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Multiple Pathways of Somatic Embryogenesis at a High Frequency in *Asparagus officinalis* L.

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Abstract

An experimental system of somatic embryogenesis at a high frequency has been established in *Asparagus officinalis* L. The best medium for asparagus somatic embryogenesis was MS medium. Sucrose was found to be the best carbon source. Optimum combination of phytohormones was 0.5 mg l^{-1} 2,4-D with 1.0 mg l^{-1} BA. More than 80% of the culture cells developed into somatic embryos when MS medium supplemented with 0.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} BA and 20 g l^{-1} sucrose was used. Interestingly, heart-shaped and mature embryos with two cotyledons, which are the typical structures of dicotyledonous embryos, were observed in this system although asparagus is a monocotyledonous species. Complete plants were regenerated from the mature embryos with one or two cotyledons at a frequency over 50%. On the basis of these results, multiple pathways of somatic embryogenesis in asparagus were proposed.

Key words: artificial seed, asparagus, high frequency, somatic embryogenesis

Abbreviations

BA-benzyladenine; GA_3-Gibberellin A_3; MS-Murashige and Skoog; 2,4-D-2,4-dichlorophenoxyacetic acid

Introduction

The ability to produce morphologically and developmentally normal embryos and, indeed, whole plantlets from undifferentiated cells in culture, via a series of morphological changes, namely, somatic embryogenesis even after their prior differentiation to specific types of cells in mature tissues, resides uniquely within plant kingdom (Zimmerman, 1993). This unique developmental ability has been recognized both as an important pathway for the regeneration of plants from cell culture systems and as a potential model for studying early regulatory and morphogenetic events in higher plants. Somatic embryogenesis has been observed in more than 56 families, 81 genera and 132 species of plants so far (Kiyosue *et al.*, 1993a) since it was reported independently by Reinert (1958) and Steward *et al.*, (1958). Among these various plants, carrot (*Dacus carota* L.) has become one of the best established model systems for attempts to elucidate the mechanisms that control embryogenesis (Nomura and Komamine 1986, Kiyosue *et al.*, 1993a).

The events of fertilization and subsequent embryo development normally occur deep within maternal tissues. The early embryo is minute and is surrounded by both endosperm and maternal cells. Although the morphological description of embryo development has been extensively recorded through microscopy, molecular and biochemical analyses of early embryogenesis have been hampered significantly by this physical inaccessibility. As a consequence, we know very little about the genes that are necessary for early embryogenesis in higher plants and even less about their regulation. In addition, once genes that are essential for embryogenesis have been identified, the subsequent analysis of their regulation would be greatly facilitated by the availability of an appropriate *in vitro* model system that is not limited in tissue quantity or accessibility. The system of somatic embryo

represents just such a model system. Using this system, a lot of researches was performed (Nomura and Komamine, 1986, Kiyosue *et al.*, 1992, 1993b, Yang *et al.*, 1992, 1993, Fukuda *et al.*, 1994) and the obtained results revealed that the processes of somatic embryogenesis closely resemble to that of zygotic embryogenesis both morphologically and molecular biologically (Zimmerman, 1993, Yang *et al.*, 1996, 1997).

High frequency and synchronous development are significant for either the production of artificial seeds (Harada *et al.*, 1990) or the use of somatic embryogenesis as a model system to clarify the regulation mechanisms of gene expression which is required for the earliest developmental events in the life of a higher plant: the development of the fertilized zygote into a mature embryo (Zimmerman, 1993). These applications would be greatly enhanced by the availability of such a high frequency and synchronous system because this kind of system makes it easy to collect samples. In this report, we describe the multiple pathways of somatic embryogenesis at a high frequency in *Asparagus officinalis* L.

