Review

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Stimulatory and Inhibitory Conditioning Factors that Regulate Cell Proliferation and Morphogenesis in Plant Cell Cultures

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Abstract

Proliferation and morphogenesis in plant cell cultures are influenced by the conditioning factors that are produced by the cultured cells themselves. Phytosulfokines (PSKs), sulfated peptide growth factors, have been identified as conditioning factors that promote cell proliferation in plant cell cultures. Our research has shown that the formation of carrot somatic embryos is regulated by both stimulatory and inhibitory conditioning factors. The stimulatory factor was found to be PSK, which markedly increased the frequency of somatic embryo formation. In contrast, the inhibitory factor was identified as 4-hydroxybenzyl alcohol (4HBA). 4HBA strongly inhibited somatic embryo formation by specifically suppressing the rapid cell division that is characteristic of the early globular stage. These findings suggest that dose-balancing of various plant growth regulators, including PSK and inhibitory factors, like 4HBA, might contribute to cell proliferation and morphogenesis in plant cell cultures.

Introduction

Since the establishment of the first plant cell and tissue culture system, many researchers have attempted to identify various chemical factors that are responsible for cell proliferation and plant morphogenesis. The finding of two key plant growth regulators, auxin and cytokinin, is closely associated with the development of a plant cell culture system. Both auxin and cytokinin have essential roles in the induction of cell division and differentiation in cultured plant cells (Riou-Khamlichi *et al.*, 1999). However, the cell proliferation and morphogenic response is frequently difficult to induce in cultures of several plant species, despite the combination of auxin and cytokinin. Therefore, many researchers have continued their efforts to identify new factors that contribute to cell proliferation and morphogenesis in plant cell and tissue cultures.

The initial density of suspension cells is another important factor in the proliferation of the cultured cells (Street, 1979; Bellincampi *et al.*, 1985). A high density of suspension cells increases cell proliferation, whereas dilution of the cultures (e.g., below approximately 5 x 10^4 cells ml^{-1}) results in markedly decreased mitogenic activity. However, the proliferation of dilute cell suspensions can be induced by the addition of liquid conditioned medium in which cells have been grown rapidly (Bellincampi and Morpurgo, 1987, 1989; Birnberg *et al.*, 1988; Schroder *et al.*, 1989; Hagimori and Nagaoka, 1992; Jorgensen *et al.*, 1992). Alternatively, many workers have used various effective techniques, such as a nurse culture system (Huang *et al.*, 1990; Schaffler and Knoop, 1990; Folling *et al.*, 1995).

Somatic embryo development is an important pathway for the regeneration of plants from cell culture systems and for the investigation of embryogenesis in higher plants (Dudits *et al.*, 1991; Zimmerman, 1993; Goldberg *et al.*, 1994). Carrot somatic embryogenesis is a useful model system for such studies (Steward *et al.*, 1958; Reinert, 1959; Fujimura and Komamine, 1979; Nomura and Komamine, 1985). An important factor in somatic embryogenesis is auxins, especially 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic cells are generated on 2,4-D-containing medium and proliferate as unorganized cell clusters in the presence of 2,4-D (Kamada and Harada, 1984). The transfer of these cell clusters to phytohormone-free medium results in the development of somatic embryos. In addition, cell density has an important effect on the formation of somatic embryogenesis (Sung and

Okimoto, 1981, 1983; Osuga *et al.*, 1993; Higashi *et al.*, 1999). When embryogenic cells are cultured in auxin-free liquid medium at high cell density, the formation of somatic embryos is strongly inhibited.

These effects of cell density on cell and tissue cultures are due to conditioning factors that are secreted by cultured cells and that accumulate in the culture medium. In this review, we describe the recent findings regarding factors that regulate cell proliferation and morphogenesis in plant cell cultures. In addition, we refer to our own research on somatic embryogenesis in carrot.

