Introduction

In animal, cell-cell interaction through signal molecules is indispensable for coordinating cell growth and differentiation in various organs. Extensive research in animal systems has revealed that intercellular communications are mainly mediated by chemical signals such as steroids, peptides and other small bioactive compounds. Among these signals, peptides are the most commonly used signal molecules. This is most likely to be a result of the features of peptide as follows; 1) the ease with which structural variation can be easily introduced; 2) availability of a universal secretion mechanism; 3) the activity of peptide signaling molecules can be controlled by common processing and modification mechanisms. However, in plant, major known phytohormones contributing for cell growth and differentiation are auxin, gibberellins, cytokinins, abscisic acid, ethylene, brassinosteroids and jasmonates, and none of them is peptidal.

The proliferation of plant cells in suspension culture is strictly dependent on the initial density of cells, and mitotic activity in low-density suspension cell cultures cannot be stimulated by supplementation of known phytohormones or defined nutrient. However, proliferation of plant cells at low density is induced by the addition of conditioned medium (CM) prepared from rapidly growing cells in culture. A possible explanation for this phenomenon is the secretion of a mitogenic factor(s) produced by the individual cells into CM [Stuart and Street, 1969; Somers et al., 1987; Birnberg et al., 1988; Bellincampi and Morpurgo, 1987, 1989; Huang et al.,

1990]. Such compounds are often referred to as conditioned medium factors (CMFs).

Since the late 1980s, various efforts have been made to detect and characterize such factors in different culture systems. According to the efforts, highly hydrophilic and neutral compounds in CM of maize cell line [Somers et al., 1987; Birnberg et al., 1988], highly hydrophilic and relatively heat-stable compounds in CM of carrot cell line [Bellincampi and Morpurgo, 1987], and pronase-resistant and relatively small compounds in CM of *Pinus radiata* cell line [Bellincampi and Morpurgo, 1989] have been reported. However, no factors have been isolated or identified, because there is no available assay method with high sensitivity, which can be done for a relatively short time [Bellincampi and Morpurgo, 1987; Birnberg et al., 1988; Schaffler and Koop, 1990].

Recently, Matsubayashi et al. (1996) established a sensitive bioassay system in which asparagus mesophyll cells at a low density were used to detect the mitogenic factor(s) released from the cultured cells at a high density. Using this bioassay system, two mitogenic factors were successfully identified from the CM prepared from asparagus mesophyll cell cultures and determined their structures to be a sulfated pentapeptide PSK-α [H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH] and its C-terminal-truncated tetrapeptide PSK-β [H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH]. The corresponding synthetic peptide had the same effect as the natural factor on

the proliferation of asparagus cells. PSK-α is the most active compound although its amount in CM is less than 20% of that of PSK-β. Recently, PSK-β was shown to be an enzymatic derivative of PSK-α [Yang et al., 1999b]. PSK-α strongly stimulates proliferation of plant cells in low-density cultures, the minimum concentration exhibiting mitogenic activity being 1.0×10^{-9} M. Although PSK-β contributes the major form in CM, it exhibits activity at a higher concentration of 1.0×10^{-8} M [Matsubayashi and Sakagami, 1996].

Truncated analogs of PSK-α without the first and second COOH-terminal amino acids retained 8 and 20% of the activity of the parent pentapeptide, respectively. In contrast, the NH_2-terminal truncated analog and an unsulfated analog exhibited little or no activity, suggesting that the NH_2-terminal tripeptide fragment is the active core [Yamakawa et al., 1998a]. Substitution of isoleucine with valine ([Val2] PSK-α), both hydrophobic, resulted in a 20-fold decrease in the ED_{50} [Matsubayashi and Sakagami, 1996, 1998]. A similar decrease in activity was observed on replacing threonine with serine ([Ser4] PSK-α), indicating that these two amino acid residues do not simply serve as spacers and are involved in the mitogenic activity of PSK-α [Yamakawa et al., 1998a].