Materials and Methods

Induction of calli

Seeds of *Asparagus officinalis* L. (cv. Mery Washington W500) purchased from Ohwa Seed Co. (Nara, Japan) were steeped in water at room temperature for 5 days, subsequently sown on moist vermiculite, and geminated at 28 ℃ in the darkness. Two-week-old seedlings were surface-sterilized with 5% NaClO for 15 minutes following by 70% EtOH for 5 minutes and then rinsed with sterilized water for several times. Young shoots, hypocotyls as well as radicles were dissected from the seedlings and inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 2 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose and 0.8% agar. The explants were incubated at 28 ℃ in light/dark cycle of 16/8 hours. Illumination (80 μmol m^{-2} s^{-1}) was supplyed by daylight fluorescent tubes (FL40SBRN, Toshiba, Tokyo, Japan).

Subculture of calli

To maintain embryogenic cell lines, calli were subcultured at 28 ℃ in light/dark cycle of 16/8 hours in fresh MS medium supplemented with 2 mg l^{-1} of 2,4-D, 1 mg l^{-1} benzyladenine (BA), 30 g l^{-1} sucrose and 0.8% agar at regular intervals of 3 weeks.

Induction of somatic embryos

To check competence for somatic embryogenesis, calli were transferred to MS liquid medium containing 2% sucrose, 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. The medium was renewed weekly by transferring of approximate 1500 mg fresh weight of cells to 30 m*l* of fresh medium in a 100 m*l* Erlenmeyer flask. The cultures were incubated for 20 days at 120 revolutions per minute on gyratory shakers. The somatic embryos formed in the cultures were collected by filtration and placed in water in a 20 × 90 mm Petrie dish. The number of somatic embryos was counted and the frequency of somatic embryogenesis was calculated according to: frequency (%) = [(number of somatic embryos)/(total number of somatic embryos, cells and cell clusters)] × 100%.

To check effects of medium composition on the frequency of somatic embryogenesis, embryogenic callus was transferred to B5 (Gamborg *et al.*, 1968), MS (Murashige and Skoog, 1962), N6 (Chu *et al.*, 1975) or White (1963) liquid medium containing 2% sucrose, 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. The somatic embryos in the cultures sampled 15, 20 and 30 days after transfer were counted as described above.

To check effects of carbon source on the frequency of somatic embryogenesis, embryogenic callus was transferred to MS liquid medium containing 2% various carbonhydrates, 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. The somatic embryos in the cultures were counted 30 days after transfer and the frequency of somatic embryogenesis was determined as described above.

To check effects of phytohormones on somatic embryogenesis, embryogenic callus was transferred to MS liquid medium containing 2% sucrose, 2,4-D and/or BA at a final concentration of 0.1, 0.5, 1.0, 2.0 or 5.0 mg l^{-1}. The somatic embryos in the cultures were counted 30 days after transfer and the frequency of somatic embryogenesis was determined as described above.

Regeneration of plantlets

Four weeks after onset of induction of somatic embryogenesis, the cultures were sieved through a stainless steel mesh with 1 mm pores to collect somatic embryos at late stages. The somatic embryos were washed 3 times with MS medium and allowed to germinate on 1/2 MS medium supplied with 1 mg l^{-1} gibberellin A_3 (GA_3), 1 mg l^{-1} BA, 1% sucrose, and 0.8% agar.

Results and Discussion

Comparison of calli derived from different explants

Quality of callus is of importance to establish a reliable somatic embryogenesis system. To induce embryogenic callus, young shoots, hypocotyls and young radicles were cultured on MS agar medium supplemented with 2 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. Ten Petrie dishes with 16 explants were tested per treatment. Three weeks after inoculation, calli were induced from 73-86% of the explants excised from the seedlings. Three kinds of calli were obtained from the cultured young shoots, hypocotyls and radicles, respectively. Although the rates of callus induction were not dramatically distinctive among the three explants, the qualities of the calli were notably different in colour, texture and shape depending upon their origins (**Table 1** and **Fig. 1A**). As summarized in **Table 1**, the calli derived from young shoots were greenish, compact and tubercular; the calli derived from hypocotyls were yellowish, friable and large granular; the calli derived from radicles were whitish, watery and appeared as free cells. Our data reported here is consistent with the previous hypothesis that the quality of callus is dependent on the nature of explant (Levi and Sink, 1991, May and Sink, 1995).