Conditioning factors that stimulate the proliferation of suspension cells

The proliferation of plant suspension-cultured cells that grow at a low cell density is promoted by supplementation with conditioned culture medium. The stimulatory effect of conditioned medium on cell division is due to substances that are secreted by cultured cells and that accumulate in the culture medium (Bellincampi and Morpurgo, 1987, 1989; Birnberg *et al.*, 1988; Schroder *et al.*, 1989; Hagimori and Nagaoka, 1992; Jorgensen *et al.*, 1992). However, supplementing with a combination of known plant hormones (e.g., auxin and cytokinin) is unable to substitute for the conditioned medium. Therefore, unknown stimulatory conditioning factors are essential for the induction of cell proliferation. Despite considarable effort by many researchers, characterizing the nature of the conditioning factors and determining their complete chemical structures remain important goals. The main obstacle in the purification and isolation of the conditioning factors is the lack of a sufficiently sensitive bioassay method (Matsubayashi and Sakagami, 1996).

To characterize conditioning factors, Matsubayashi and Sakagami (1996) established a highly sensitive bioassay system by using mechanically dispersed mesophyll cells prepared from *Asparagus officinalis* L. By using this bioassay system, two conditioning factors were purified and isolated from conditioned medium of *Asparagus* suspension cultures. These conditioning factors, designated phytosulfokines (PSKs), are peptidyl growth factors that were first chemically characterized in higher plants. Their structures were determined to be the disulfated pentapeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (PSK-α) and its carboxy-terminally truncated tetrapeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH (PSK-β). The minimum molecule required for the mitogenic activity of PSK is a sulfated tripeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-OH, and the amino-terminal Tyr(SO_3H) residue has an essential role in this activity (Matsubayashi *et al.*, 1996). At a minimum concentration of 1.0 x 10^{-9} M, PSK-α can induce the cell division of *Asparagus* mesophyll cells, and the ED_{50} of this peptide is 3.8 x 10^{-9} M.

Interspecific stimulatory effects of conditioned medium on cell proliferation have often been observed even between very distinct species (Bellincampi and Morpurgo, 1987; Somers *et al.*, 1987). This observation suggests that identical or similar molecules act as conditioning factors in cell suspension cultures of various plants. PSKs exist throughout the plant kingdom (Yang *et al.*, 2000b). PSK-α and its truncated peptides were found in the conditioned medium of several plant families, both monocotyledons and dicotyledons, including carrot (Matsubayashi and Sakagami, 1996, 1999; Hanai *et al.*, 2000). The amino-acid sequences of PSKs are completely conserved among these plants (Yang *et al.*, 2000b). In addition, PSKs stimulate the proliferation of suspension-cultured cells and protoplasts of these plants. These results suggest that PSKs may be conditioning factors common to all plant cell cultures.

Conditioning effect on somatic embryo formation

The formation of somatic embryos from carrot cell suspension cultures is strongly inhibited at high density of embryogenic cell clusters (**Fig. 1**). The inhibition of somatic embryogenesis in high-cell-density cultures is caused by some chemical factors that are released from cells and accumulate in the culture medium cells (Osuga *et al.*, 1993; Higashi *et al.*, 1999). The addition of activated charcoal into the culture medium improved the formation of somatic embryos (Fridborg and Eriksson, 1975). This recovery of somatic embryo formation is due to the absorption of the inhibitory substances from the cultures. The accumulation of the inhibitory factors in the culture medium was corroborated by an experiment using conditioned medium prepared from the high-cell-density cultures (Higashi *et al.*, 1999). The inhibition was not associated with depletion of nutrients or with the bringing of 2,4-D into the fresh medium by embryogenic cells themselves. When somatic embryogenesis was induced at a low cell density (many somatic embryos formed under these conditions), the formation of somatic embryos was strongly inhibited by the addition of conditioned medium from high-cell-density cultures.

