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Precocious *In-Vitro* Flowering of Perennial Asparagus (*Asparagus officinalis* L.) Regenerants with a Chemical Inducer

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Abstract

A precocious flowering system of regenerants in asparagus (*Asparagus officinalis*) was achieved by treatment with a chemical inducer. Somatic embryos withered completely by being processed for 8 - 12 days with 200 μM *n*-propyl *N*-(3,4-dichlorophenyl)carbamate that had been dissolved in distilled water. In contrast, precocious flowering occurred at an extremely low rate (3.4%) when somatic embryos were processed in carbamate dissolved in Murashige and Skoog's liquid medium. To encapsulate the female and male embryos, we surveyed the optimum conditions of viscosity and concentration of sodium alginate for encapsulating the seeds, and we screened the values of 80 - 120 cps and 2% - 3%, respectively. The synthetic seeds produced also withered when they were processed with the carbamate dissolved in distilled water. However, when Murashige and Skoog's liquid medium was used for the solvent, the flowering frequency of the synthetic seeds was enhanced (13.3%). Based on our morphological and histological observations, female and male regenerants that were processed with the carbamate solution produced individual flower organs. The conversion of sex expression did not occur. A precocious flowering system would allow a significant reduction in the time required for perennial seedlings to flower and can, therefore, save time required for further experiments that employ floral homeotic mutants.

Keywords

Asparagus, Carbamate, Encapsulation, Precocious Flowering, Somatic Embryo

1. Introduction

Asparagus (*Asparagus officinalis*), an economically important horticultural crop, is a

dioecious species with separate unisexual individuals producing female or male flowers. The flowers start their development as hermaphrodites that later become unisexual. It is well known that the stamens in female flowers stop growing and degenerate, and that the ovaries in male flowers stop growing without associated degeneration [1] [2]. Thus, sex differentiation appears to be the result of selective abortion of the opposite flower organ. In higher plants, the development and specification of flower organs are controlled by a limited set of genes. Mutant studies of *Arabidopsis* and *Antirrhinum* produced the ABC model of floral organ determination, in which there is combinatorial action of three regulatory functions [3]. The ABC model became more complex, and now includes another class of genes referred to as the D-function genes [4] [5] and the E-function genes [6] [7]. The study of the molecular genetic mechanisms that control flower development in asparagus has advanced recently [8]-[16]. Toward a confirmation of the functional analyses of isolates, it was hoped that the transgenic asparagus plant could be brought out to early flowering for estimation of its morphological and histological aspects, since asparagus usually requires several years from seed to flower.

Precocious flowering of asparagus seedlings was first reported by Abe and Kameya [17]. They were able to induce flowers in one-month-old seedlings by treatment with atrazine and diuron, and they subsequently showed the effectiveness of inducing flowers with four kinds of atrazine analogues [18]. Other related compounds, such as *N*-phenylcarbamates [19] [20], *n*-propyl *N*-(3,4-dichlorophenyl)carbamate [21]-[23], *N*-phenylalkanamides [24], *N*-alkylbenzamides [24], *n*-phenyl-*N'*-alkoxyformamidines [25], *N*-(3,4-Methylenedioxy-phenyl)carbamates [26], thiocarbamates [27], and *n*-(4-chloro-2-trifluoromethylphenyl)-*N'*-propoxyacetamidine [28] have also been reported to induce a greater percentage of seedlings to flower. Apart from regenerants that include transformants of asparagus, findings regarding treatment parameters such as timing, dose, duration, and environmental conditions have accumulated, and these findings have been used to successfully induce the early flowering of seedlings. Here, we determined the essential parameters for *in vitro* flowering and we describe the realization of precocious flowering of asparagus regenerants encapsulated with an alginate matrix.