Detection of competence of calli for somatic embryogenesis

To assess competence for somatic embryogenesis, calli (130 clusters per treatment) were transplanted to flasks containing the liquid MS medium supplemented with 0.5 mg l^{-1} 2,4-D, 1 mg l^{-1} BA and 2% sucrose. Three weeks after transfer, explants were collected and the number of somatic embryos within the explants was counted. The callus derived from radicle was failing to produce somatic embryo. Although the calli derived from both young shoots and hypocotyls possessed the competence for somatic embryogenesis, their frequencies of somatic embryogenesis were significantly different: while more than 80% of the cells or cell clusters derived from hypocotyls converted to somatic embryos (**Fig. 1B**), only 5% of the cells or cell clusters derived from young shoots developed into somatic embryos, suggesting that the physiological state of explants significantly affects the competence for somatic embryogenesis, and that

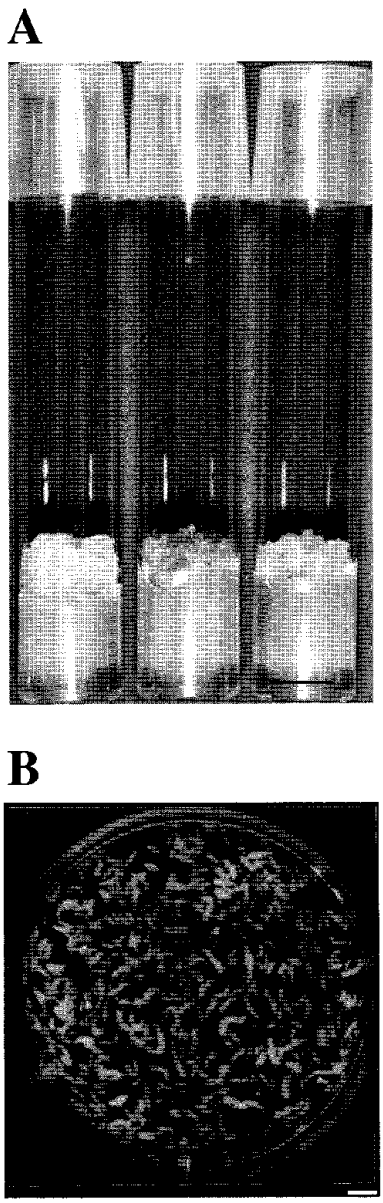

Fig. 1. (A) Callus derived from radicle (left), young shoot (centre) and hypocotyl (right), respectively. The yellowish, friable and smaller granular callus (right) produced somatic embryos at the highest frequency. Bar equals 1.5 cm. (B) Somatic embryos at different stages induced from the yellowish callus. Bar equals 12 mm.

Table 1. Comparison of calli derived from different explants

Explant	Characters of callus			Frequency of callus induction (%)	Frequency of somatic embryogenesis (%)
	Color	Texture	Shape		
Radicle	whitish	watery	unregular	73 (109/149)	0 (0/136)
Hypocotyl	yellowish	friable	small granular	86 (138/160)	83 (102/123)
Young shoot	greenish	compact	large tubercular	81 (126/156)	5 (7/132)

hypocotyls were a superior source for induction of embryogenic callus than either green shoots or radicles in asparagus (**Table 1**). The embryogenic callus that produced somatic embryos at the highest frequency was subcultured and maintained on the MS medium supplemented with 2 mg l^{-1} of 2,4-D, 1 mg l^{-1} BA, 30 g l^{-1} sucrose and 0.8% agar at regular intervals of 3 weeks for next analyses.