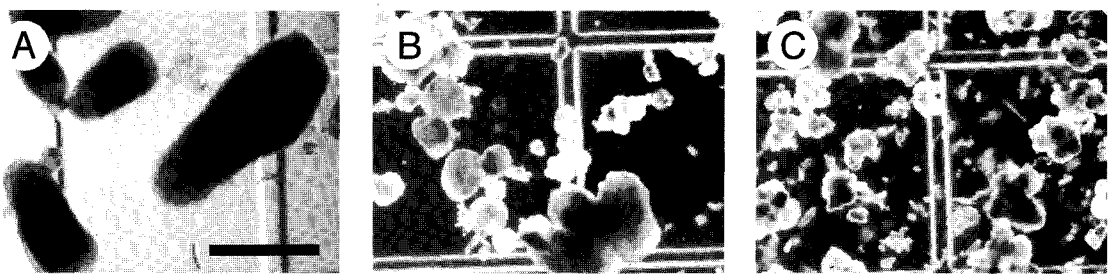

Fig. 1 Morphology of carrot somatic embryos induced at various initial cell densities in phytohormone-free MS medium and cultured for two weeks. Cell density was defined in term of the packed cell volume (PCV), measured in ml, after centrifugation at 100 g of one litter of culture (m*l* PCV l^{-1}). A: 0.2 m*l* PCV ml^{-1}. B: 1.0 m*l* PCV l^{-1}. C: 5.0 m*l* PCV l^{-1}. Bar in A = 500 μm for A to C.

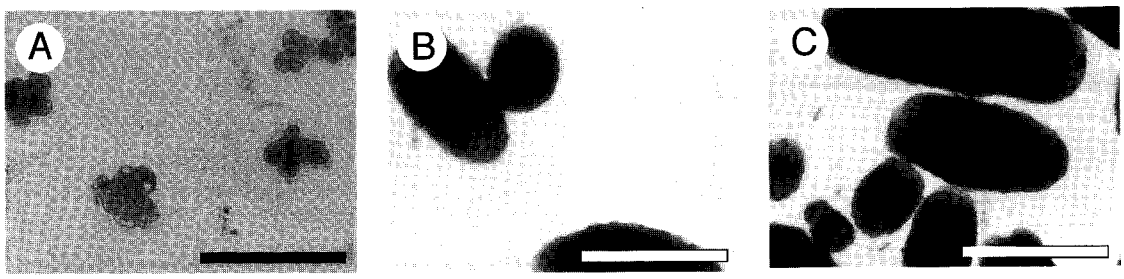

Fig. 2 Morphology of carrot cell cultures that treated with or without PSK-α. A: Embryogenic cell clusters before induction of somatic embryo formation. Bar = 200 μm. B and C: Somatic embryos cultured without (B) or with PSK-α at 10^{-7} M (C) for two weeks. Bar = 500 μm.

Moreover, dialysation analysis of the conditioned medium showed that the inhibitory factors had molecular weights of less than 3500.

Factors that stimulate somatic embryogenesis in carrot have been recognized in conditioned medium (Halperin, 1967; Ammirato and Steward, 1971; Hari, 1980). Carrot somatic embryogenesis is stimulated by the addition of conditioned medium derived from a culture in which somatic embryos have been growing vigorously (Hari, 1980; Kobayashi *et al.*, unpublished result). This result suggests the presence of factors that stimulate somatic embryo formation in the conditioned medium. These stimulatory factors seemed to have relatively high molecular weights, compared with those of inhibitory factors (Higashi *et al.*, 1999). Therefore, somatic embryogenesis in carrot might be affected by the dose-balance of both stimulatory and inhibitory conditioning factors.

Conditioning factors that stimulate somatic embryogenesis

Conditioning factors that stimulate carrot somatic embryogenesis were purified from the culture medium in which a high-competence cell line had vigorously formed somatic embryos (Hanai *et al.*, 2000). The active principles were identified to be PSKs.

PSK stimulated the formation of somatic embryos in carrot (Kobayashi *et al.*, 1999a). The number of somatic embryos increased remarkably with a rise in PSK-α concentrations, whereas the size of somatic embryos treated with PSK-α was almost the same as that of the controls (**Fig. 2**). This observation suggests that PSKs increase the population of cell clusters that develop into somatic embryos, rather than stimulate the development of each somatic embryo. In addition, PSK-α enhanced cell proliferation in cultures of somatic embryos. Because PSKs typically stimulate proliferation of carrot suspension-cultured cells (e.g., embryogenic cells under unorganized growth and non-embryogenic cells that have lost embryogenic competence), PSKs might stimulate somatic embryogenesis by promoting the cell division that enables each cell cluster to initiate somatic embryo development (Hanai *et al.*, 2000).

Quantification studies using a competition enzyme-linked immunosorbent assay system with an anti-PSK-α polyclonal antibody demonstrated that the increase in PSK during somatic embryogenesis contributes to an increase in the cell number rather than to the development of somatic embryos (Hanai *et al.*, 2000). However, the relationship between the progression of somatic embryo development and the endogenous PSK level remains to be clarified.

