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A Secreted Peptide Growth Factor, Phytosulfokine, Acting as a Stimulatory Factor of Carrot Somatic Embryo Formation

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Somatic embryogenesis of the carrot (*Daucus carota* L.) depends on a set of factors, some of which accumulate in culture medium (conditioned medium, CM). When embryogenic cell clusters were transferred to an embryo-inducing medium, addition of CM derived from somatic embryo culture markedly stimulated somatic embryo formation. The active principles were purified using a simple bioassay system and identified to be phytosulfokines (PSKs), sulfated oligopeptide growth factors originally isolated from a CM derived from asparagus (*Asparagus officinalis* L.) mesophyll culture. Quantification studies using a competition ELISA system employing an anti-PSK-α polyclonal antibody showed that PSK production might be related to growth of cells, rather than development of somatic embryos. Thus the stimulatory effect of PSK on somatic embryo formation might be due to promotion of cell proliferation.

Key words: *Daucus* — Phytosulfokine-α — Somatic embryogenesis — Sulfated peptide.

Conditioning effects are widely observed in various plant culture systems. It has long been known that proliferation of plant cells at a low density is induced by addition of a conditioned medium (CM) prepared from rapidly growing cells in culture (Stuart and Street 1969). In 1996, we established a sensitive bioassay system to detect mitogenic activity of CM employing a primary culture system of mesophyll cells prepared from *Asparagus officinalis* L. (Matsubayashi and Sakagami 1996). Using this system, we previously isolated the active principles and determined their chemical structures to be a disulfated pentapeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (phytosulfokine-α, PSK-α), and its C-terminal-truncated tetrapeptide (PSK-β). These factors are distinguishable from known plant growth regulators and are distributed in several plant families (Matsubayashi et al. 1997, 1999b).

Somatic embryogenesis is a model system for investigating alterations in the early development of plant embryogenesis. Among several plant species, the carrot (*Daucus carota* L.) has been the most widely used for studies of somatic embryogenesis. Morphological and physiological studies demonstrate that differentiation of carrot cells into somatic embryos can be separated into at least two phases. The first phase is thought to involve acquisition of embryogenic competence and the second is characterized by somatic embryo formation (Nomura and Komamine 1985).

Several factors have been shown to influence the second phase of carrot somatic embryogenesis. One is initial cell density. Thus somatic embryo formation is known to be inhibited when embryogenic cells are cultured at a high density (Fridborg et al. 1978, Hari 1980, Sung and Okimoto 1981, 1983). This inhibition is considered to be due to chemical factors secreted into the culture medium rather than depletion of nutrients. Other chemical factors, which stimulate somatic embryo formation, have also been suggested to be secreted into the culture medium (Kato and Takeuchi 1963, Halperin 1967, Hari 1980). Recently, researchers have revealed the chemical properties of these endogenous, conditioned medium factors (CMFs). Treatment with brefeldin A, a specific inhibitor of protein secretion, results in complete arrest of the embryogenic process, but addition of CM partially abrogates this inhibition (Capitanio et al. 1997). Higashi et al. (1998) showed that the inhibition of somatic embryo formation at a high cell density was due to endogenous factors released into the CM, whose molecular weights were below 3,500. Moreover, they suggested that a high molecular weight factor, which stimulated somatic embryo formation, was also secreted.

Here we describe the purification of the active principles of CM, which stimulate the formation of carrot somatic embryos and their identification as PSK.

Materials and Methods

Plant materials and culture conditions—Embryogenic carrot calli were produced and somatic embryogenesis was induced as

Abbreviations: CM, conditioned medium; EC, high embryogenic competent cell; LC, low embryogenic competent cell; pcv, packed cell volume at $100 \times g$; PSK, phytosulfokine; TFA, trifluoroacetic acid.

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described previously (Satoh et al. 1986) with slight modification. Carrot (*Daucus carota* L. cv. US-Harumakigosun) seedlings were germinated and their hypocotyl segments were inoculated on Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) supplemented with 4.5×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified with 0.2% Gelrite. One-month-old yellowish embryogenic calli were obtained, and small pieces were suspended in 100 ml of MS liquid medium supplemented with 4.5×10^{-6} M 2,4-D in a 300-ml Erlenmeyer flask and allowed to grow on a rotary shaker (70 rpm) at 25°C in darkness. After two weeks, the suspension was sieved through a stainless steel mesh (1 mm in pore size), and cell clusters remaining on the mesh were transferred to 100 ml of fresh medium supplemented with 2,4-D. This operation was repeated every two weeks, and the resulting cells were designated as high embryogenic competent cells (ECs). Other cells which had embryogenic competence but generated fewer embryos at a low cell density, were designated as low embryogenic competent cells (LCs). Each cell line was classified into EC or LC by determination of the embryogenic competence (see below).