Multiple pathways of asparagus somatic embryogenesis

Light microscopic observations revealed that asparagus somatic embryos were sequentially differentiated through globular, pear-shaped, banana-shaped and cotyledon-differentiated to mature embryos (**Fig. 2**). Noticeably, the typical heart-shaped (**Fig. 2C**) and mature somatic embryos with two cotyledons (**Fig. 2F, H**), which appear characteristically during dicotyledonous embryogenesis *in vivo* as well as *in vitro*, were formed frequently during somatic embryogenesis although asparagus is a monocotyledonous species. The heart-shaped and mature somatic embryos with two cotyledons were also observed in some other monocotyledons (Yang and Cheng, 1991). These findings may be considered as an unique evidence of experimental embryology to strongly support the hypothesis that monocotyledons are originated from dicotyledons. The similar event might occur during monocotyledonous zygotic embryogenesis *in vitro* but at an extremely low frequency.

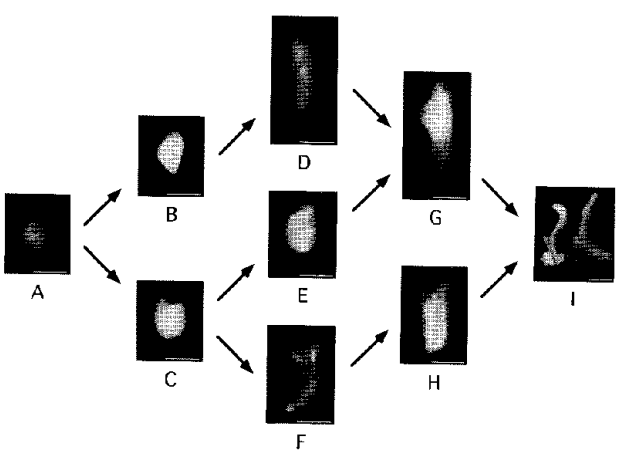

Fig. 2. Multiple pathways of somatic embryogenesis in *Asparagus officinalis*. A-B-D-G-I: a typi cal pathway of monocotyledonous embryogenesis; A-C-F-H-I: a pathway similar to dicotyledonous embryogenesis process; A-C-E-G-I: an intermediate pathway between the monocotyledonous and dicotyledonous embryogenesis processes. A: globular, B: pear-shaped, C: heart-shaped, D. banana-shaped, E: early mature (single cotyledon), F: early mature (double cotyledons), G: late mature (single cotyledon), H: late mature (double cotyledons) and I: germination of somatic embryos. Bar equals 1 mm (A to H) or 2.5 mm (I).

On the basis of the observations above, pathways of somatic embryogenesis in asparagus can be proposed as shown in **Fig. 2**. The most somatic embryos developed through the pathway of monocotyledonous embryogenesis (**Fig. 2: A-B-D-G-I**): globular, pear-shaped, banana-shaped and cotyledon-differentiated to mature embryos. Some globular somatic embryos did not develop into pear-shaped but heart-shaped somatic embryos, and the later differentiated into mature somatic embryos with two cotyledons through the pathway of dicotyledonous embryogenesis (**Fig. 2: A-C-F-H-I**). The third pathway (**Fig. 2: A-C-E-G-I**) was found to be an intermediate between the pathways of monocotyledonous and dicotyledonous embryogenesis, in which one of the two cotyledon primordia on the heart-shaped somatic embryos failed to develop, finally, the mature embryos with one cotyledon were formed. The mature embryos with either one or two cotyledons could germinate and convert to normal plantlets. The regenerated plants were transplanted to soil and most of them could survive. Although somatic embryogenesis in *Asparagus officinalis* was previously reported by several groups (Saito *et al.*, 1991, Kohmura *et al.*, 1994), this article is the first one to have described the multiple pathways of somatic embryogenesis at a high frequency.