PSK-α at a concentration above 1.0×10^{-9} M induced proliferation of the asparagus mesophyll cells cultured at a density of 4.0×10^{4} cells/ml [Matsubayashi and Sakagami, 1996]. The percentage of divided cells,

however, fell steeply below the initial cell density of 1.0 x 10^4 cell/ml in proportion to a decrease in cell density, even if a sufficient amount of PSK-α was added in the culture medium (unpublished data) (personal communication to Dr. Sakagami). One possibility is that the medium successfully used for cell cultures at a density of 4.0 x 10^4 cells/ml is not optimal for cell cultures at an extremely low density. These factors are distinguishable from known plant growth regulators and are distributed in several plant families [Matsubayashi et al., 1997, 1999b]. PSK- α was also identified in the CM of rice Oc culture cells [Matsubayashi et al., 1997] and was found to strongly stimulate proliferation of plant cells in low density cultures at concentrations as low as 1.0 x 10^{-9} M [Matsubayashi et al., 1996, 1997, 1999b; Hanai et al., 2000]. Besides of asparagus and rice, PSK- α has also been identified in CM derived from maize [Matsubayashi et al., 1997], zinnia [Matsubayashi et al., 1999a], and carrot [Hanai et al., 2000] cell cultures, suggesting the universal existence in plant kingdom. The amino acid sequence of PSK- α is perfectly conserved among different species.

Besides of the stimulatory effect of PSK- α on the cell proliferation, several other biological activities of PSK- α have also been reported. In cucumber, PSK- α promoted the formation of adventitious roots on hypocotyls. The number of emerging adventitious roots increased 2- to 2.5-fold upon treatment with PSK- α and the expression of RCC, a gene for a

root specific cystatin of cucumber, was also induced by PSK- α at the time as adventitious roots were formed [Yamakawa et al., 1998a]. Moreover, PSK-α also stimulated the chlorophyll formation in etiolated cotyledon of cucumber [Yamakawa et al., 1998b]. There was no difference between the PSK-α treatment and water controls on increasing rate of fresh weights of cotyledons, suggesting that PSK-α has the effect of elevating the chlorophyll content in the cells. This is the first report showing that PSK- α has organogenetic activity in differentiated plant organs.

In *Arabidopsis*, PSK-α promoted the growth and chlorophyll content of *Arabidopsis* seedlings under high night-time temperature conditions [Yamakawa et al., 1999]. When the seedlings were exposed to high nigh-time temperature, the fresh weight and chlorophyll content were markedly reduced. However, in the plants supplied with PSK- α the fresh weight and chlorophyll content remained at the same levels as those of non-treated controls which did not exposed to high-night time temperature, suggesting that PSK- α may aid plants in their tolerance against heat stress.

In *Zinnia elegans*, dispersed mesophyll cells could not differentiate into tracheary elements (TEs) at low cell density conditions even if auxin and cytokinin are present in the medium. However, when zinnia cells were cultured at a low density in TE-induction medium in the presence of PSK-α, TE differentiation was strongly stimulated in a dose-dependent manner [Matsubayashi et al., 1999a]. The result suggested that PSK- α is a factor

responsible for TE differentiation of zinnia mesophyll cells.

In *Antirrhinum majus*, PSK- α promoted the formation of adventitious buds induced from hypocotyls-derived callus [Yang et al., 1999a]. In *Nicotiana tobacum* L., PSK- α stimulated pollen germination in the low-density culture, indicating that PSK- α is an important regulator involved in the pollen population effect [Chen et al., 2000].

In carrot, when embryogenic cell clusters were transferred to an embryo-inducing medium, addition of CM derived from somatic embryo culture markedly stimulated somatic embryo formation [Kobayashi et al., 1999]. Quantification studies using a competition ELISA showed that PSK production might be related to growth of cells [Hanai et al., 2000]. Exogenously applied PSK- α also promoted the formation of somatic embryos [Hanai et al., 2000; Kobayashi et al., 1999]. The chemical analogues [2-5]PSK- α and tyrosine sulphate ester [Tyr-SO_3H] had no effect on the somatic embryo formation. Moreover, the proliferation of cells during somatic embryogenesis was also enhanced by PSK- α, suggesting that the stimulatory effect of PSK- α on somatic embryogenesis might be due to the promotion of cell proliferation [Kobayashi et al., 1999].

