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Tissue Culture Note

Establishment of a tissue culture system for somatic embryogenesis from germinating embryos of *Arabidopsis thaliana*

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Abstract Somatic embryogenesis is a valuable tool for investigating the totipotency of plant cells. We have established a simple culture system for inducing somatic embryogenesis from germinating embryos of *Arabidopsis thaliana*. One day after sowing, germinating embryos of the Nossen accession were placed on agar-solidified Gamborg's B5 medium supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid. After 14 days, light-green somatic embryos had formed in the region around the shoot apical meristems. After transfer to phytohormone-free medium, the somatic embryos grew into seedlings with cotyledon-like organs and roots, and finally into mature plants. The expression of several embryo-specific genes was detected in somatic embryos, suggesting that the somatic embryos retain embryonic features. We examined the effect of genotype on the formation of somatic embryos from germinating embryos of 352 *Arabidopsis* accessions. The frequency of somatic embryo formation differed markedly between the accessions and ranged from 0 to 92%. This result indicates that somatic embryogenesis from *Arabidopsis* germinating embryos is strongly affected by genotype.

Key words: *Arabidopsis thaliana*, 2,4-dichlorophenoxyacetic acid, germinating embryos, natural accessions, somatic embryogenesis.

The ability of plant cells to undergo somatic embryogenesis is experimental evidence of their totipotency. *In vitro* culture systems to induce somatic embryogenesis are useful not only for physiological, biochemical, and molecular biological studies of totipotency and embryogenesis, but also for agricultural applications such as clonal propagation of plants, production of artificial seeds, and regeneration of transgenic plants. Carrot has long served as a model system to investigate molecular mechanisms of somatic embryogenesis (Raghavan 2006). Several groups have established efficient methods for inducing somatic embryogenesis in the model plant *Arabidopsis thaliana* (Gaj 2001; Ikeda-Iwai et al. 2002; Pillon et al. 1996).

The establishment of culture systems for somatic embryogenesis from a variety of tissues and organs is important for evaluating the embryogenic competence of plant cells. *Arabidopsis* somatic embryogenesis is effectively induced from immature zygotic embryos (Gaj 2001; Ikeda-Iwai et al. 2002; Mordhorst et al. 1998; Pillon et al. 1996; Sangwan et al. 1992; Wu et al. 1992). Clusters that consist of many somatic embryos and embryogenic calli are formed from immature zygotic embryos in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D). Ikeda-Iwai et al. (2003) established another culture system for inducing somatic embryogenesis from vegetative shoot tips using stress treatment: somatic embryos are formed from shoot-tip explants excised from seedlings by first culturing on phytohormone-free medium under stress, and then transferring to 2,4-D-containing medium and culturing in the absence of the stress. In addition, somatic embryogenesis has been induced from mesophyll protoplasts (Luo and Koop 1997; O'Neill and Mathias 1993).

Arabidopsis developmental mutants, such as phytohormone response mutants and shoot apical meristem mutants, have been used as valuable tools for the investigation of gene functions in somatic embryogenesis (Gaj et al. 2006; Mordhorst et al. 1998, 2002). Furthermore, natural variations in *Arabidopsis* have been used extensively for molecular genetic studies of developmental and physiological processes (Alonso-Blanco et al. 2009; Koornneef et al. 2004). Natural accession lines that differ in phenotype are selected from stock

Abbreviations: Col, Columbia; 2,4-D, 2,4-dichlorophenoxyacetic acid; Ler, Landsberg *erecta*; No, Nossen; Ws, Wassilewskija.
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collection and used for quantitative trait loci analysis of seed germinability (Meng et al. 2008), flowering time (Werner et al. 2005), light sensitivity (Maloof et al. 2001), and leaf architecture (Pérez-Pérez et al. 2002). Because somatic embryogenesis is strongly influenced by genotype in many plant species (Brown and Atanassov 1985; Hiraga et al. 2007; Sharma and Rajam 1995; Zhang et al. 2008), characterization of *Arabidopsis* accessions with differing capacities for somatic embryos is helpful for investigating molecular mechanisms of somatic embryogenesis. RIKEN BioResource Center preserves over 340 natural accessions collected worldwide and is characterizing morphological and physiological properties of all individual accessions. In this study, we established a simple culture system for inducing somatic embryogenesis from *Arabidopsis* germinating embryos and examined the formation of somatic embryos in a large number of natural accessions.