Optimized medium for asparagus somatic embryogenesis

To screen the optimized medium for asparagus somatic embryogenesis, the calli derived from hypocotyls were transferred to and cultured in a series of the defined media, namely, B5, MS, N6 and white media supplemented with 0.5 mg l^{-1} 2,4-D, 1 mg l^{-1} BA and 20 g l-1^{-1} sucrose. The number of somatic embryos was counted on 15, 20 and 30 days after transplanting. As shown in **Fig. 3**, the cells or cell clusters cultured in MS medium developed into somatic embryos with the highest efficiency up to 80%. The cells or cell clusters incubated in N6 medium exhibited a lower rate (about 50%) of somatic embryogenesis. In contrast, the cells or cell clusters cultured in ether B5 or White medium initiated somatic embryos at the lowest frequencies of only 2~10%. Furthermore, more than 50% of the cells or cell clusters in MS medium initiated somatic embryogenesis within the first 15 days of culture, faster than in N6 or any other media tested (**Fig. 3**). These data suggest that

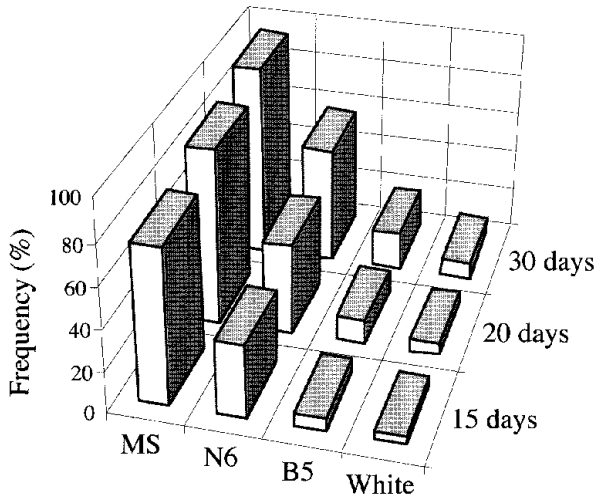

Fig. 3. Effects of medium compositions on frequency of somatic embryogenesis. After 15, 20 or 30 days of culture in the media indicated, the number of somatic embryos was counted. The frequency is presented in percentage as described in the Materials and Methods. Averages of three independent experiments are given.

MS medium is the best one for asparagus somatic embryogenesis. The earliest widely used inorganic salt formulation was that of White medium (1943). However, most researchers have been using hight-salt formulation of MS medium or its derivatives, e.g., B5 since 1960s. The major difference of these high-salt media lies in the amount and form of nitrogen, plus the relative amounts of some of the microelements. Since MS medium contains a much higher concentration of the reduced nitrogen, ammonium salt (1650 mg l^{-1} NH_4NO_3), as nitrogen source than the other media, it can be considered that the ammoniacal nitrogen is critical for somatic embryogenesis in asparagus.

Effects of phytohormones on frequency of asparagus somatic embryogenesis

Auxins and cytokinins are the two types of phytohormones which are most often required in tissue cultures. Their concentration and ratio in medium often control the pattern of differentiation in tissue culture (Skoog and Miller, 1957). Although the removal or decrease of auxin is the trigger of somatic embryo development in most cases, explants or cells require exposure to exogenous auxin for a few days before they become competent to undergo embryogenesis upon auxin removal. To evaluate effects of plant phytohormones on somatic embryogenesis in asparagus, auxin or cytokinin at a final concentration of 0, 0.1, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}, or both of them at various concentrations were added to MS medium and then the frequency of somatic embryogenesis was detected. As shown in **Fig. 4**, the rate of somatic embryogenesis was lowered according to the higher concentrations of phytohormones, showing that phytohormones at higher concentrations were inhibitory for somatic embryogenesis. Somatic embryogenesis could be induced but at lower frequencies in the presence of 2,4-D at lower concentrations. BA was more effective than 2,4-D to induce somatic embryos in asparagus as reported in other species. Addition of 0.5 mg l^{-1} BA in combination with 2,4-D at different concentrations significantly improved the frequency of somatic embryogenesis. BA at 1.0 mg l^{-1} in combination with 2,4-D at 0.5 mg l^{-1} displayed the highest frequency of embryo formation. In contrast, exogenous auxin is not required for somatic embryogenesis in the carrot system, suggesting that the role of exogenous auxin in somatic embryo induction depends on the nature of the explant used in the experiment.