2. Materials and Methods

2.1. Survey of the Chemical Inducer for Early Flowering of Seedlings

Seeds of asparagus cv. Welcome (Sakata Seed Co., Japan) were treated with 200 μM of (2-chloroethyl)-trimethylammonium chloride (CCC; chlormequat), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine(atrazine) and *n*-propyl *N*-(3,4-dichlorophenyl)carbamate. Treatment solutions were made by first dissolving compounds in a small volume of dimethyl sulfoxide (DMSO) and diluting with distilled water so that the final concentration of DMSO was 0.5%. Twenty-five seeds without surface sterilized were placed on a filter paper in a

plastic petri dish (90 mm diameter, 20 mm high) and soaked with 20 ml of the treatment solutions for 12 days at 25˚C under a 16-h photoperiod from fluorescent lamps (40 μmol·m^{-2}·s^{-1}). After incubation, the seeds and seedlings were washed in running water and then transplanted to soil in plastic trays in a greenhouse at ca. 25˚C under long-day condition. Fifty days later, the germination and flowering rate (percent) was scored as (no. of plants germinated/no. of seeds sown) × 100 and (no. of plants that flowered/no. of seeds sown) × 100, respectively. Each experiment was replicated twice.

2.2. Somatic Embryogenesis

Young spears were harvested individually from female and male plants of asparagus, and were sterilized for 5 min with a sodium hypochlorite solution (3% active chlorine) containing 0.1% Tween® 20. The meristems excised from the apical region of aseptic spears were used as explants. The explants were placed onto dedifferentiation medium composed of Murashige and Skoog's (MS) basal salts [29], 3% sucrose, 2 mg·l^{-1} 2,4-D and 1% gelrite. All media were adjusted to pH 5.8 prior to autoclaving. Cultures were kept at 25˚C with a 16-h photoperiod under fluorescent lamps (40 μmol·m^{-2}·s^{-1}). After 2 months of culture, the calli were subcultured at 4-week intervals in the same medium in order to obtain embryogenic calli. Approximately 1 g of embryogenic calli was precultured in 100 ml of liquid hormone-free dedifferentiation medium in a 300-ml Erlenmeyer flask. The flasks were placed on a rotary shaker at 100 rpm in a dim light condition at 25˚C. After 1 week of preculture, the suspensions were sieved with a stainless steel mesh (φ 220 μm), and the passed through cells were gathered and then plated with 0.1 ml packed cell volume on regeneration medium composed of MS basal salts, 0.1 M maltose and 1% gelrite in a plastic petri dish (90 mm diameter, 20 mm high). Somatic embryos of different developmental stages were isolated after 4 weeks of culture.

2.3. Encapsulation

Sodium alginate solution was used as an encapsulation matrix. The disinfected seeds were suspended in calcium-free MS inorganic medium supplemented with 2% − 4% (w/v) sodium alginate [80 - 120 cps (Wako, Japan), 300 - 400 cps (Wako) or 500 cps (Sigma, USA)] and 0.4 M sucrose. The mixture including seeds was dropped with a sterile pipette (5 mm internal diameter) into a solution of 0.1 M $CaCl_2$·$2H_2O$ with 0.4 M sucrose added. Each bead containing a single seed was maintained in $CaCl_2$ solution for 30 min at room temperature. The beads were placed on a hormone-free medium composed of MS basal salts, 88 mM sucrose and 0.2% gelrite. The cultures were kept at 25˚C with a 16-h photoperiod under fluorescent lamps (40 μmol·m^{-2}·s^{-1}), and the percentage of rooting and shooting [(no. of plants rooted or shooted/no. of seeds sown) × 100] was measured 45 days after incubation. The somatic embryos were picked up using a sterile forceps, and then suspended in calcium-free MS inorganic medium supplemented with 3% sodium alginate (80 - 120 cps) and 0.4 M sucrose. The procedure

for the production of beads containing a single embryo and the culture conditions were the same as those used for the encapsulation of the sterile seeds. The beads were placed on a hormone-free medium, and the percentages of rooting, shooting, and flowering were scored at 45 days of culture. Each experiment was replicated two to three times.

2.4. Flower Induction of Somatic Embryos and Synthetic Seeds

Somatic embryos and synthetic seeds were treated with 200 μM *n*-propyl *N*-(3,4-dichlorophenyl)carbamate. The compounds were dissolved in a small volume of DMSO (the final concentration was 0.5%) and diluted with distilled water or MS liquid medium. The other procedures were performed as described above.