Bioassay procedures—MS medium without 2,4-D prepared at a 3-fold concentration was sterilized by filtration, then gently dispensed into 24-well microplates at a volume of 200 μl per well. Various CM sample solutions (400 μl) were sterilized by filtration, then added to the medium in each well. These bioassays were performed at an initial density of 0.5–0.1 ml packed cell volume (pcv) ($100 \times g$) cell clusters per liter. Cell clusters were obtained from the two-week-old LC cultures by successive sieving through meshes. Those which passed through a 63 μm but remained on a 37 μm mesh were suspended in MS medium without 2,4-D and centrifuged at $100 \times g$ for 5 min. The precipitated cell clusters were rinsed five times with the same medium, and the resulting cell suspension was diluted, followed by dispensing into the microplate at a volume of 200 μl per well. The plates were sealed with Parafilm to avoid evaporation of the medium and incubated in the dark at 25°C with continuous rotary shaking at 120 rpm. Stimulatory activity in terms of somatic embryo formation was determined on the 10th to 14th day of culture by counting the numbers of embryos (globular, heart-shaped and torpedo-shaped embryos) under an inverted microscope. All of the treatments were performed in duplicate or triplicate and errors calculated as standard deviations. The embryogenic competence of each cell was determined by the procedure mentioned above, except that the initial density was at 0.01, 0.05, 0.1, 0.5, 1 and 5 ml pcv liter^{-1}, and sterile water was substituted for CM samples.

Preparation of CM—CM was prepared by the following procedure. Embryogenic cell clusters obtained from EC cultures by the successive sieving described above, were suspended in 150 ml of culture medium without 2,4-D at a density of 0.2 ml pcv liter^{-1}. This suspension was cultured in a 500-ml Erlenmeyer flask in the dark at 25°C with rotary shaking at 70 rpm. For the purification of active substances, 14-day CM was prepared and stored at −30°C. In addition, CM from cultures with 2,4-D was prepared by filtering culture medium of EC or LC and stored at −30°C.

Purification of active principles—CM (600 ml) was concentrated to 200 ml under reduced pressure, adjusted to pH 8.0 with 6.0 M KOH, and then applied to a DEAE-Sephadex A-25 column (3.2 × 20 cm, Pharmacia) that had been equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The column was washed with 300 ml of equilibration buffer, and fractions were eluted successively with 300 ml of this buffer containing 400, 800, 1,200 or 1,600 mM KCl. For desalting, each fraction was concentrated to 100 ml and then applied to Sep-Pak Vac C_{18} cartridges (2 g × 2, Waters) that had been equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The columns were washed with 40 ml of the equilibration solution, and fractions were eluted with 40 ml of 30% acetonitrile containing 0.1% TFA. Desalted active fractions recovered from the DEAE Sephadex column (800 and 1,200 mM KCl fractions) were lyophilized, dissolved in 500 μl of 20 mM KH_2PO_4-KOH (pH 5.8), and then applied to a Bio-Gel P-2 extra fine column (1.6 × 48 cm, Bio-Rad), which had been equilibrated with the same buffer. Fractions were eluted with the buffer at a flow rate of 15 ml h^{-1} with absorbance monitored at UV 220 nm. Five-milliliter fractions were collected, and each was bioassayed. The fractions recovered from the Bio-Gel column were lyophilized, dissolved in 100 μl of water, and further analyzed by liquid chromatography/mass spectrometry (LC/MS).

