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Regeneration of Plants from EMS-treated Immature Embryo Cultures in Soybean [*Glycine max* (L.) Merr.]

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Abstract

Since somatic embryogenesis combined with ethylmethane sulfonate (EMS) treatments is the most efficient technique for mutagenesis, the embryogenic capacity of four soybean cultivars was evaluated at different EMS concentrations, treatment times, and preculture durations. Two to 4 mm long immature cotyledons were placed in induction medium after EMS treatment, and the numbers of somatic embryos formed per explant were counted four weeks after culture initiation. We observed genotypic differences in the efficiency of somatic embryogenesis from immature embryos among four cultivars treated with different concentrations of EMS for six hours. Cultivars, Sinpaldalkong 2 and Jack, displayed highly efficient somatic embryogenesis regardless of EMS concentration, whereas very low efficiency or no survival was observed in Jinju 1 and Iksannamulkong cultivars. Preculture duration did not influence the efficiency of somatic embryogenesis. Because Sinpaldalkong 2 exhibited the best somatic embryogenesis, much higher concentrations of EMS were used to test somatic embryo formation under different periods of time in this cultivar. Three and six hour treatments with both 1 and 2 mM EMS yielded higher embryo formation than longer periods of time. Increasing the time with embryos in 2 mM EMS caused a reduction in somatic embryogenesis in Sinpaldalkong 2, but many chlorophyll-deficient soybean variants were identified in the M_1R_0 and M_2R_1 generations. In addition to Jack, Sinpaldalkong 2 is a good genotype for plant regeneration from EMS-treated immature embryo cultures.

Key words: EMS, mutagenesis, somatic embryogenesis, soybean

Introduction

Tissue culture and plant regeneration are required to generate of transgenic plants, and these techniques open new possibilities for improving soybean [*Glycine max* (L.) Merr.] (Hildebrand et al. 1991; Kita et al. 2007). *In vitro* regeneration via somatic embryogenesis has drawn more attention than other methods because it can produce a large number of plants in a relatively short time (Wu et al. 2007). Somatic embryogenesis is defined as asexual reproduction in which a bipolar structure, resembling a zygotic embryo, is induced from a non-zygotic cell without vascular connection in the original tissue (Namasivayam 2007; Zimmerman 1993). In addition to a high number of regenerates, somatic embryogenesis is more attractive than organogenesis as a plant regeneration system due to the low frequency of chimeras and limited level of somaclonal variation (Ahloowalia 1991; Gaj 2001; Henry et al. 1998).

Regeneration of soybean via somatic embryogenesis was first attempted by Beversdorf and Bingham (1977), but somatic embryos have only been obtained sporadically since then. Christianson et al. (1983) induced adventive somatic embryos from immature soybean cotyledons on medium containing moderately high auxin concentrations. Immature and meristematic soybean tissues are the most suitable explants for somatic embryogenesis. The selection of the explant is a critical factor that determines the success of most tissue culture experiments. Plant regeneration has been achieved via somatic embryogenesis from the immature cotyledons of developing seeds in soybean (Amberger et al. 1992; Bailey et al. 1993; Lazzeri et al. 1988; Liu et al. 1992; Walker and Parrott 2001). Thus, one of the most

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suitable targets for genetic manipulation in soybean is embryogenic tissue (Sato et al. 1993).

High regeneration efficiency is essential when using somatic embryogenesis to identify mutants of targeted genomic lesions in reverse genetics studies (Santos et al. 1997). Since the mutation frequency may differ depending on many factors, considerable research has been conducted on the variables influencing somatic embryogenesis (Bonacin et al. 2000; Lazzeri et al. 1987a, 1987b; Parrott et al. 1989; Santos et al. 1997). For example, the level of 2, 4-dichlorophenoxyacetic acid (2, 4-D) influences the level of induction of soybean somatic embryos from immature cotyledons on solid culture medium and proliferation in liquid or solid media (Christianson et al. 1983; Hiraga et al. 2007; Kita et al. 2007). In addition, various soybean genotypes show different potentials for forming proliferative embryonic tissue (Bailey et al. 1993; Hiraga et al. 2007; Kita et al. 2007; Ko et al. 2004; Parrott et al. 1989).