Seeds of *Arabidopsis thaliana* (L.) Heynh. accession Nossen (No) were surface-sterilized with 1% sodium hypochlorite solution for 5 min. The sterilized seeds were sown on germination medium [Murashige and Skoog salts (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), 10 g l^{-1} sucrose, and 8 g l^{-1} agar] and incubated at 21°C under continuous light at 100 μmol photons m^{-2} s^{-1} for 1 day. Seeds that had begun to germinate by 1 day after sowing were used for the tissue culture experiments (Figure 1A). Seed coats were removed from the germinating seeds with forceps and a scalpel under a stereomicroscope (Figure 1B). The germinating embryos were put on semi-solid (8 g l^{-1} agar) B5 medium supplemented with 4.5 µM 2,4-D and incubated at 21°C under continuous light (Gaj 2001; Ikeda-Iwai et al. 2002; Pillon et al. 1996). After 14 days, the putative somatic embryos were separated from the explants and cultured on phytohormone-free semi-solid B5 medium under continuous light at 21°C. In order to propagate somatic embryos, clusters of somatic embryos were separated from the seedling explants and transferred onto semi-solid B5 medium containing 9.0 µM 2,4-D (Pillon et al. 1996). The somatic embryos were incubated at 21°C under continuous light and subcultured every 10 days.

Somatic embryo-like structures developed from germinating embryos cultured in the presence of 2,4-D. These structures were light green with a smooth surface and formed in the region around the shoot apical meristems, between the cotyledons (Figure 1C). Their morphology was different from that of undifferentiated non-embryogenic callus (Figure 1D) or normal first leaves (Figure 1E), but similar to that of somatic embryos derived from immature zygotic embryos (Gaj 2001; Ikeda-Iwai et al. 2002; Pillon et al. 1996) or from shoot tips under stress treatment (Ikeda-Iwai et al. 2003). The somatic embryo-like structures displayed bipolar morphology at the time they were transferred to phytohormone-free medium (Figure 1F, G). They grew into seedlings that had roots, fused cotyledons without