Hanai *et al.* (2000) presumed that differences in embryogenic competence between embryogenic cell lines were due to differences in the potential for PSK production or of sensitivity to PSK.

Conditioning factors that inhibit somatic embryogenesis

The improvement of carrot somatic embryogenesis by activated charcoal is due to the absorption of inhibitory substances secreted into the culture medium (Fridborg and Eriksson, 1975). Mass spectrometric analysis showed that media without charcoal contained high amounts of phenylacetic acid, benzoic acid, and 4-hydroxybenzoic acid, whereas media with activated charcoal did not (Fridborg *et al.*, 1978). Of these phenolic compounds, 4-hydroxybenzoic acid at a concentration of 10^{-4} M had inhibitory effects on the formation of somatic embryos. Therefore, certain phenolic compounds might accumulate in the culture medium and inhibit somatic embryogenesis in carrot.

We purified and isolated the inhibitory factors that are involved in the suppression of somatic embryogenesis in high-cell-density carrot cultures (Kobayashi *et al.*, 2000a). The inhibitory factors consisted of at least two distinct compounds. The inhibitory factor with the strongest apparent activit was identified as 4-hydroxybenzyl alcohol (4HBA). At a concentration equal to that in high-cell-density cultures (10^{-6} M), synthetic 4HBA strongly inhibited the formation of somatic embryos (Kobayashi *et al.*, 2000b). The inhibitory effect of 4HBA was stronger than that of analogous molecules, such as benzyl alcohol, 2-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, and 4-hydroxybenzaldehyde (**Fig. 3**). It is noteworthy that 4HBA had a much greater inhibitory effect on somatic embryo formation than 4-hydroxybenzoic acid had. This observation suggests that 4HBA is essential for the inhibition of carrot somatic embryogenesis in high-cell-density cultures.

Quantification analysis of 4HBA using high-performance liquid chromatography demonstrated that the concentration of 4HBA in the culture medium was higher in high-cell-density cultures than in low-cell-density cultures (Kobayashi *et al.*, 2001). In high-cell-density cultures, the amount of 4HBA during the early days of culture rapidly increased to a concentration sufficient to inhibit somatic embryo formation. These results indicate that the rapid accumulation of 4HBA during early developmental stage might result in the strong inhibition of somatic embryogenesis in high-cell-density cultures.

Stage-specific inhibition of somatic embryogenesis by 4-hydroxybenzyl alcohol

The inhibition of carrot somatic embryogenesis in high-cell-density cultures has been proposed to be due to suppression of the transition from the globular stage to the heart-shaped stage (Sung and Okimoto, 1981, 1983; Osuga *et al.*, 1993). However, experiments using high-cell-density conditioned medium showed that the inhibition is caused by the suppression of cell division at the early globular stage (Kobayashi *et al.*, 1999b). In addition, the proliferation of both undifferentiated embryogenic cells and non-embryogenic cells was not