Induced mutation may broaden genetic variants and provide materials for plant improvement. Ethylmethane sulfonate (EMS) is typically used to induce mutations, because it causes mispairing between complementary bases by formation of adducts with nucleotides, leading to base changes after replication (Ashburner 1990; Greene et al. 2003; Haughn and Somerville 1987). EMS mutagenesis is a standard technique for induction of point mutations (Greene et al. 2003). TILLING (Targeting Induced Local Lesions IN Genomics) is now a popular technology to screen point mutations with EMS-mutagenized plants (McCallum et al. 2000a, b). EMS has been also used to induce mutations in mature seed and cell suspension cultures of soybean (Fujii and Tano 1986; Sung 1976; Wilcox et al. 1984). Further, Van et al. (2005) describe the generation of many super-hypernodulating soybean mutants by EMS mutagenesis with mature seeds from three different soybean genotypes. The induction of mutations in embryogenic cultures combined with EMS treatment in soybeans has not yet been reported.

Since EMS mutagenesis by somatic embryogenesis would be a great source of useful germplasm in soybean breeding programs, we attempted to generate soybean mutants with immature embryonic cultures via somatic embryogenesis. The objectives of this study were to determine the optimal conditions for EMS treatment and establish an efficient method for EMS mutagenesis of immature soybean embryo cultures.

Materials and Methods

Plant materials

Four soybean genotypes were used: 'Sinpaldalkong 2', 'Jack', 'Iksannamulkong', and 'Jinju 1'. Jack is the standard genotype for somatic embryogenesis (Kita et al. 2007;Tomlin et al. 2002). Sinpaldalkong 2 and Iksannamulkong are recommended soybean varieties in Korea, and Jinju 1 is a local cultivar. These two Korean genotypes Sinpaldalkong 2 and Jinju 1 displayed high regeneration efficiency from immature embryo cultures in our preliminary studies. After plants were grown in pots under greenhouse conditions, immature pods containing immature 3-5 mm cotyledons were harvested 2 to 3 weeks after flowering.

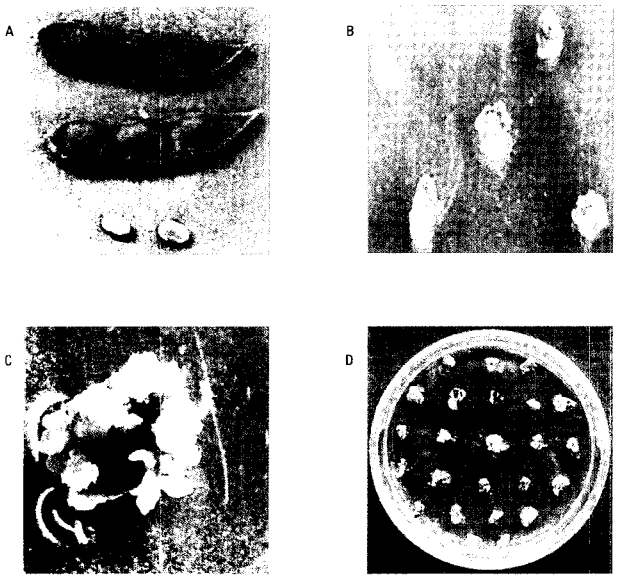

Fig. 1. Induction of direct somatic embryos from cotyledon explants on MSD 40 and MSD 20 medium. (A) immature zygotic embryos (B) browning and expansion of immature zygotic embryos (C) somatic embryo directly induced on immature embryos (D) proliferation of somatic embryos on MSD 20 medium at four weeks

Mutagenic treatments

The chemical mutagen EMS was applied for explant treatment. Four different concentrations (0.0, 0.2, 0.4 and 0.8 mM) were used in 6 hr treatments (Carroll et al. 1985; Lee et al. 1997). Two additional concentrations (1.0 and 2.0 mM) were used for EMS treatments at seven different time points (0, 3, 6, 9, 12, 15 and 18 hours) in Sinpaldalkong 2. Preculture was also performed for 0, 1, 2, 3, 4 and 5 days. Somatic embryos were immersed in a 50 ml Falcon conical tube containing 20 ml of the mutagenic solution (MSB5 medium supplemented with appropriate concentrations of EMS, Gamborg et al. 1968; Murashige and Skoog 1962), after which each tube was kept in a shaker at 100 rpm. Following EMS treatment, the somatic embryos were rinsed three times in sterilized water and then transferred onto induction medium.