Effects of carbon sources on efficiency of asparagus somatic embryogenesis

The major constituents that require for successful growth, organogenesis and embryogenesis are carbon and energy source besides plant phytohormones. The carbon and energy requirements are usually met with sucrose, although this can sometimes be replaced by glucose. To identify which one is better for somatic embryogenesis in *Asparagus*

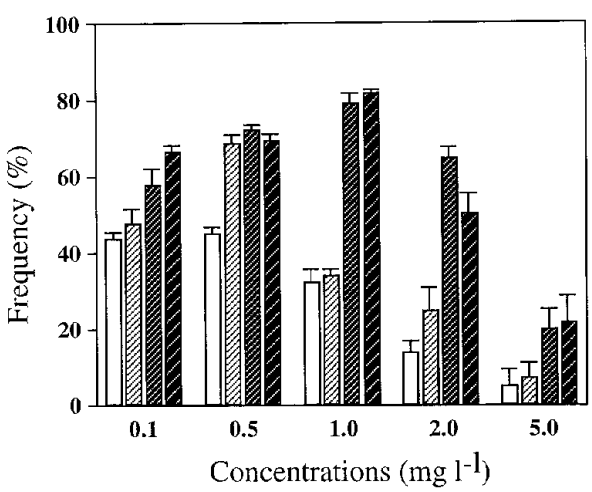

Fig. 4. Effects of plant growth regulators on frequency of somatic embryogenesis. After 30 days of culture in the media indicated, the number of somatic embryos was counted. The frequency is presented in percentages as described above. Averages of three independent experiments are given with the standard deviation (□: 0.1~5.0 mg/*l* 2,4-D; ▨: 0.1~5.0 mg/*l* 2,4-D+0.5 mg/*l* BA; ▩: 0.1~5.0 mg/*l* BA; ■: 0.1~5.0 mg/*l* BA+0.5 mg/*l* 2,4-D).

officinalis, the effects of various carbohydrates on somatic embryogenesis were analyzed. Fructose, galactose, glucose, sucrose, or starch was supplemented to the MS medium at a final concentration of 20 g l^{-1} with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D. As shown in **Fig. 5**, the average frequency of somatic embryogenesis was 91, 42, 39, 30, and 12% for sucrose, glucose, starch, fructose, and galactose, respectively. Although galactose, glucose and fructose are monosaccharides which are supposed to be easily decomposed, their effects on somatic embryogenesis were worse than sucrose, a disaccharide consisting of glucose and fructose, suggesting that supplying glucose in combination of fructose might be better than using only one of them. To check this suggestion, we simultaneously added 1% glucose and 1% fructose into the induction medium, and an additive effect was observed (data not shown). A possible explanation is that fructose is an intermediary product of glucose catabolism, both glucose and fructose can simultaneously join glycolysis at different steps and supply somatic embryogenesis with carbon source and energy. Starch is homopolysaccharide that can not be utilized by glycolysis before it is degraded into glucose, therefore its effect on somatic embryogenesis was worse than that of glucose. Galactose is an epimer of glucose. The worst effect of galactose on somatic embryogenesis might result from very less epimerase in plant cells. In general, glucose promotes root growth, fructose promotes shoot development, while sucrose promotes both shoot development and root growth (Li and Wolyn, 1997). Since somatic embryogenesis is related to the growth of a bipolar structure, sucrose is the best carbon and energy sources for somatic embryogenesis in asparagus as reported for carrot.