LC/MS Experiments and amino acid sequence analysis—The active fraction of CM was dissolved in 100 μl of water and separated on a Develosil ODS-5 column (4.6 × 250 mm, Nomura Chemical) with 10% acetonitrile containing 0.1% TFA at 1.0 ml min^{-1}. Mass spectra were obtained using a Fisons VG platform quadrupole mass spectrometer equipped to perform electrospray ionization, interfaced to a Jasco PU 980 HPLC system. The HPLC split 1 : 9 so that 100 μl min^{-1} flowed to the mass spectrometer during the separation. The source temperature was maintained at 70°C, and the m/z 50–1,500 range was scanned over 2.9 s at unit resolution. One-milliliter fractions were collected, lyophilized and bioassayed. Amino acid sequences of the active compounds were determined by Edman degradation with an Applied Biosystems model 491 sequencer. Phenylthiohydantoin derivatives of amino acids obtained at each cycle of the Edman degradation were determined by reverse-phase HPLC on an Applied Biosystems Brownlee C-18 column.

Synthesis of PSK—Preparation of synthetic PSK-α and -β was performed with a peptide synthesizer (Applied Biosystems Model 433A) as previously described (Matsubayashi et al. 1996).

Competition ELISA system—Competition ELISA and quantification of PSK were performed according to Matsubayashi et al. (1999a). For quantification of PSK, aliquots of the CM were concentrated under reduced pressure and then purified using a reverse-phase HPLC column. Fractions corresponding to those of PSK-α and -β were collected and lyophilized, followed by competition ELISA. A standard curve was obtained by addition of known amounts of PSK-α, and the concentration of PSK in each sample was calculated as that of PSK-α using the standard curve. The identification limit of this system is about 0.1 nM.

Time course of PSK production and somatic embryo formation—Induction of somatic embryogenesis was carried out as mentioned above. Ten milliliters of suspension were collected every other day from embryo-inducing cultures. Five hundred microliters of each aliquot were transferred into a counting chamber and the numbers of somatic embryos were counted. The cells or embryos and CM were then separated by centrifugation at $100 \times g$ for 10 min and the amount of PSK in each CM measured by competition ELISA. The packed cells and embryos were dried in an evaporator for measurement of their dry weight.

Results

Establishment of the bioassay system—In carrot somatic embryogenesis, somatic embryo formation is inhibited at a high cell density. The CM under such conditions contains low molecular weight inhibitors which are

not retained by dialysis (Higashi et al. 1998). The CM also contained higher molecular weight factors. While the higher molecular weight factors stimulated somatic embryo formation, the high competence for somatic embryogenesis of high embryogenic competent cells (ECs) in our bioassay meant that stimulatory effects were not clearly observed (data not shown). The EC which had been subcultured at two-week intervals for more than four months decreased the embryogenic competence. The decline of embryogenic competence (below one-tenth as compared to the newly established EC) enabled of observation of the stimulatory effect of CM derived from the fresh EC culture. However, the old EC had lost the embryogenic competence completely after one or two months and could no longer form somatic embryos even if the CM was added. Thus we concluded that the old EC was not suitable for use in the bioassay and we had to establish another cell line to purify the stimulatory factor in the CM. In screening of embryogenic cell lines, we selected a low embryogenic competent cells (LCs) whose embryo formation frequency (25 embryos per 800 μl culture at an initial density of 0.1 ml pcv liter^{-1}) was lower than that of fresh EC (143 embryos) and dependent on its initial cell density at 0.01–0.5 ml pcv liter^{-1} (Fig. 1). The formation of somatic embryos in the LC culture was markedly stimulated by addition of the CM derived from the EC culture (Fig. 1). Stimulatory effects of CM were first observed on the 6th day of culture and embryo formation attained about 7-fold that of controls on the 14th day. The stimulatory effects were observed stably by use of LC for about four months.

Identification of PSK in carrot CM—The chemical properties of the active principles in the carrot CM resembled those of PSK (Matsubayashi and Sakagami 1996). Following the purification procedure for PSK-α and -β (Matsubayashi and Sakagami 1996), with two steps using ion exchange and gel permeation columns, active fractions were obtained in the same fractions that eluted PSK. For the final purification, the active fractions recovered from the gel permeation were injected into a reverse-phase HPLC column, and a part (10%) of the eluate was directly

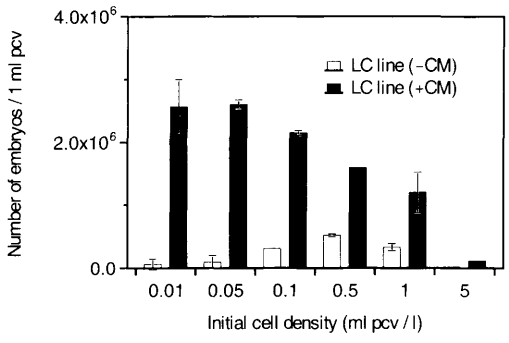

Fig. 1 Induction of somatic embryogenesis in LC cultures at several initial cell densities. Cell clusters (38–63 μm) were collected and transferred to embryo-inducing medium with or without addition of conditioned medium (CM). The total number of somatic embryos was counted on the 14th day of culture and the number of embryos per 1 ml pcv was calculated by dividing the total number of embryos of each well by the initial packed cell volume dispensed into each well.