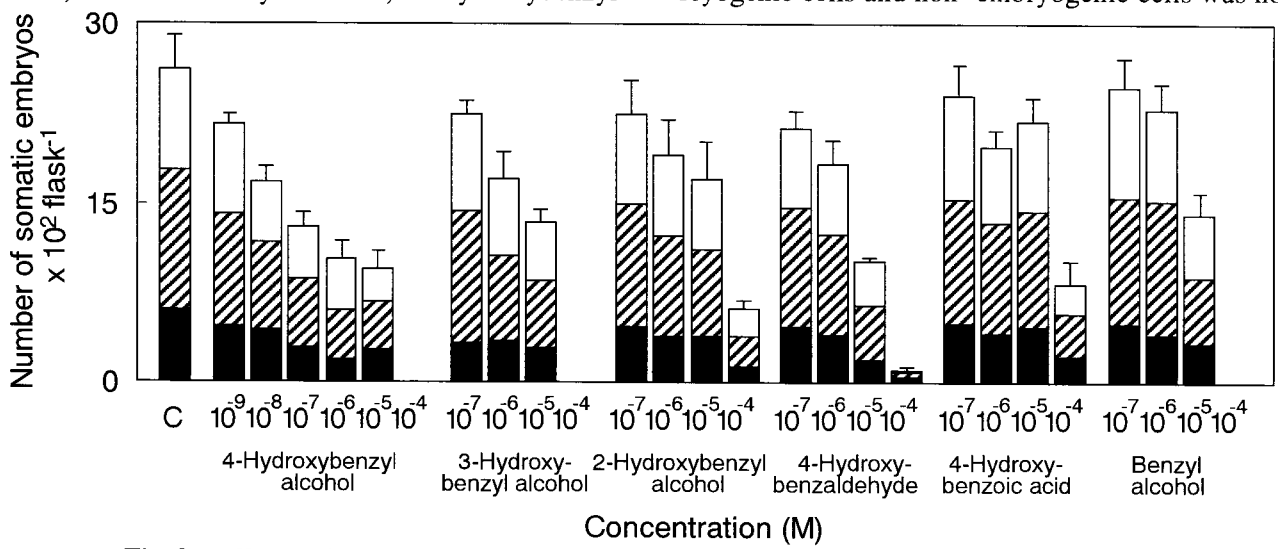

Fig. 3 Effects of 4HBA and its analogs on somatic embryogenesis in carrot. Somatic embryogenesis was induced in the medium contained 4HBA or an analog, and somatic embryos formed were counted after two weeks. Closed boxes, globular embryos; stripped boxes, heart-shaped embryos; open boxes, torpedo-shaped embryos.

inhibited by addition of the conditioned medium. Synthetic 4HBA caused similar inhibitory effects on the formation of somatic embryos (Kobayashi *et al.*, 2000b). Several reports suggested that exceptionally rapid division of cells specifically occurred during the early globular stage (Bayliss, 1975, 1977; Fujimura and Komamine, 1980). These observations suggest that the inhibition of somatic embryogenesis in high-cell-density cultures is caused by the suppression of only the rapid cell division that is specific to the early globular stage of development.

The production of 4HBA is developmentally regulated during somatic embryogenesis (Kobayashi *et al.*, 2001). The globular and heart-shaped somatic embryos produced higher amounts of 4HBA than did torpedo-shaped somatic embryos. Simple phenolic compounds, including 4HBA, originate from phenylpropanoid synthesis (Dixon and Paiva, 1995). Sugano *et al.* (1975) demonstrated that the activity of phenylalanine ammonia lyase, which catalyzes a rate-limiting step of this pathway, increased during early stage of somatic embryogenesis.

This rapid cell division during the early globular stage plays an important role in the differentiation and pattern formation of somatic embryos in carrot (Fujimura and Komamine, 1980). The duration of the cell cycle in cultures undergoing somatic embryo development is approximately half of that in cultures of undifferentiated cells (Bayliss, 1977). This rapid division of cell might be due to the changes of chromatin structures. Chromatin after the induction of somatic embryo formation showed higher template activity, DNA and RNA synthesis with chromatic as the template, than that of undifferentiated embryogenic cells (Matsumoto *et al.*, 1975). The histone and chromosomal non-histone proteins that are associated with chromatin appeared to contribute to the regulation of DNA duplication and transcription. 4HBA is produced in quantity during the early stage of somatic embryogenesis, and its accumulation results in a strong suppression of development at the early globular stage (Kobayashi *et al.*, 2000b, 2001). These observations suggest that 4HBA plays a physiological role in the regulation of somatic embryogenesis.

Perspectives

PSK stimulates cell proliferation in suspension cultures. Matsubayashi *et al.* (1999) suggested that the simultaneous presence of auxin, cytokinin and PSK is essential for the induction and progression of cell division cycle. However, the nature of the interaction between the three remains to be elucidated. *OsPSK* cDNA encoding the PSK-α precursor has been isolated from rice (Yang *et al.*, 1999b, 2000a, c). Further, specific PSK-α-binding proteins were identified in rice suspension-cultured cells (Matsubayashi *et al.*, 1997; Matsubayashi and Sakagami, 1999, 2000). The isolation and characterization of the PSK-α precursor and receptor genes will provide new insight into the mechanism that controls the growth and differentiation of plants.

Carrot somatic embryogenesis is a model system for the morphologic, biochemical, and molecular biological aspects of plant embryogenesis (Zimmerman, 1993). We found that the interaction between PSK and 4HBA is responsible for somatic embryo formation (Kobayashi *et al.*, unpublished result). Therefore, further analysis is needed to elucidate whether the findings are relevant only to the somatic embryo system or whether they are also applicable to zygotic embryogenesis.