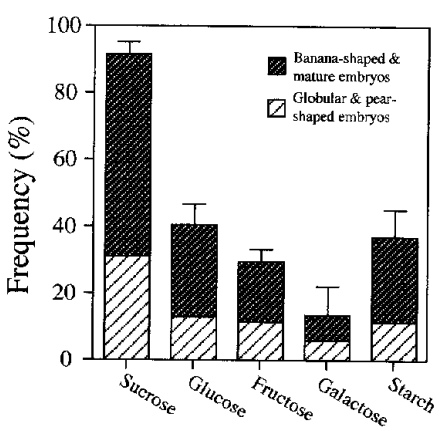

Fig. 5. Effects of carbon sources on frequency of somatic embryogenesis. After 30 days of culture in the media indicated, the number of somatic embryos was counted. The frequency is presented in percentages as described in the Materials and Methods. Averages of three independent experiments are given with the standard deviation.

Stability of asparagus embryogenic calli

Somaclonal variation may affect growth, differentiation and embryogenic competence of calli or culture cells (Yang *et al.*, 1999). Most of embryogenic cell lines have a limited embryonic lifespan (usually in the range of 1 to 2 years), after which it may become impossible to generate any embryos. This is why long-term calli or culture cells usually lose their embryogenic competence in carrot system (Harada *et al.*, 1990). Although the embryogenic callus in our system reported here was routinely maintained over the course of 2 years by subculturing them in the fresh MS medium supplemented with 2 mg l^{-1} 2,4-D and 1 mg l^{-1} BA at 3 weeks intervals, it could continuously produce somatic embryos at a higher frequency under the conditions for induction of somatic embryogenesis, suggesting that it was genetically stable during long-term subculture. Another long-term embryogenic line that produces numerous somatic embryos on phytohormone-free medium, some of them converting to whole plants, was reported in asparagus by Delbreil *et al.*, (1994). Moreover, their regenerated plants exhibited an increased embryogenic response compared to the parent plants; e.g. apex culture produce somatic embryos without any auxin treatments. For one of the embryogenic lines, a genetic analysis showed that the improved embryogenic response of regenerated plants was controlled by a mendelian dominant monogenic mutation (Delbreil and Jullien, 1994). These results and other previous observations suggested that somaclonal variation may affect somatic embryogenesis in a negative or positive way.

Regeneration of plantlets from asparagus somatic embryos

The bipolar mature embryos recovered from the embryo induction medium were placed on germi nation medium (1/2 MS medium containing 1 mg l^{-1} BA, 1 mg l^{-1} GA3, 1% sucrose and 0.8% agar) for conversion to plantlets. Fifty to sixty percent of them germinated normally and grew into plantlets for 3 to 4 weeks after transplanting (**Fig. 2I**). The mature embryos with either one cotyledon or two cotyledons could germinate and convert to plantlets. The regenerated plants were transferred to soil. Most of them could survive and showed normal phenotypes for over 2 years (**Fig. 6**). Chromosome number confirmed the diploid status (2n=20) of plants regenerated with somatic embryogenesis (data not shown). In micropropagated plants, the

Fig. 6. An asparagus plantlet regenerated from somatic embryo at 3 months after transplanting to soil.

flower types were identical with the parent and flowering occurred simultaneously. The quality of young spears, such as head shape and colors resembled the parent. Spear size and shoot diameter of mother stalks, yield and other growth characteristics of them were more uniform than those of seed propagated cultivars. Thus, micropropagation of superior asparagus clones through somatic embryogenesis may be used as an economical means of producing high quality and high yield spears (Kohmura *et al.*, 1996).

Our studies focused on various factors affecting development of somatic embryos to maximize the frequency of somatic embryogenesis in asparagus. The optimized protocol for somatic embryo induction at a high frequency in asparagus was developed through a series of experiments using several defined media. In this system, embryogenic cells or cell clusters in the embryo induction medium underwent somatic embryogenesis with higher efficiencies up to 80%. Therefore, this system can generate essentially unlimited quantities of embryos at defined stages through relatively simple manipulations for biochemical and molecular biological approaches on embryogenesis. This ability holds great promise for unravelling the complex process of plant embryo development.

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