Recently, *OsPSK* gene encoding a precursor of PSK- α was isolated from monocot rice [Yang et al., 1999b, 2000]. The cDNA was 725 base pairs longs, and the 89-aa product, preprophytosulfokine (PP-PSK), has a 22-aa hydrophobic region that resembles a cleavable leader peptide at its

NH_2 terminus [Yang et al., 1999b]. PP-PSK mRNA was detected at a constitutive level in rice Oc culture cells on RNA blot analysis. The expression pattern of *OsPSK* gene in Oc culture cells corresponds to the accumulation curve of PSK secreted into CM and the proliferation pattern [Yang et al., 1999b]. Furthermore, although PSK- α molecules have never been identified in any intact plants with chemical and immunological methods, RT-PCR analysis demonstrated that *OsPSK* transcripts accumulated in entire seedlings, whereas the PP-PSK mRNA was most abundant in fragments containing shoot or root apexes where cells proliferate vigorously, indicating that PSK-α molecules may be also produced in intact plants, and that PSK-α may be important for cell proliferation not only *in vitro* but also *in* vivo [Yang et al., 1999b].

Also, in *Arabidopsis*, four genes that encode precursor of PSK-α have been identified [Yang et al., 2001]. Two cDNA of them, *AtPSK2* and *AtPSK3*, showed that the predicted precursors have N-terminal signal peptides and only a single PSK-α sequence located close to their carboxyl terminal. Both precursors contained dibasic processing sites flanking PSK, analogous to animal and yeast prohormones. The precursors bear very limited similarity among *Arabidopsis* and rice (*Oryza sativa*), suggesting a new level of diversity among polypeptides that are processed into the same signaling molecule in plants, a scenario not found in animals and yeast [Yang et al., 2001]. The expression of *AtPSK2* and *AtPSK3* was observed

not only in cultured cells but also in intact plants, suggesting that PSK-α may be essential for plant cell proliferation *in vivo* as well as *in vitro* [Yang et al., 2001]. Overexpression of either precursor gene allowed the transgenic calli to grow twice as large as the controls [Yang et al., 2001].

By analogy to animal systems, it can be proposed that the interaction of PSK-α and its receptor results in activation of genes and leads to cell proliferation. Evidence for the existence of high-affinity binding sites for PSK-α in rice plasma membrane was provided by using [^{35}S] PSK-α [Matsubayashi et al., 1997], and, latter, [^{3}H] PSK-α [Matsubayashi and Sakagami, 1999]. The observed binding was saturable and reversible, and localized to the outer surface of the plasma membrane. Recently, a high-affinity binding protein for PSK-α was identified in the plasma membrane of suspension cultured rice cells by photoaffinity labeling and the putative receptor proteins for PSK were 120 kDa and 160 kDa glycosylated membrane proteins, suggesting that PSK-α is involved in plant cell proliferation through a novel signal transduction pathway with similarities to those of mammalian peptide hormones and growth factors [Matsubayashi and Sakagami, 2000]. Matsubayashi et al. (2002) also purified a 120-kDa membrane protein, specifically interacting with PSK-α, from carrot microsomal fractions. The corresponding complementary DNA encodes a 1021-amino acid receptor kinase that contains extracellular leucine-rich repeats, a single transmembrane domain, and a cytoplasmic

kinase domain. Overexpression of this receptor kinase in carrot cells caused enhanced callus growth in response to PSK-α and a substantial increase in the number of tritium-labeled PSK binding site, suggesting that PSK-α and the receptor kinase act as a ligand-receptor pair.