2.5. Histological Study

Histological specimens (*in vitro* and *in vivo* flowers) were fixed for 24 h in Carnoy's solution (ethanol:chloroform:acetic acid = 60:30:10, v/v) at room temperature. After dehydration in butanol, the samples were embedded in paraffin, and 10-μm-thick sections were made using a rotary microtome (HM340E, Zeiss, Germany). The sections were stained with toluidine blue O and then observed under a light microscope.

3. Results

3.1. Early Flowering of Somatic Embryos

Effective chemical inducers were selected according to the flowering inducement at the early stage of the asparagus seedlings, and the survival rate and flowering frequency of the seed were different depending on the kind of chemical inducer used (Table 1). The survival rate was the lowest at 28% with the use of diuron. When atrazine or carbamate was used as the inducer, flowering was induced, although flowering plants were not observed as in the case of diuron or CCC. The flowering rate was the highest, approx. 29%, with carbamate. These results regarding the procedure period (8 - 12 days of application) and optimum concentration (200 μM) of carbamate correspond to those of previous reports (data not shown) [19] [22]. The somatic embryos withered completely regardless of the concentration of carbamate when they were processed under optimum conditions for early flowering of seedlings, that is, when redifferentiated individuals were processed for 8 - 12 days with 200 μM carbamate dissolved in distilled water (Table 2). In contrast, when the somatic embryos were treated with carbamate dis-

Table 1. Precocious flowering of asparagus seeds treated with chemical inducers.

Chemicals	Germination (%)	Precocious flowering (%)
Diuron	28.0 ± 4.0	0.0 ± 0.0
Atrazine	58.0 ± 6.0	3.9 ± 3.8
Carbamate	70.0 ± 2.0	28.9 ± 6.6
CCC	78.0 ± 6.0	0.0 ± 0.0
Distilled Water + 0.5% DMSO	78.0 ± 2.0	0.0 ± 0.0

Data are means from two independent experiments with 25 seeds (±standard error).

Table 2. Effect of carbamate and its solvent on the precocious flowering of somatic embryos.

Carbamate	Solvent	Germination (%)	Precocious flowering (%)
–	DW	46.7 ± 20.0	0.0 ± 0.0
–	MS	40.0 ± 13.3	0.0 ± 0.0
+	DW	0.0 ± 0.0	0.0 ± 0.0
+	MS	26.7 ± 0.0	3.4 ± 3.3

Distilled water (DW) and Murashige and Skoog's basal medium (MS) were used for the solvent. Data are means from two independent experiments with 15 seeds (±standard error).

solved in MS basal medium, precocious flowering was observed at an extremely low rate (3.4%), although survival rates were decreased compare to the controls.

3.2. Early Flowering of Synthetic Seeds

The root and shoot formation rates of the seeds encapsulated by the sodium alginate decreased as the viscosity and concentration of sodium alginate rose (Table 3). Both the shoot and root formation rates were 100% in encapsulated seeds that had 500 cps of high-viscosity sodium alginate added. This was due to the fact that the gel tended to crack when germination and rooting of the involved seed began, and because the plantlets were directly immersed in the carbamate solution at an early developmental stage. The operations of dissolution and encapsulation of the sodium alginate were complicated for the density to rise. All the encapsulated somatic embryos (Figure 1(a)) were withered when they were processed with the carbamate that dissolved in distilled water. The result was similar to that obtained with the non-encapsulated somatic embryos with the carbamate solution, since MS liquid media was then used for the solvent, the flowering inducement rate was 13.3% (Figure 1(b), Table 4).

3.3. Morphological and Histological Observations

Morphological views of an asparagus flower are shown in Figure 2. A developing ovule and pistil were observed in the female flowers of the asparagus seedlings (Figure 2(a)). The petal was composed of six pieces, and a degenerating androecium was observed in the base. In the male flowers, the anther could be seen on the point of its respective filament, and there was an aborted ovule in the base (Figure 2(b)). In contrast, the morphology of the flower organs that were precociously induced by carbamate processing were almost the same as that of the control, although the petals developed into a verdure that looked like the calyxes (Figure 2(c) and Figure 2(d)). We observed that ovules, pistils, petals that looked like calyxes, and degenerating androecia were present in the flowers induced to female regenerants (Figure 2(e)). The anther was observed in the point of six filaments individually in the flowers induced to male regenerants, although the petals looked like calyxes, too (Figure 2(f)). Female and male regenerants that flowered precociously following the application of carbamate produced a female flower and a male flower, respectively, and thus conversion of the character expression did not occur.