Embryo induction

Somatic embryogenesis was conducted as previously described, with slight modifications (Finer and Nagasawa 1988; Parrott et al. 1989; Samoylov et al. 1998; Santarém et al. 1997). Pods were surface-sterilized by immersion for 1 min in 70%

Table 1. Effect of genotype on somatic embryogenesis and plant regeneration after treatment with various concentrations of EMS for six hours.

EMS concentrations (mM)		Genotypes				*P*
		Sinpaldalkong 2	Jack	Iksannamulkong	Jinju 1	
0.0	% of explants forming somatic embryos[a]	35.1	61.5	1.5	13.6	< 0.0001
	% of explants regenerating plants	102.4	-	-	-	_
0.2	% of explants forming somatic embryos[a]	61.1	69.4	1.6	1.4	< 0.0001
	% of explants regenerating plants[a]	152.8	156.5	4.9	1.4	< 0.0001
0.4	% of explants forming somatic embryos[a]	65.0	64.4	2.6	1.8	< 0.0001
	% of explants regenerating plants[a]	155.6	196.6	0.0	0.0	< 0.0001
0.8	% of explants forming somatic embryos[a]	81.8	51.3	6.5	4.1	< 0.0001
	% of explants regenerating plants[a]	172.7	54.1	0.0	0.0	< 0.0001

[a]Data (percentages within a line) were subjected to Chi-Square analysis.

isopropyl alcohol and 15 min in a 1% solution of sodium hypochloride and then rinsed three times in sterile water. In a laminar flow hood, immature cotyledons were aseptically removed from the pods and the end containing the embryonic axis was cut off and discarded.

After the seed coats were removed, the two cotyledons were separated and the abaxial side was placed on MSD40 medium (MS basal and B5 medium with 2, 4-D (40 mg l^{-1}), sucrose (30 g l^{-1}), and Gelrite (2 g l^{-1}) adjusted to pH 7.0 and autoclaved at 121 °C for 15 min). Twenty cotyledons of each cultivar were placed on each 85 x 15 mm Petri dish and incubated in a 18/6-h light/dark photoperiod at approximately 25 °C ±1 °C. Somatic embryos were counted as the number of embryos formed per explant four weeks after culture initiation.

Embryo proliferation, histodifferentiation, and maturation

Somatic embryo clusters of globular stage or embryogenic tissues were harvested from plant tissues 28 days after incubation and proliferated in MSD20 medium (MSB5 medium, 20 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose, and 2 g l^{-1} Gelrite, pH 5.8). The proliferated embryos were transferred onto 20-25 ml of MSM6AC medium, containing MSB5 medium, 60 g l^{-1} maltose, 5 g l^{-1} activated charcoal, and 2 g ℓ^{-1} Gelrite, pH 5.8, for histodifferentiation and maturation.

Desiccation, germination, and conversion

After four weeks on MSM6AC, the mature embryos were desiccated in an empty Petri dish containing a small piece of MSO medium. A small piece of auxin-free medium was placed at the edge of each Petri dish away from the embryos and the Petri dish was then kept in a Vitro Vent container (96 x 96 x 90 mm, Duchefa, Germany) for three to five days.

Partially desiccated embryos were placed on MSO medium (MSB5 medium, 30 g l^{-1} sucrose and 2 g l^{-1} Gelrite, pH 5.8) for germination induction. Embryos that produced roots and a shoot within three weeks were scored as germinated. Upon conversion, the plantlets were transferred to a Vitro Vent container containing MSO medium for further growth. Once the root system was established, the plants were transferred to a Vitro Vent container filled with sterilized horticultural bed soil and gradually acclimated to a lower humidity environment by progressively opening the lid of the container. Upon complete acclimatization, plants were maintained in a greenhouse for flowering and seed setting.

Data collection and analysis

Efficiency of somatic embryogenesis was determined by scoring the percentage of explants forming somatic embryos four weeks after culture. Somatic embryo conversion into plants was expressed as a percentage of number of regenerated plants to the total number of cotyledon explants cultured. For statistical analysis, the data for percentage of explants forming somatic embryos or regenerating plants were compared with the average of those percentages from the four soybean genotypes using Chi-Square analysis performed with Statistical Analysis System Software (SAS 2001).