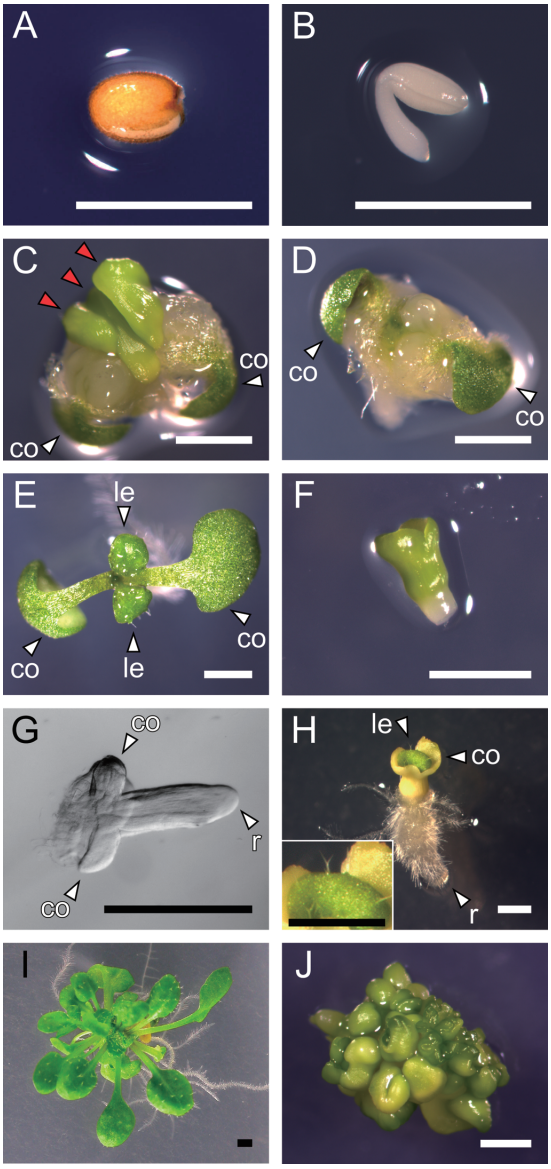

Figure 1. Formation of somatic embryos from germinating embryos of *Arabidopsis* and regeneration of plants. (A) A seed of accession No, 1 day after sowing. (B) A germinating embryo with the seed coat removed. (C) Somatic embryos (red arrowheads) after 14 days of culture on B5 medium containing 4.5 µM 2,4-D. (D) An undifferentiated non-embryogenic callus with no somatic embryos after 14 days of culture on B5 medium containing 4.5 µM 2,4-D. (E) A seedling without treatment grown 6 days on germination medium. (F) A somatic embryo isolated from the explant and transferred to phytohormone-free B5 medium after 14 days of culture. (G) Bright-field view of a somatic embryo. A somatic embryo was fixed in ethanol : acetic acid (9 : 1) solution and cleared with chloral hydrate solution (Yadegari et al. 1994). (H) A seedling grown from a somatic embryo 14 days after transfer to phytohormone-free medium. The inset shows a magnified view of the first leaf with trichomes. (I) A plantlet 28 days after transfer to phytohormone-free medium. (J) A cluster of somatic embryos maintained on B5 medium containing 9.0 µM 2,4-D. co, cotyledon; le, leaf; r, root. Bars = 1 mm.

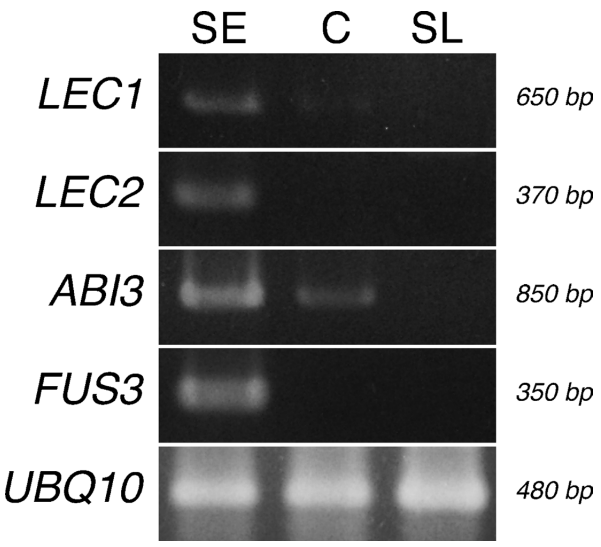

Figure 2. Expression of embryo-specific genes. Germinating embryos of accession No were cultured on B5 medium containing 4.5 μM 2,4-D for 14 days. The light-green somatic embryos were isolated from the explants, and the remaining undifferentiated non-embryogenic calli were collected. Total RNA was prepared from the samples by using TRIzol Reagent (Invitrogen, CA, USA), and used for RT-PCR analysis of *LEC1*, *LEC2*, *ABI3*, and *FUS3* with the PrimeScript RT-PCR kit (Takara Bio Inc., Shiga, Japan). Each gene was amplified with specific primers designed by Tanaka et al. (2008) under the following conditions: 94°C for 2 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min; and 72°C for 5 min. The PCR products were separated by electrophoresis in 1% agarose gel and stained with SYBR Green I. Gel images were visualized on a Dark Reader UV transilluminator (Clare Chemicals, CO, USA). *UBQ10* was used as an internal standard. SE, somatic embryos; C, undifferentiated non-embryogenic calli; SL, 14-day-old seedlings.

trichomes, and leaves with trichomes (Figure 1H), and eventually developed into plantlets (Figure 1I). This result suggested that the structures formed from the germinating embryos were somatic embryos. Some somatic embryos were propagated on medium supplemented with 9.0 μM 2,4-D and maintained for at least 3 months (Figure 1J). Similar to embryogenic callus derived from immature zygotic embryos (Ikeda-Iwai et al. 2002; Pillon et al. 1996), these somatic embryo clusters might consist of a mixture of somatic embryos and embryogenic cells, capable of generating new somatic embryos.

To confirm that the light-green structures were true somatic embryos, we monitored the expression of several embryo-specific genes. Somatic embryos and undifferentiated non-embryogenic calli were collected separately and used for RT-PCR analysis. Four transcription factor genes, *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, *ABSCISIC ACID INSENSITIVE3* (*ABI3*), and *FUSCA3* (*FUS3*), were selected as representative embryo-specific genes (Ikeda-Iwai et al. 2002, 2003; Ledwoń and Gaj 2009; Tanaka et al. 2008). Strong expression of *LEC1*, *LEC2*, *ABI3*, and *FUS3* was detected in somatic embryos, but expression levels were much lower in undifferentiated non-embryogenic calli and seedlings (Figure 2). This result reveals that the somatic embryos retain embryonic features.