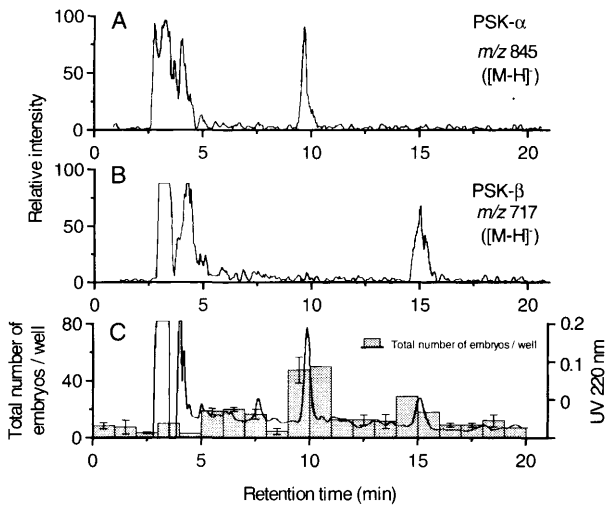

Fig. 2 Purification of the active principles. Active fractions eluted from the Bio-Gel P-2 column were dissolved in 100 μl of water and separated on a Develosil ODS-5 column (4.6 × 250 mm) with 10% acetonitrile containing 0.1% TFA at 1.0 ml min^{-1}. The HPLC eluate was split 1 : 9 so that 100 μl min^{-1} flowed to the mass spectrometer during separation. (A, B) Mass chromatograms of each molecular ion of PSK-α and -β are shown. (C) Absorbance was monitored at UV 220 nm (solid line). The stimulatory activity of each fraction was determined after 13 d of culture (left scale, dotted bars).

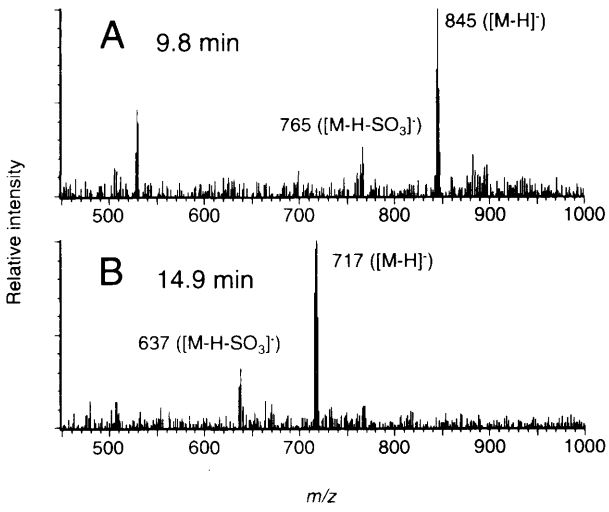

Fig. 3 Mass spectra of the active principles. (A) Mass spectrum of the active compound eluting at 9.8 min. (B) Mass spectrum of the active compound eluting at 14.9 min.

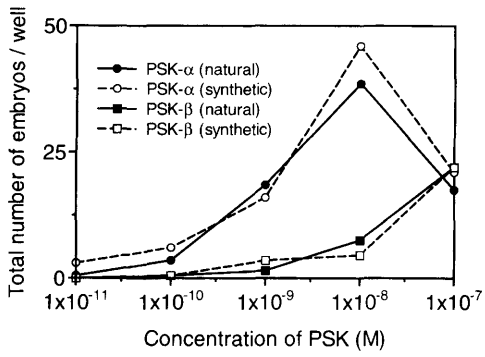

Fig. 4 Stimulation of somatic embryo formation by natural or synthetic PSK-α and -β. Cell clusters (38–63 μm) from LC cultures were collected and transferred to embryo-inducing medium at a density of 0.2 ml pcv liter^{-1} with various concentrations of natural or synthetic PSK-α and -β. The total number of somatic embryos was counted on the 14th day of culture. All the assays were performed in duplicate. Closed circles, natural PSK-α; open circles, synthetic PSK-α; closed squares, natural PSK-β; open squares, synthetic PSK-β.