Because the promotive effect of activated charcoal on somatic embryo formation was observed in several plant species, various phenolic compounds like 4HBA may be involved in the inhibition of somatic embryogenesis (Johansson, 1983; Kobayashi *et al.*, 1985; Gland *et al.*, 1988; Huang *et al.*, 1990; Folling *et al.*, 1995). However, the presence of 4HBA in these plants and its effect on somatic embryogenesis remains to be accessed. Further, because PSK-α has stimulatory effects on adventitious bud and root formation as well as on cell proliferation and somatic embryogenesis (Yamakawa *et al.*, 1998; Yang *et al.*, 1999a), PSK might stimulate plant regeneration from cell cultures.

Cell proliferation and morphogenesis in plant cell cultures are affected by the dose-balance between various plant growth regulators (e.g., auxin, cytokinin, and PSK) and inhibitory factors (e.g., 4HBA). The cell proliferation and morphogenic response is frequently difficult to induce in cultures of several plant species. The availability of stimulatory and inhibitory conditioning factors might facilitate efficient establishment of plant cell culture and plant regeneration systems.

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References

Ammirato, P. V., Steward, F. C., 1971. Some effects on environment on the development of embryos from cultures free cells. Bot. Gaz., **132**: 149-158.

Bayliss, M. W., 1975. The duration of the cell cycle of *Daucus carota* L. *in vivo* and *in vitro*. Exp. Cell Res., **92**: 31-38.

Bayliss, M. W., 1977. The effects of 2,4-D on growth and mitosis in suspension cultures of *Daucus carota*. Plant Sci. Lett., **8**: 99-103.

Bellincampi, D., Baduri, N., Morpurgo, G., 1985. High plating efficiency with plant cell cultures. Plant Cell Rep., **4**: 155-157.

Bellincampi, D., Morpurgo, G., 1987. Conditioning factor affecting growth in plant cells in culture. Plant Sci., **51**: 83-91.

Bellincampi, D., Morpurgo, G., 1989. Evidence for the presence of a second conditioning factor in plant cell cultures. Plant Sci., **65**: 125-130.

Birnberg, P. R., Somers, D. A., Brenner, M. L., 1988. Characterization of conditioning factors that increase colony formation from black Mexican sweet corn protoplasts. J. Plant Physiol., **132**: 316-321.

Dudits, D., Bogre, L., Gyorgyey, J., 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. J. Cell Sci., **99**: 475-484.

Dixon, R. A., Paiva, N. L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell, **7**: 1085-1097.

Folling, M., Madsen, S., Olesen, A., 1995. Effect of nurse culture and conditioned medium on colony formation and plant regeneration from *Lolium perenne* protoplasts. Plant Sci., **108**: 229-239.

Fridborg, G., Eriksson, T., 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant., **34**: 306-308.

Fridborg, G., Pedersen, M., Landstrom, L.-E., Eriksson, T., 1978. The effect of activated charcoal on tissue cultures: Adsorption of metabolites inhibiting morphogenesis. Physiol. Plant., **43**: 104-106.

Fujimura, T., Komamine, A., 1979. Synchronization of somatic embryogenesis in carrot cell suspension culture. Plant Physiol., **64**: 1162-1167.

Fujimura, T., Komamine, A., 1980. The serial observation of embryogenesis in a carrot cell suspension culture. New Phytol., **86**: 213-218.

Gland, A., Lichter, R., Schweiger, H.-G., 1988. Genetic and exogenous factors affecting embryogenesis in isolated microspore cultures of *Brassica napus* L. J. Plant Physiol., **132**: 613-617.

Hagimori, M., Nagaoka, M., 1992. Nurse culture of Japanese radish (*Raphanus sativus* L.) mesophyll protoplasts. Plant Sci., **86**: 105-113.

Halperin, W., 1967. Population density effects on embryogenesis in carrot-cell cultures. Exp. Cell Res., **48**: 170-173.