Results

Induction of somatic embryogenesis by mutagenic treatment with explants

As zygotic cotyledons from immature embryos are suitable for the induction of somatic embryogenesis (Fig. 1A), immature zygotic embryos were treated with different concentrations of EMS on MSB5 medium. These mutagenized immature embryos were induced directly to somatic embryos on MSD40 medium after browning and expansion of immature zygotic embryos (Fig. 1B-C). Somatic embryos were visualized on MSM6AC medium four weeks after EMS mutagenesis (Fig. 1D).

Different concentrations of EMS and treatment times were

applied to explore their influence on the frequency of somatic embryo formation in culture. Somatic embryos were counted as the number of embryos formed per explant four weeks after culture initiation. The efficiency of somatic embryogenesis from immature embryos treated with EMS for six hours was different in the four cultivars depending on the EMS concentration (Table 1). There were significant differences in somatic embryo production among cultivars. Sinpaldalkong 2 and Jack displayed highly efficient somatic embryogenesis under all EMS concentrations. In contrast, very low efficiency or no survival was observed in Iksannamulkong and Jinju 1.

Since high efficiency was observed in Sinpaldalkong 2 and Jack following treatment with 0.2 and 0.4 mM EMS, those concentrations were used to evaluate the appropriate preculture duration with all four soybean genotypes. The cotyledons from zygotic embryos of all four cultivars were initially cultured on MSO medium for zero to five days. Comparison of means for somatic embryo production after treatment with 0.2 mM EMS for 18 hours confirmed that Sinpaldalkong 2 and Jack performed well for induction of somatic embryo formation. Further, no differences were observed in somatic embryo production under the different preculture durations with 0.2 mM EMS treatment for 18 hours (data not shown). The efficiency of somatic embryos was slightly different, however, depending on preculture duration when the embryos were treated with 0.4 mM EMS for 18 hours. Cultivar 'Jack' showed the highest efficiency of embryogenic mass formation with two-day precultured embryos. In contrast to somatic embryogenesis by genotype, statistical analysis suggested that preculture duration did not significantly impact the efficiency of somatic embryogenesis in the mutagenic treatments (0.2 or 0.4 mM for 18 hr) with immature embryos (data not shown).

Conversion of somatic embryos to plants

Like the efficiency of somatic embryogenesis, the frequency of plant development was also genotype-dependent (Table 1). Sinpaldalkong 2 exhibited highly efficient regeneration under all EMS concentrations. However, Jack showed much lower regeneration efficiency after 0.8 mM EMS treatment. In addition, induced somatic embryos of Jack did not germinate or grow normally, although they produced a higher frequency of embryogenesis.

The efficiency of regeneration with respect to preculture duration was also investigated with 0.2 mM EMS treatment for 18 hours. No differences were observed in regeneration of plants among the preculture durations (data not shown). Thus, preculture duration did not influence the efficiency of plant regeneration following mutagenic treatment in immature embryos.

Sinpaldalkong 2 and Jack produced a reasonable number of somatic embryos and regenerated plants after treatment with various concentrations of EMS. A total of 110 and 113 M_1R_0 plants were isolated from EMS-mutagenized Sinpaldalkong 2 and Jack, respectively. Mutant soybean populations composed of a total of 35 and 43 M_2R_1 lines from Sinpaldalkong 2 and Jack, respectively, were maintained. Accordingly, Sinpaldalkong 2 and Jack are good soybean genotypes for EMS mutagenesis via somatic embryogenesis.

Sinpaldalkong 2 as a genotype for EMS mutagenesis via somatic embryogenesis

Since Sinpaldalkong 2 showed good embryogenesis, much higher concentrations of EMS (1 and 2 mM EMS) were applied to test somatic embryo formation under a short period of time. Higher embryo formation occurred 3 and 6 hours after 1 mM EMS treatment than after any other longer period of time (data not shown). A high efficiency of embryo formation was also observed at 3 and 6 hours after treatment with 2 mM EMS (Table 2). Increasing treatment time with 2 mM EMS caused a reduction in somatic embryogenesis in Sinpaldalkong 2 (Table 2). The efficiency of plant regeneration was also estimated by different treatment times with 1 or 2 mM EMS in Sinpaldalkong 2. Reasonable efficiencies of regeneration of Sinpaldalkong 2 plants were observed after 1 mM EMS treatment for 3 and 6 hours (data not shown). With 2 mM EMS treatment, the efficiency of forming regenerated plants dramatically decreased as treatment time increased (Table 2). Neither somatic embryos nor regenerated plants formed after treatment for longer than 12 hours. A total of 519 Sinpaldalkong 2 M_1R_0 plants were obtained after treatment with 2 mM EMS (Table 2).

