentiated in similar yields (Bailey et al. 1993), more than 50% of 'Yuuzuru' and 'Yumeyutaka' embryos would be converted to plantlets. The conversion efficiency may be improved by replacement of the differentiation medium from MSM6AC (Bailey et al. 1993, this study) to a new liquid medium which is modified for differentiation and maturation of soybean SEs (Schmidt et al. 2005). In addition, efficiency of SE induction may be improved by modifications of culture conditions such as 2,4-D concentrations in the culture medium. Thus, further improvements in experimental conditions may optimize the procedures of somatic embryogenesis in 'Yuuzuru' and 'Yumeyutaka'. We are now examining the conditions for efficient transformation and plant regeneration using these Japanese soybean cultivars.

Acknowledgments

We whish to thank Mses. M Ogawa and S Kawai for their help in maintenance of plant materials, and Drs. M Ishimoto, T Kubo and Y Ohashi for helpful discussions. Toyomusume, Yuuzuru and Wase Midori seeds were kindly distributed from Hokkaido Central Agricultural Experiment Station. We thank Dr. RL Nelson at USDA/ARS Univ. of Illinois for supplying Jack seeds. Seeds of other cultivars were generously provided by MAFF Genebank at National Institute of Agrobiological Sciences. This work was supported by Pioneer Research Project funds of Ministry of Agriculture, Forestry and Fisheries, Japan.

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