Hanai, H., Matsuno, T., Yamamoto, M., Matsubayashi, Y., Kobayashi, T., Kamada, H., Sakagami, Y., 2000. A secreted peptide growth factor, phytosulfokine, acting as a stimulatory factor of carrot somatic embryo formation. Plant Cell Physiol., **41**: 27-32.

Hari, V., 1980. Effect of cell density changes and conditioned media on carrot cell embryogenesis. Z. Pflanzenphysiol., **96**: 227-231.

Higashi, K., Daita, M., Kobayashi, T., Sasaki, K., Harada, H., Kamada, H., 1999. Inhibitory conditioning for carrot somatic embryogenesis in high-cell-density cultures. Plant Cell Rep., **18**: 2-6.

Huang, B., Bird, S., Kemble, R., Simmonds, D., Keller, W., Miki, B., 1990. Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. Plant Cell Rep., **8**: 594-597.

Johansson, L., 1983. Effects of activated charcoal in anthor cultures. Physiol. Plant., **59**: 397-403.

Jorgensen, R. B., Andersen, B., Andersen, J. M., 1992. Effects and characterization of the conditioning medium that increase colony formation from barley (*Hordeum vulgare* L.) protoplasts. J. Plant Physiol., **140**: 328-333.

Kamada, H., Harada, H., 1984. Studies on nitrogen metabolism during somatic embryogenesis in carrot. I. Utilization of alanin as a nitrogen source. Plant Sci. Lett., **33**: 7-13.

Kobayashi, S., Ikeda, I., Uchimiya, H., 1985. Conditions for high frequency embryogenesis from orange (*Citrus sinensis* Osb.) protoplasts. Plant Cell Tiss. Org. Cult., **4**: 249-259.

Kobayashi, T., Eun, C.-H., Hanai, H., Matsubayashi, Y., Sakagami, Y., Kamada, H., 1999a. Phytosulfokine-α, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. J. Exp. Bot., **50**: 1123-1128.

Kobayashi, T., Higashi, K., Kamada, H., 2000b. Inhibitory effects of *p*-hydroxybenzyl alcohol on somatic embryogenesis in carrot cell cultures. Plant Biotech., **17**: 87-92.

Kobayashi, T., Higashi, K., Kamada, H., 2001. 4-Hydroxybenzyl alcohol accumulates in suspension-cell cultures and inhibits somatic embryogenesis in carrot. Physiol. Plant., in press.

Kobayashi, T., Higashi, K., Saitou, T., Kamada, H., 1999b. Physiological properties of inhibitory conditioning factor(s), inhibitory to somatic embryogenesis, in high-cell-density cell cultures of carrot. Plant Sci., **144**: 69-75.

Kobayashi, T., Higashi, K., Sasaki, K., Asami, T., Yoshida, S., Kamada, H., 2000a. Purification from conditioned medium and chemical identification of a factor that inhibits somatic embryogenesis in carrot. Plant Cell Physiol., **41**: 268-273.

Matsubayashi, Y., Hanai, H., Hara, O., Sakagami, Y., 1996. Active fragments and analogs of the plant growth factor, phytosulfokine: structure-activity relationships. Biochem. Biophys. Res. Comm., **255**: 209-214.

Matsubayashi, Y., Morita, A., Matsunaga, E., Furuya, A.,

Hanai, H., Sakagami, Y., 1999. Physiological relationships between auxin, cytokinin, and a peptide growth factor, phytosulfokine-α, in stimulation of asparagus cell proliferation. Planta, **207**: 559-565.

Matsubayashi, Y., Sakagami, Y., 1996. Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. Proc. Natl. Acad. Sci. USA, **96**: 7623-7627.

Matsubayashi, Y., Sakagami, Y., 1999. Characterization of specific binding sites for an endogenous sulfated pentapeptide, phytosulfokine-α, in the plasma membrane fraction derived from *Oryza sativa* L. Eur. J. Biochem., **262**: 666-671.

Matsubayashi, Y., Sakagami, Y., 2000. 120- and 160-kDa receptors for endogenous mitogenic peptide, phytosulfokine-α, in rice plasma membrane. J. Biol. Chem., **275**: 15520-15525.

Matsubayashi, Y., Takagi, L., Sakagami, Y., 1997. Phytosulfokine-α, a sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high- and low-affinity binding sites. Proc. Natl. Acad. Sci. USA, **94**: 13357-13362.