Table 3 summarizes the number and percentage of individuals in the M_1R_0 generation depending on EMS concentration in Sinpaldalkong 2. The highest efficiency was observed after 0.4 mM EMS treatment for six hours. As with the efficiency of forming regenerated plants with 2 mM EMS, the efficiency generally decreased as EMS concentration increased. Even so, an efficiency of at least 50% in producing the M_1R_0 generation was observed at high concentrations of EMS (1 and 2 mM EMS) (Table 3).

Discussion

Reverse genetics using TILLING is now a commonly used strategy for studying plant functional genomics with EMS-mutagenized M_2 populations, such as Arabidopsis and *Lotus japonicus* (McCallum et al. 2000b; Perry et al. 2003). Mutation techniques have also been used widely in breeding to identify the function of genes (Greene et al. 2003; Perry et al. 2003). The combination of chemical mutagenesis of explants and somatic embryogenesis represents an attractive *in vitro* technique for mutagenesis (Ahloowalia 1998; Deane et al. 1995; Gaj 2002).

Table 2. Effect of duration of 2 mM EMS treatment on somatic embryogenesis and plant regeneration in Sinpaldalkong 2.

Time (hour)	Number of explants	% of explants forming somatic embryos	% of explants regenerating plants
0	123	36.5	102.4
3	149	66.4	207.4
6	368	38.8	76.4
9	109	9.2	56.9
12	105	13.3	60.0
15	68	0.0	0.0
18	210	0.0	0.0

Table 3. Effect of treatment with various concentrations of EMS for six hours on the number and percentage of individuals in the Sinpaldalkong 2 M_1R_0 generation.

EMS conc. (mM)	Number of explants	Number of individuals in M_1R_0 generation	% of individuals in M_1R_0 generation
0.0	123	67	54.5
0.2	112	94	83.9
0.4	97	116	119.6
0.8	29	11	37.9
1.0	45	23	51.1
2.0	368	198	53.8
Total	774	509	65.8

Chemical mutagens such as EMS or N-nitroso, N-methyl urea (NMU) have been used to induce mutagenesis to broaden the genetic base of germplasm because they are very effective mutagens (Gaj 2002; Greene et al. 2003). These mutant lines have been directly used as new varieties or as sources of new variation in cross-breeding programs (Henikoff and Comai 2003). This new high-throughput application requires a high efficiency of mutation in the entire crop genome, although the precise nature of mutation induction has not been clarified.

Gentoype selection is one of the most important factors in the success of tissue culture experiments for both somatic embryogenesis and EMS mutagenesis (Kita et al. 2007; Ko et al. 2004; Santos et al. 1997). In this study, different conditions were tested with soybean immature embryo cultures to generate mutant populations by treatment with EMS. Since Jack has frequently been used for efficient induction of somatic embryogenesis, organogenesis, and transformation in previous studies (Jang et al. 2001; Samoylov et al. 1998; Santarém et al. 1998; Walker and Parrott 2001), this genotype was selected for EMS mutagenesis. Sinpaldalkong 2 and Jinju 1 were also used to test somatic embryogenesis because these soybean genotypes of Korean origin displayed high regeneration efficiency from immature embryo cultures in our preliminary studies.