We next examined the effects of explant developmental stage and 2,4-D concentration on the formation of somatic embryos. Seeds started to germinate 1 day after sowing (germinating embryos) and developed into seedlings after 2–4 days (Figure 3A–D). As the seedlings grew older, higher concentrations of 2,4-D were required for the induction of somatic embryogenesis (Figure 3I). The addition of 4.5, 9.0, and 18 μM 2,4-D promoted the formation of somatic embryos from germinating embryos, 2-day-old seedlings, and 3-day-old seedlings, respectively, and the frequency of somatic embryo formation reached approximately 70% at each of these three ages. In contrast, few 4-day-old seedlings formed somatic embryos (Figure 3H, I). The formation of somatic embryos from the germinating embryos was more rapid than from the 2- and 3-day-old seedlings (Figure 3J). Both germinating embryos and 2-day-old seedlings produced somatic embryos distinct from the undifferentiated non-embryogenic callus, whereas 3-day-old seedlings produced small somatic embryos surrounded by large amount of callus (Figure 3E–G). These results demonstrate that somatic embryogenesis of accession No was most efficiently induced in germinating embryos cultured in the presence of 4.5 μM 2,4-D.

Using this method, we induced somatic embryogenesis from a wide variety of *Arabidopsis* accessions. These included four major accessions used in laboratory research—No, Columbia (Col), Landsberg *erecta* (Ler), and Wassilewskija (Ws)—which were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK), and 348 natural accessions deposited in the RIKEN BioResource Center, which is a participant in the National Bio-Resource Project of Ministry of Education, Culture, Sports, Science and Technology, Japan. The seeds were sown on germination medium, stored at 4°C in the dark for 7 days to break dormancy, and then incubated at 21°C under continuous light. Most of the accessions started to germinate after 6 h. The germinating embryos were cultured on medium containing 4.5 μM 2,4-D for 14 days.

The capacity to form somatic embryos differed markedly between *Arabidopsis* accessions. The frequency of somatic embryo formation ranged from 0 to 92%, with the greatest number of accessions having a frequency between 10% and 20% (Figure 4, Supplemental Table 1). When 10 embryos of each accession were cultured on phytohormone-free medium, almost all of them grew normally (data not shown). These results indicate that somatic embryogenesis from germinating embryos is strongly affected by genotype. In contrast,