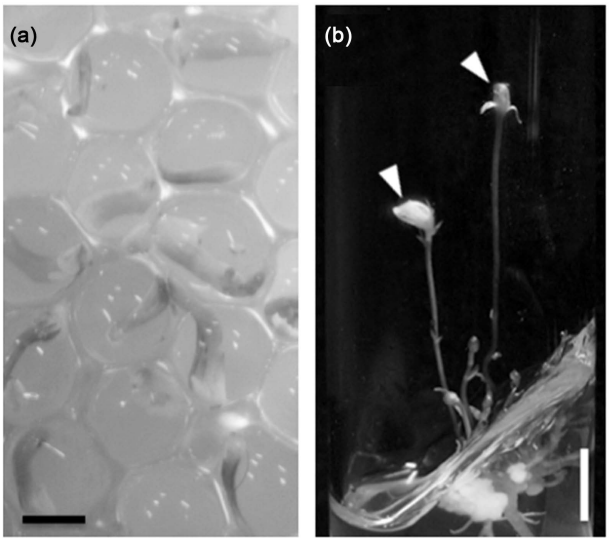

Figure 1. Encapsulation and early flowering of somatic embryos of asparagus (*Asparagus officinalis*). (a) Encapsulation of torpedo embryos. (b) Synthetic seeds that were encapsulated with the sodium alginate of 80 - 120 cps by 3% flowered early *in vitro* (arrowheads) when they were treated with 200 μM of *n*-propyl *N*-(3,4-dichlorophenyl)carbamate. Scale bars in a = 5 mm; in b = 10 mm.

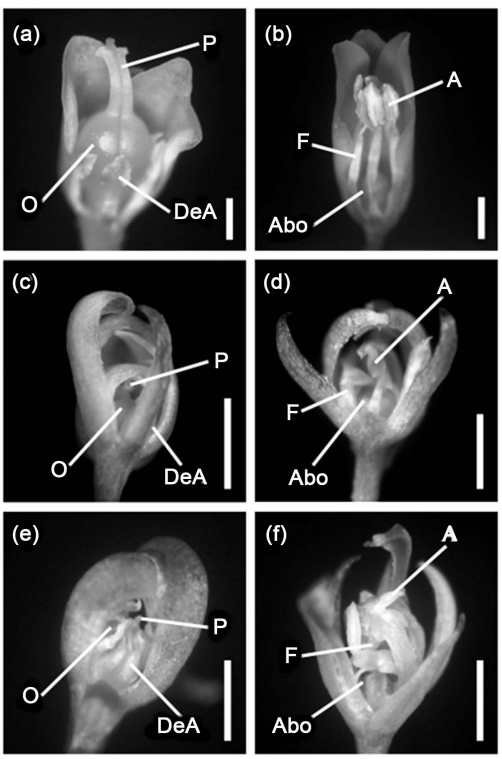

Figure 2. Appearance of asparagus flowers (cv. Welcome) flowered on a seedling ((a), (b)), a seedling processed with carbamate ((c), (d)), and a regenerant with chemical treatment ((e), (f)). The female flower ((a), (c), (e)) has a pistil (P), ovary (O) and degenerating androecium (DeA), whereas the male flower ((b), (d), (f)) has six anthers (A) and filaments (F) and an aborted ovule (AbO). Scale bars = 1 mm.

Table 3. Effect of viscosity and concentration of sodium alginate on the root and shoot formation of encapsulated seeds.

Sodium alginate viscosity (cps)	Conc. (%)	Root formation (%)	Shoot formation (%)
80 - 120	2	100.0 ± 0.0	77.8 ± 18.1
	3	88.9 ± 0.0	55.6 ± 0.0
	4	88.9 ± 0.0	55.6 ± 9.1
300 - 400	2	100.0 ± 0.0	100.0 ± 0.0
	3	100.0 ± 0.0	78.0 ± 9.1
	4	77.8 ± 9.1	44.0 ± 0.0
500	2	88.9 ± 9.1	55.6 ± 0.0
	3	88.9 ± 0.0	33.3 ± 0.0
	4	100.0 ± 0.0	100.0 ± 0.0

Data are means from three independent experiments with nine beads (±standard error).