The induction of somatic embryos, formation of stable proliferating embryogenic cultures, and regeneration of whole plants are three key steps for evaluating the success of soybean somatic embryogenesis (Kita et al. 2007; Simmonds and Donalson 2000; Tomlin et al. 2002). Table 1 gives a clear picture of the best genotypes for somatic embryogenesis by EMS mutagenesis. Very low efficiency was observed in Iksannamulkong and Jinju 1, while the efficiency was high in Sinpaldalkong 2 and Jack. Efficiency increased substantially with EMS concentration in Sinpaldalkong 2, but efficiency in Jack declined after the highest peak at 0.2 mM EMS. Overall, Sinpaldalkong 2 performed the best. A high efficiency of explants forming somatic embryos does not always correlate with high efficiency in regeneration of plants by proliferative embryogenesis (Simmonds and Donaldson 2000). In addition, maturation competency for regenerating plants is also dependent on soybean genotype (Komatsuda and Ohyama 1998; Tomlin et al. 2002). Comparing Sinpaldalkong 2 and Jack, the highest efficiency of somatic embryogenesis (81.8%) was observed following 0.8 mM EMS treatment in Sinpaldalkong 2. Nevertheless, Jack showed the highest efficiency (196.6%) in regenerating plants at 0.4 mM EMS (Table 1).

Higher efficiencies in embryo formation and regeneration of explants were observed in EMS treatments compared to controls. EMS is one of many stress elements, and its stimulative effect on plant regeneration in the culture of somatic tissue has been reported (Pius et al. 1994). Stress treatment stimulates initiation of embryogenesis in microspore cultures (Touraev et al. 1997). Gaj (2002) also suggested that enhanced mutagenesis than controls were not directly affected by EMS, because this chemical could induce similar stress conditions. Thus, EMS treatment led to greater efficiency in somatic embryogenesis than controls. Our study demonstrated the enhancement of somatic embryogenesis by EMS treatment in Sinpaldalkong 2 and Jack.

The length of the preculture period (0 to 6 days) was vital for determining the efficiency of gene delivery by particle gun in *A. thaliana leaves* (Seki et al. 1991). Evidence for a preculture requirement has been described (Brown et al. 1995), and somatic embryos from cotton and coffee have been precultured (Fuentes et al. 2000; Price and Smith 1997). Even soybean cotyledon halves require a preculture period for further growth (Rajasekaran and Pellow 1997), and preculture on 2, 4-D helps increase subsequent soybean embryogenesis efficiency on α-naphthalene acetic acid medium (Lazzeri et al. 1987a). Of the six different preculture periods evaluated in this study, none of them increased the efficiency of embryo formation or regeneration of explants. Thus, preculture duration did not influence the efficiency of somatic embryogenesis in mutagenic treatment with immature soybean embryos.

Since Sinpaldalkong 2 yielded the best somatic embryogenesis with EMS treatment, very high concentrations of EMS (1 and 2 mM) were applied to that genotype for seven different durations (Table 2). The highest efficiencies were observed in both embryo formation and regeneration following 2 mM EMS

treatment for three hours. Efficiency rapidly declined after three hours in both embryo formation and regeneration (Table 2). The frequencies of chlorophyll-deficient variants in the M_1R_0 and M_2R_1 plants were evaluated, because many chlorophyll-deficiency variants were generated by the EMS treatments. EMS is the most efficient mutagen in inducing chlorophyll-deficient variants, and chlorophyll-deficient sectors on EMS-generated M_1 plants are an indicator of mutation frequency (Carroll et al. 1985, 1986). In this study, chlorophyll-deficient variants generated from Sinpaldalkong 2 and Jack were identified in the M1R0 generation. Soybean mutant populations composed of 35 and 43 M_2R_1 lines from Sinpaldalkong 2 and Jack, respectively, were constructed and maintained. Sinpaldalkong 2 performed better than Jack and is, therefore, the best genotype for EMS mutagenesis in somatic embryogenesis.

In the present study, Sinpaldalkong 2 and Jack displayed high efficiencies for somatic embryogenesis and regeneration following various EMS treatments. To detect the point-mutated regions, several M_2 or M_3 lines generated by this study could be used for large-scale random sequencing by GS-FLX (Emrich et al. 2007), along with SS2-2 generated by EMS mutagenesis from Sinpaldalkong 2 (Kim et al. 2005; Lee et al. 1997; Van et al. 2005). Generation of soybean mutants by EMS mutagenesis via somatic embryogenesis can provide geneticists and plant breeders with information about the application of somatic embryo cultures for induction of mutations. This methodology can also be used to generate novel genetic variability to improve crop performance, and enhance our knowledge of plant genome structure. Additional studies are currently in progress to elucidate the combined effect of somatic embryogenesis and EMS treatment on the induction of mutants at the DNA sequence level.

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