To date, beside of PSK-α, several peptide signaling molecules also has been identified in plants; such as tomato systemin, ENOD40, CLV3, SCR and tobacco systemin. Tomato systemin is the first identified plant signaling peptide. It is composed of 18 amino acids and is able to confer a systemic wounding response in tomato plants attacked by herbivores [Pearce et al., 1991; McGurl et al., 1992]. It was known that tomato systemin could activate a mitogen-activated protein (MAP) kinase in suspension culture cells and induce an alkalinization of the medium [Scheer and Ryan, 1999]. In tomato plants, tomato systemin is able to induce expression of proteinase-inhibitor protein-encoding genes [Pearce et al., 1991]. Recently, two systemic signal peptides, Tob sysI and Tob sysII, have also been isolated from tobacco leaves [Pearce et al., 2001a]. Both peptides contained 18 amino acids and activate the proteinase-inhibitor genes in a manner similar to that of tomato systemin, but they have no homology to it. This means that structurally different peptides might serve the same functions in different plant species. From tobacco, other peptide signal, RALF (rapid alkalinization factor), was identified in a bioassay involving a suspension culture of tobacco cells [Pearce et al., 2001b].

RALF is a 5-kDa peptide that is located at the C-terminus of a 115-aa long preproprotein. RALF is able to activate MAP kinase and to induce alkalinization of the medium, like systemin [Pearce et al., 2001b]. In contrast to Tob sysI and Tob sysII, RALF did not induce proteinase-inhibitor gene expression in plants [Pearce et al., 2001b] and thus, is not a systemin.

The early nodulin ENOD40 is a plant-encoded peptide expressed in response to Nod factors and is thought to be a classical phytohormone such as auxin and cytokinin in legumes and a non-legume [e.g. Horvath et al., 1993; Fang and Hirsch, 1998; for a review see Cohn et al., 1988]. In mature nodules, the expression of *ENOD40* has been detected in cells surrounding vascular bundles [Kouchi and Hata, 1993; Yang et al., 1993]. In addition, this gene also is expressed at low levels in stem and root cells [Kouchi and Hata, 1993]. Recent work [Kouchi et al., 1999] has revealed that ENOD40 is therefore widespread in plant kingdom, suggesting that it may have a general biological function.

Genetic studies showed that *CLAVATA1 (CLV1)* and *CLAVATA3 (CLV3)* genes of *Arabidopsis* are both required for correct organization of the shoot apical meristem. The *CLV3* gene encodes a 96 amino acid polypeptide, with a predicted signal peptide at the amino terminus and is expressed in the stem cells and the shoot and floral meristems [Fletcher et al., 1999; Brand et al., 2000]. It has been proposed that the CLV3 protein is

secreted from the outermost meristem cell layers and interacts with the CLV1 for making the CLV1-CLV3 receptor complex in deeper cell layers to restrict the size of the stem cell population. Recently, a large family of genes that share homology with *CLV3* has been found by database searches in several plants [Cock and McCormick, 2001]. Most of the predicted polypeptides have N-terminal signal sequences and thus seem to be secreted peptides. Because CLV1 is a member of the LRR receptor kinase family, some of CLV3 homologs are expected to be ligands for orphan LRR receptors [Clark et al., 1997; Trotochaud et al., 2000].

In *Brassica oleracea*, *SCR (SP II)* genes, as receptor kinase ligand genes, encode highly polymorphic peptides (74-77 amino acids) containing the putative signal peptide cleavage site, and were expressed in the tapetum of the anther, a site consistent with sporophytic control of self-incompatibility (SI) [Schopfer et al., 1999; Takayama et al., 2000]. The *SCR (SP II)* gene product is necessary and sufficient for determining pollen self-incompatibility specificity [Schopfer et al., 1999]. Recent study has identified a large family of SCR-like genes in *Arabidopsis* [Vanoosthuyse et al., 2001].

In this thesis, to clarify how PSK-α leads to the stimulation of cell proliferation, using carrot non-embryogenic cells which had lost the ability to form somatic embryos and only occur cell division, I will represent as follows; 1) Effect of PSK-α on cell proliferation in the presence of auxin,

2) Independent effect of PSK-α on the cell proliferation, 3) Effect of auxin on the production of PSK-α, 4) Effect of PSK-α on the promotion of cell cycle progression, and 5) Effect of PSK-α on re-entry into the cell cycle from the quiescent state.