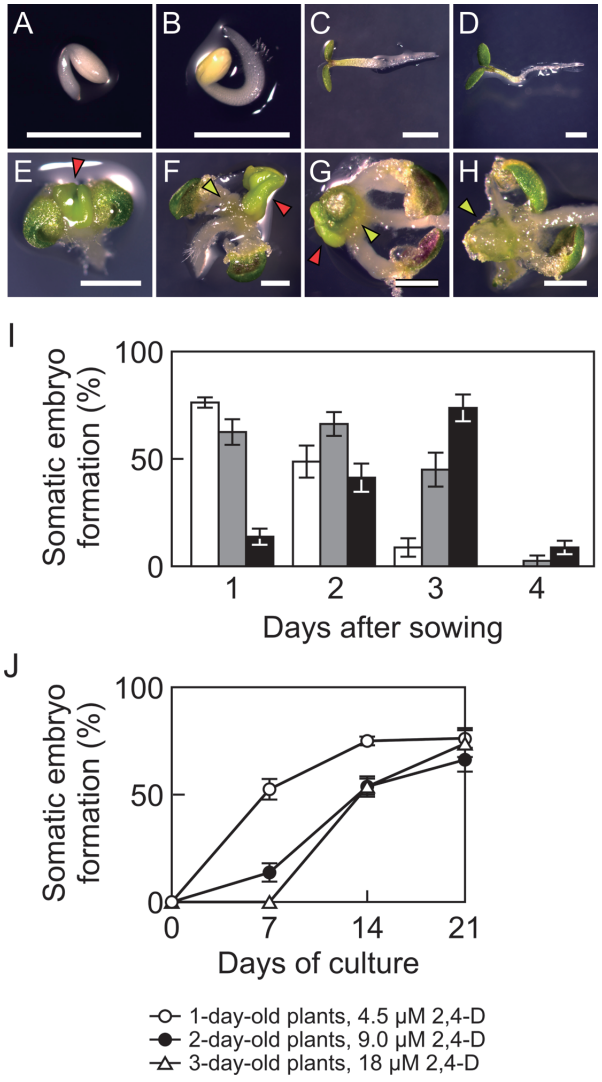

Figure 3. (A–D) Plants of accession No, 1 day (A), 2 days (B), 3 days (C), and 4 days (D) after sowing. One-day-old plants were classified as germinating embryos; 2- to 4-day-old plants were classified as seedlings. (E–H) Tissue cultures derived from plants 1 day (E), 2 days (F), 3 days (G), and 4 days (H) after sowing. Culture period and 2,4-D concentration were as follows: (E) 7 days at 4.5 μM; (F) 14 days at 9.0 μM; (G) 14 days at 18 μM; (H) 14 days at 18 μM. Red and yellow arrowheads indicate somatic embryos and undifferentiated non-embryogenic calli, respectively. Bars in A–H = 1 mm. (I) Effect of developmental stage and 2,4-D concentration on the formation of somatic embryos. Plants 1, 2, 3, or 4 days after sowing were cultured for 21 days in the presence of 2,4-D at 4.5 μM (open column), 9.0 μM (gray column), or 18 μM (closed column). Twenty plants were used for each treatment, and the frequency of somatic embryo formation was calculated as (number of plants that formed somatic embryos)/(total number of plants treated)×100%. Results represent the means and standard errors of four independent experiments. (J) Time course of somatic embryo formation from 1-, 2-, and 3-day-old plants. Open circles, 1-day-old plants (germinating embryos) cultured in the presence of 4.5 μM 2,4-D; closed circles, 2-day-old plants (seedlings) cultured in the presence of 9.0 μM 2,4-D; open triangles, 3-day-old plants (seedlings) cultured in the presence of 18 μM 2,4-D.

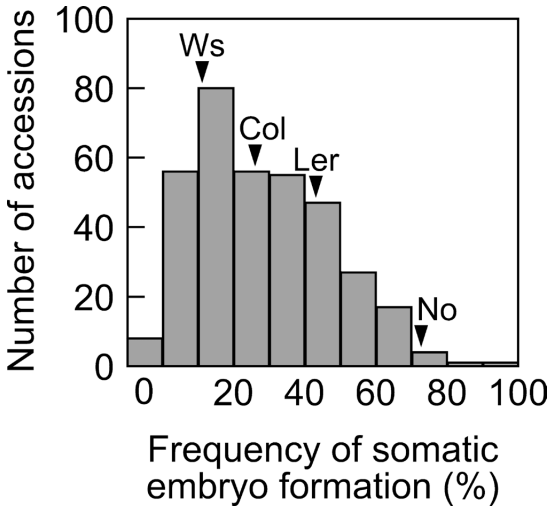

Figure 4. Frequency distribution of somatic embryo formation by 352 *Arabidopsis* accessions. Germinating embryos of four major accessions (No, Col, Ler, and Ws) and 348 natural accessions were cultured on B5 medium containing 4.5 μM 2,4-D for 14 days. Twenty embryos were used for each experiment. The means of the frequency of somatic embryo formation were calculated from at least three independent experiments. Detailed results are presented in Supplemental Table 1. The mean frequencies for Col, Ler, Ws, and No are indicated.

Gaj (2001) tested six accessions (Col, Ws, Ler, C-24, RLD, and Wilna) and found that the frequency of somatic embryo formation from immature zygotic embryos did not differ markedly between them (86.7–96.0%). The frequency of stress-induced somatic embryogenesis from shoot tips was approximately 20% and similar between accessions Col, Ws, and Ler (Ikeda-Iwai et al. 2003). The effect of genotype on somatic embryogenesis may vary depending on the tissues or organs used as explants and the culture systems.