Table 4. Precocious flowering of encapsulated somatic embryos treated with carbamate dissolved in distilled water (DW) and Murashige and Skoog's basal medium (MS).

Carbamate	Solvent	Root formation (%)	Shoot formation (%)	Precocious flowering (%)
+	DW	0	0	0
+	MS	90.0 ± 9.9	23.3 ± 9.9	13.3 ± 0.0

Data are means from two independent experiments with 15 beads (±standard error).

Histological views of the asparagus flowers are shown in **Figure 3**. In the female flowers, embryo sacs were observed at the top and bottom of the ovules. In the male flowers, six anthers were observed, and a significant amount of pollen was formed in each anther. In the flowers that were induced precociously by carbamate processing to both seeds and somatic embryos, the structure in the ovules of the female flowers looked like seedlings. In the male flowers that flowered early, innumerable pollens were observed in the anther as well as the male flowers of the seedlings. Conversion of the character expression was not confirmed by histology.

4. Discussion

Somatic embryos flowered precociously at a low frequency (3.4%) when they were directly treated with carbamate dissolved in MS basal medium. It was reported that the frequency of embryogenesis could be controlled by alteration of the concentration of sugars and basal medium in order to produce encapsulatable units [30], and that vigorous embryos were obtained by treatment with plant growth regulators such as IAA, kinetin, ancimidol and ABA [31]. The use of these methods may lead to a high survival rate of embryos and result in an increased frequency of precocious flowering in asparagus. In this study, the concentration of sodium alginate needed for encapsulation and easy handling varied among the different viscosities. The viscosity and concentration of

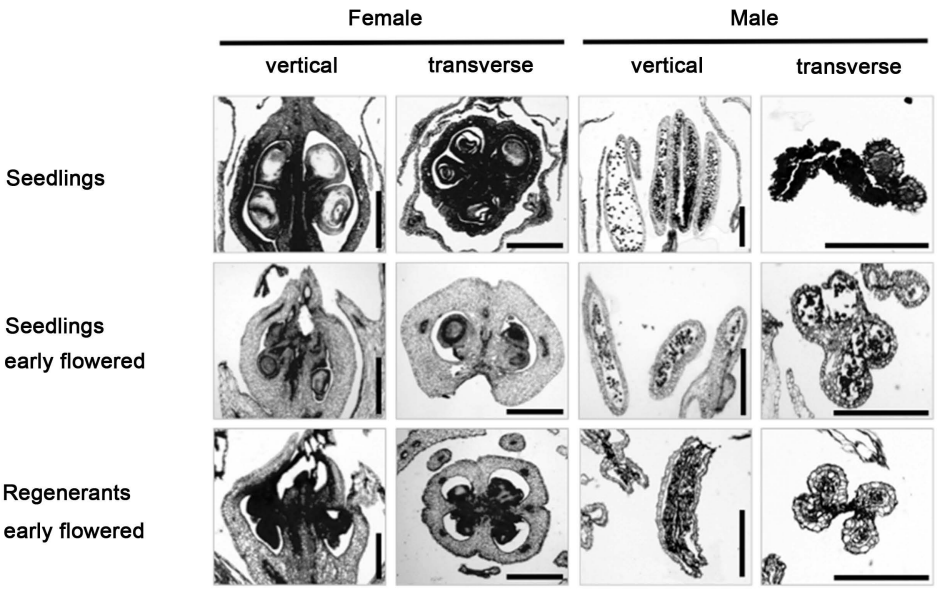

Figure 3. Photomicrographs of flowers of asparagus. Longitudinal and transverse sections of a female flower indicate embryo sacs in the upper and bottom portion of the ovule whether treated with the chemical inducer or untreated. Histological sections of a male flower show that innumerable pollens were produced in the anther whether the plant was treated with the chemical inducer or not. Scale bars represent 500 μm.

alginic acid also influenced the frequency of shoot formation due to the hardness factor of the beads. A differential conversion response with respect to the concentration of sodium alginate has been reported for asparagus [32], but no information on viscosity was provided in that report. In the present study, the suitable viscosity and concentration for encapsulating the seeds were 80 - 120 cps and 2% - 3%, respectively. These parameters permitted embryo development, shoot and root formation of embryos, and easy handling.