Matsumoto, H., Gregor, D., Reinert, J., 1975. Changes in chromatin of *Daucus carota* cells during embryogenesis. Phytochemistry, **14**: 41-47.

Nomura, K., Komamine, A., 1985. Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol., **79**: 988-991.

Osuga, K., Kamada, H., Komamine, A., 1993. Cell density is an important factor for synchronization of the late stage of somatic embryogenesis at high frequency. Plant Tiss. Cult. Lett., **10**: 180-183.

Reinert, J. 1959. Uber die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebenkulturen aus Karotten. Planta, **53**: 318-333.

Riou-Khamlichi, C., Huntley, R., Jacquard, A., Murray J. A. H., 1999. Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science, **283**: 1541-1544.

Schaffler, E., Knoop, H.-U., 1990. Single cell nurse culture of tobacco protoplasts: Physiological analysis of conditioning factors. J. Plant Physiol., **137**: 95-101.

Schroder, R., Gartner, F., Steinbrenner, B., Knoop, B., Beiderbeck, R., 1989. Viability factors in plant suspension cultures: some properties. J. Plant Physiol., **135**: 422-427.

Somers, D. A., Birnberg, P. R., Petersen, W. L., Brenner, M. L., 1987. The effect of conditioned medium on colony formation from black Mexican sweet Corn protoplasts. Plant Sci., **53**: 249-256.

Steward, F. C., Mapes, M. O., Mears, K., 1958. Growth and organized development of cultured cells. I Growth and division of freely suspended cells. Am. J. Bot., **45**: 693-703.

Street, H. E., 1979. Plant tissue and cell culture. Blackwell Scientific Publications, Oxford.

Sugano, N., Iwata, R., Nishi, A., 1975. Formation of phenolic acid in carrot cells in suspension cultures. Phytochemistry, **14**: 1205-1207.

Sung, Z. R., Okimoto, R., 1981. Embryonic proteins in somatic embryos of carrot. Proc. Natl. Acad. Sci. USA, **78**: 3683-3687.

Sung, Z. R., Okimoto, R., 1983. Coordinate gene expression during somatic embryogenesis in carrots. Proc. Natl. Acad. Sci. USA, **80**: 2661-2665.

Yamakawa, S., Sakuta, C., Matsubayashi, Y., Sakagami, Y., Kamada, H., Satoh, S., 1998. The promotive effects of a peptidyl plant growth factor, phytosulfokine-α, on the formation of adventitious roots and expression of a gene for a root-specific cystatin in cucumber hypocotyls. J. Plant Res., **111**: 453-458.

Yang, G., Shen, S., Kobayashi, T., Matsubayashi, Y., Sakagami, Y., Kamada, H., 1999a. Stimulatory effects of a novel peptidyl plant growth factor, phytosulfokine-α, on the adventitious bud formation from callus of *Antirrhinum majus*. Plant Biotech., **16**: 231-234.

Yang, H., Matsubayashi, Y., Nakamura, K., Sakagami, Y., 1999b. *Oryza sativa PSK* encodes a precursor of phytosulfokine-α, a sulfated peptide growth factor found in plants. Proc. Natl. Acad. Sci. USA, **96**: 13560-13565.

Yang, H., Matsubayashi, Y., Hanai, H., Nakamura, K., Sakagami, Y., 2000a. Molecular cloning and characterization of *OsPSK*, a gene encoding a precursor for phytosulfokine-α, required for rice cell proliferation. Plant Mol. Biol., **635**: 635-647.

Yang, H., Matsubayashi, Y., Hanai, H., Sakagami, Y., 2000b. Phytosulfokine-α, a peptide growth factor found in higher plants: Its structure, functions, precursor and receptors. Plant Cell Physiol., **41**: 825-830.

Yang, H., Morita, A., Matsubayashi, Y., Nakamura, K., Sakagami, Y., 2000c. A rapid and efficient system of *Agrobacterium* infection-mediated transient gene expression in rice Oc cells and its appication for analysis of the expression and antisense suppression of prepro-phytosulfokine-α, encoded by *OsPSK* gene. Plant Cell Physiol., **41**: 811-816.

Zimmerman, J. L., 1993. Somatic embryogenesis: A model for early development in higher plants. Plant Cell, **5**: 1411-1423.