We further examined the effect of 2,4-D concentration on the formation of somatic embryos in the four major accessions and eight natural accessions that had high embryogenic competence. The addition of 4.5 μM 2,4-D was effective for the formation of somatic embryos in most accessions, but some accessions exhibited slightly different responses to 2,4-D (Figure 5). The effect of 9.0 μM 2,4-D on the formation of somatic embryos in JA2 was the same as that of 4.5 μM 2,4-D. In contrast, the addition of 2.3 and 4.5 μM 2,4-D had similar effect on the formation of somatic embryos in Col, JA192, and JA335. Somatic embryogenesis in Ws was most effectively induced in the presence of 2.3 μM 2,4-D.

The culture system for somatic embryogenesis using *Arabidopsis* germinating embryos has advantages over with previous systems. Preparation of immature zygotic embryos requires large space for plant growth and more than 2 months for flowering and seed set (Gaj 2001; Ikeda-Iwai et al. 2002; Pillon et al. 1996). Moreover, it is difficult to obtain immature zygotic embryos from

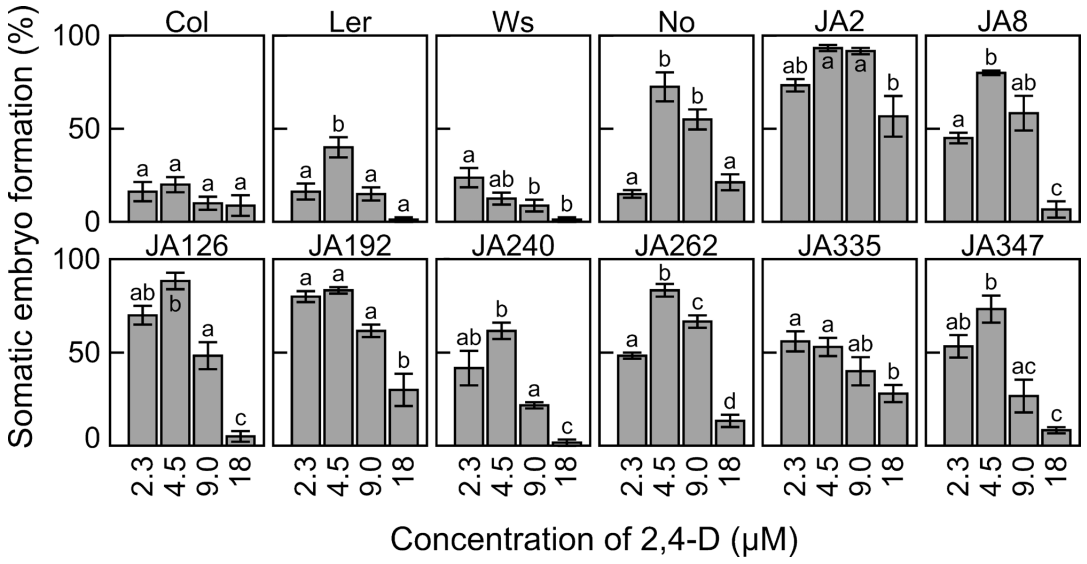

Figure 5. Effect of 2,4-D on the formation of somatic embryos in selected accessions. Germinating embryos of 12 accessions were cultured on B5 medium containing 2.3, 4.5, 9.0 or 18 μM 2,4-D for 14 days. Twenty embryos were used for each treatment. Results represent the means and standard errors of three independent experiments. Columns labeled with different characters are statistically significant as determined by Tukey's multiple comparison tests ($P<0.05$).

several accessions that have a late-flowering phenotype (*e.g.*, JA4, JA46, and JA219). In contrast, germinating embryos are easily obtained 1 day after sowing of mature seed (Figure 1). The culture system using stress-induced somatic embryogenesis from shoot tips is also complicated (Ikeda-Iwai et al. 2003): small shoot tips excised from 5-day-old seedlings are first treated with a stress, and then transferred to 2,4-D-containing medium and grown in the absence of the stress. Somatic embryogenesis from germinating embryos requires no stress treatment. Another advantage of using germinating embryos is that the method can be used with large numbers of samples. In this study, we were able to examine the frequency of somatic embryo formation in 352 accessions (Supplemental Table 1). This simple culture system for inducing somatic embryogenesis from *Arabidopsis* germinating embryos, combined with the availability of natural accessions with a broad range of embryogenic potential, will be a valuable tool for investigating molecular mechanisms of somatic embryogenesis.

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