When we treated encapsulated embryos with the carbamate dissolved in MS basal medium, the precocious flowering rate showed a fourfold increase compared to the naked embryos. Crucial differences were observed in plantlet development between the non-encapsulated (**Table 2**) and encapsulated (**Table 4**) embryos. This may be due to the nature of the alginate or to the physical handling of the embryos. Unlike the encapsulated embryos, the non-encapsulated embryos were in direct contact with the carbamate solution. Dissolving the carbamate into basal medium that was used to encapsulate the embryos was an important factor, enabling them to survive and flower. In an earlier study, when encapsulated embryos of asparagus were cultured on filter paper soaked with various basal media, the MS basal medium gave the highest conversion frequency [32]. A particular basal medium also promoted the conversion frequency of artificial seeds in eggplant (*Solanum melongena*) [33]. These results indicate that asparagus embryos must be treated indirectly with carbamate dissolved in a complete nutrient medium, in order to induce precocious flowering of the embryos. Additionally, encapsulated embryos derived from Kinnow mandarin (*Citrus nobilis* Lour × *C. delici-*

osa Tenora) failed to germinate on MS basal medium without plant growth regulators, but medium supplemented with 9.29 μM kinetin allowed embryo germination [34]. The frequencies of survival and precocious flowering in encapsulated asparagus embryos may be accelerated by the addition of plant growth regulators to the solvent.

The developmental stages of the encapsulated somatic embryos may affect the degree to which they react to a chemical inducer. Synchronization of the growth of embryogenic calli and embryogenesis has been investigated in detail. In a suspension culture of Madagascar periwinkle (*Catharanthus roseus*), a synchronous cell division system was established using the double phosphate starvation method [35]. It has been discussed how a phosphate-limited population of suspension cultures of sycamore (*Acer pseudoplatanus*) in the stationary phase showed high viability even in the late stationary phase compared to the use of limited carbohydrate and nitrogen sources [36]. Another report described how carrot (*Daucus carota*) suspension cultures were obtained by fractionation through sieving with nylon screens, and then density-gradient centrifugation using Percoll solutions synchronously differentiated the embryos when they were cultured in a medium supplemented with a proper combination of plant growth regulator [37]. Precocious flowering regenerants might be highly obtainable, if we could identify the developmental stage of asparagus embryogenic calli and embryos that are most sensitive to a chemical inducer, and then synchronize them with the partial phase.

In our study, in light of the morphological and histological findings, the sex expression of precocious flowering regenerants derived from female or male plants expressed femaleness or maleness, respectively. Like asparagus, spinach (*Spinacia oleracea*) is a dioecious species, and the sex expression seems to be influenced by tissue culture sequences. Four to 13.2% of the regenerants originated from male explants showed andromonoecy, although the plants regenerated from female explants did not change their sex expression [38] [39]. The stable sex expression of a female phenotype in spinach could be changed by irradiation with ion particles [40]. Based on comparative analyses using transgenic plants, the stable expression of sex in regenerants is considered to be essential. In the present study, no sex alteration was observed in asparagus regenerants that flowered precociously, and a flowering system thus established would be useful in functional studies of the genes controlling the development of floral organs.

In the genus *Asparagus*, MADS-box genes correlating with the development of flower organs have been isolated and characterized: *AGAMOUS*-like genes (*AVAG1* [12], *AVAG2* [13]), *DEFICIENS*-like genes (*AODEF* [9] [14]), *GLOBOSA*-like genes (*AOGLOA* and *AOGLOB* [10]), and *SEPALLATA*-like genes (*AOM1* [8], *AOM3* and *AOM4* [11], *AOMADS1*, *AOMADS2* and *AOMADS3* [15], and *AVMADS1*, *AVMADS2* and *AVMADS3* [15]). Ectopic expression experiments that employed floral homeotic mutants would provide information regarding the molecular dynamics that depend on these genes. A precocious flowering system of asparagus regenerants will contribute to a reduction in the amount of time required for the flowering of perennial transgenic plants.

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