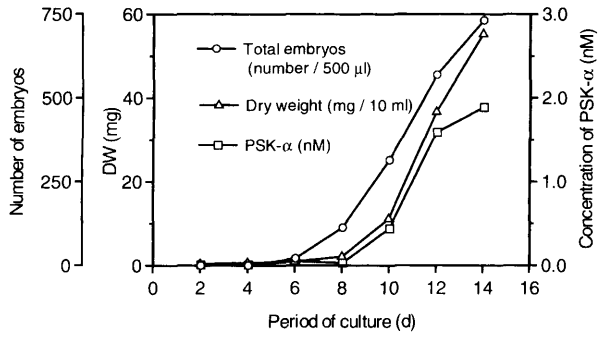

Fig. 5 Time course of PSK production and somatic embryo formation. Cell clusters obtained from EC suspension cultures were induced to undergo formation of somatic embryos. Ten milliliters of the culture suspension were collected every other day. Total numbers of embryos (left scale, open circles), their dry weights (left scale, open triangles) and accumulation of PSK in CM (right scale, open squares) are shown.

introduced into a mass spectrometer. Two major UV peaks eluted at 9.8 and 14.9 min were coincident with the peaks on mass chromatograms of *m/z* 845 (consistent with a quasi molecular ion of PSK-α) and 717 (that of PSK-β) (Fig. 2A, B). Biological activities were observed principally in the fractions of both peaks (Fig. 2C). Mass spectra of these compounds were also identical with those of PSK-α and -β (Fig. 3). Amino acid sequence analysis revealed primary sequences of Tyr-Ile-Tyr-Thr-Gln and Tyr-Ile-Tyr-Thr, allowing the conclusion that the active principles of the CM were PSK-α and -β. Total yields of PSK-α and -β from 600 ml of CM were 0.5 μg and 0.3 μg, respectively.

Exogenous PSK-α and -β stimulate somatic embryo formation—When somatic embryo formation was examined in the presence of synthetic PSK-α or -β at several concentrations, the frequency increased in a dose dependent manner (Fig. 4). Minimum concentrations of PSK-α and -β exhibiting stimulatory activity were 0.1 nM and 1 nM, respectively. The dose responses with natural PSK-α and -β isolated from the CM of EC closely corresponded to those with synthetic PSK-α and -β. At concentrations of more than 10 nM, however, PSK-α exhibited weak activity (Fig. 4).

PSK production of EC and LC in the presence or absence of 2,4-D—To investigate the relation between PSK production and embryogenic competence, we measured the amounts of PSK in CM derived from EC and LC cultures, with or without 2,4-D. For this purpose, we adopted a recently developed competition ELISA system using an anti-PSK-α antibody (Matsubayashi et al. 1999a). When cultured with 2,4-D, both cell lines attained 6- to 10-fold increase in their dry weights during the culture for 14 d and produced large amounts of PSK (Table 1). When cultured without 2,4-D (embryo-inducing condition), cell clusters of EC formed many somatic embryos and the concentration of PSK in the culture was 3.3 nM (Table 1). The amount of PSK in LC culture was, however, less than the detection limit. In this case, the cell growth of LC, which attained a 3-fold increase, was much less than that of EC (183-fold) (Table 1).

Relation between production of PSK and formation

Table 1 Quantification of PSK amounts in CM derived from different cultures

Culture condition	+2,4-D (callus condition)		−2,4-D (embryo-inducing condition)	
Cell line	EC	LC	EC	LC
Concentration of PSK-α (nM)	17.4	24.6	3.3	<0.1
DW (initial, g (100 ml)$^{-1}$)	0.08	0.06	0.003	0.013
DW (final, g (100 ml)$^{-1}$)	0.50	0.62	0.55	0.041

The amounts of PSK in four varieties of CM obtained on the 14th day of culture were quantified using competition ELISA. Two milliliters of each CM was partially purified by a reverse-phase HPLC column, followed by competition ELISA. The concentration of PSK was calculated to be that of PSK-α, with an identification limit of 0.1 nM. Experiments were performed in duplicate.

of somatic embryos—The amount of PSK accumulating in the CM prepared from the embryo cultures of EC was assessed by competition ELISA (Fig. 5). On the 6th day of culture, the first morphogenetic alterations were observed; certain cells in the embryogenic cell clusters rapidly divided and then developed into globular embryos. On the 14th day of culture, the total number of later stage embryos, such as heart- and torpedo-shaped embryos, was 35-fold that on the 6th day of culture. The dry weight of cells reached a maximum on the 14th day of culture, which was a 180-fold increase from the initial weight. PSK was detected from the 10th day of culture and increased rapidly with an increase in dry weight from the 8th to the 12th day of culture.

Discussion

Carrot somatic embryogenesis consists of two distinct phases, the first phase resulting in acquisition of embryogenic competence in the presence of auxin and the second phase being formation of the somatic embryo from the embryogenic cell in the absence of auxin (Nomura and Komamine 1985). The trigger for transition to the second phase is accepted as elimination of auxin in the medium. Although there is a body of evidence that secretory factors in CM might stimulate the formation of somatic embryos during the second phase, it is only recently that the chemical properties have been elucidated (Kato and Takeuchi 1963, Halperin 1967, Hari 1980, Higashi et al. 1998). In this study, we selected two cell lines, one a high competence cell line (EC) for preparation of CM and the other a low competence cell line (LC) for detection of factors stimulating somatic embryo formation. Thus we established a simple bioassay system employing LC to facilitate purification of the stimulatory factors (Fig. 1), these then being identified as PSK-α and -β (Fig. 2). The confirmation that they exhibited the same activities as synthetic PSK-α and -β (Fig. 4) demonstrated that PSK is the major active principle in the CM.

An important question is whether the capacity for PSK production is related to the embryogenic competence of the carrot cell. As shown in Table 1, the embryogenic competence of carrot cells seemed to be unrelated to the ability of PSK production in the presence of 2,4-D. When cultured without 2,4-D, in contrast, a high amount of PSK was detected in the CM of EC. Growth of LC was much less than that of EC under this condition, but prior to the logarithmic growth phase, or before the formation of globular embryos (before the 8th day of culture) in Fig. 5, the amount of PSK in the CM of EC was also still low. These data imply that the low amount of PSK in LC culture under auxin-free conditions is not a reflection of the low embryogenic competence, but a result of poor growth of cells. We concluded that the ability to produce PSK does not directly relate to the embryogenic competence.

We investigated the role of PSK on somatic embryogenesis. PSK is not capable of inducing embryogenic competence in non-embryogenic cells and also does not trigger the transition to the second phase of somatic embryogenesis (data not shown). Addition of exogenous PSK-α to EC culture in the presence of 2,4-D stimulated proliferation of the cells, and not stimulated differentiation into the somatic embryos (Kobayashi et al. 1999). On the other hand, addition of PSK-α to an embryo-inducing culture of old EC markedly accelerated somatic embryo formation and increase of the cell number. There were, however, few alterations in the rate of globular, heart-shaped and torpedo-shaped embryos and the cell number per somatic embryo in each treatment of PSK-α (Kobayashi et al. 1999). We assumed that exogenous PSK-α stimulated cell division of certain cell clusters in old EC or LC, which had embryogenic competence but could not grow into somatic embryos without addition of exogenous PSK-α or high cell density culture. The cell division once stimulated, the cell clusters of old EC or LC would allow formation of the same somatic embryos as that of newly established EC. It is well known that extremely rapid cell division occurs during an early stage of somatic embryogenesis (Fujimura and Komamine 1980). Thus exogenous PSK-α and, probably also endogenous PSK might stimulate such specific cell division, and as a consequence, embryogenic cell clusters could form somatic embryos.

The increase in amount of PSK in the CM of EC was observed from the 10th day of culture (Fig. 5). Although production of PSK was still low (less than 0.1 nM) on the 6th day of culture when rapid cell division might occur, it appeared to be enough to grow the cell clusters of EC into somatic embryos. We presumed that the difference of embryogenic competence between fresh EC and old EC or LC is due to a difference in the potential of PSK production and/or sensitivity to PSK. Further investigation will be required to clarify the function of PSK on the somatic embryogenesis.

In summary, we have identified the principle of CM activity stimulating the formation of carrot somatic embryos as an oligopeptide growth factor, PSK, whose production appears to be correlated with somatic embryo growth. These results imply that PSK could improve the frequency of somatic embryo formation in plants when this is difficult to achieve.

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