Discussion

Carrot cell culture is classified into three culture systems: somatic embryogenesis, embryogenic cell culture, and non-embryogenic cell culture. Transferring embryogenic cells to phytohormone-free medium induces somatic embryos. Embryogenic cells, which are competent to form somatic embryos, proliferate in and are sustained by culture in medium containing high concentrations of 2,4-D (4.5×10^{-6} M). Non-embryogenic cells, which have lost somatic embryogenesis ability, also multiply in and are maintained by the same culture conditions as embryogenic cells.

Stimulatory effect of PSK- α on the formation of somatic embryos and the proliferation of embryogenic cells

My previous work collaborated with Dr. Kobayashi showed that PSK-α stimulates the formation of somatic embryos and the proliferation of embryogenic cells, suggesting that the promotion of somatic embryogenesis by PSK-α results from stimulation of the rapid cell division that is specific to the early stage of somatic embryogenesis [Kobayashi et al., 1999]. As additional evidence, somatic embryogenesis was stimulated more by PSK-α treatment in the first half than in the latter half of the induction of somatic embryos (my unpublished data).

Effect of PSK-α on the proliferation of non-embryogenic cells in the presence of 2,4-D

PSK-α did not promote non-embryogenic cell proliferation in medium containing high concentrations of 2,4-D, but did stimulate proliferation in the presence of low concentrations of 2,4-D (Fig. 1). However, embryogenic cell proliferation was stimulated by the addition of PSK-α in the presence of high concentrations of 2,4-D [Kobayashi et al., 1999]. Together, these results suggest that the two cell lines have different sensitivities to cell-division activity of PSK-α. The biochemical and molecular biological properties of embryogenic and non-embryogenic cells have been compared [Kiyosue et al., 1990, 1991; Nagata et al., 1993], and it was reported that the two cell types have different endogenous auxin metabolisms [Sasaki et al., 1994; Michalczuk et al., 1992]. This may be a possible reason for the different sensitivities of the two cell lines.

The apparent absence of a stimulatory effect by PSK-α on non-embryogenic cell culture at high concentrations of 2,4-D could be caused by the fully active cell division, because, in fact, the cells produce enough PSKs for division activity (Fig. 3). PSK-α did not stimulate somatic embryogenesis when a newly established cell line was used and almost all cells in the line developed into somatic embryos even in the absence of PSK-α (my unpublished data). The reason remains to be clarified.

PSK- α shows general effect to auxin on the cell proliferation

An important question is whether PSK-α stimulation of cell proliferation is a specific response to 2,4-D or a general response to auxin. As shown in Fig. 2, when cells were cultured in medium containing IAA or NAA, cell division activity was similar for high and low IAA concentrations (the same concentrations as for 2,4-D). However, cell-division activity at lower concentrations of IAA or NAA was reduced. In this starved condition, cell proliferation was significantly stimulated by PSK-α, depending on its concentration. These results strongly indicate that the promotion of cell division by PSK-α is a general effect to auxin, and that PSK-α stimulates cell division under starved conditions.

Auxin participates in PSK-α production

The reason for the difference in the effect of PSK-α at high and low auxin concentrations was unclear. I assumed that auxin may induce PSK-α production. Quantification of PSK-α in CM derived from non-embryogenic cell suspension culture containing high or low auxin concentrations revealed that auxin is required for PSK-α production (Fig. 3). In asparagus mesophyll cell culture, both auxin and cytokinin are prerequisite for PSK-α production [Matsubayashi et al., 1999b]. PSK-α-specific binding on plasma membrane is unaffected by exogenous auxin and cytokinin, suggesting that a novel signal transduction pathway mediated by PSK-α

might be independent of phytohormones [Matsubayashi et al., 1997].

PSK- α requires auxin to stimulate cell proliferation

As shown in Fig. 4, proliferation was not stimulated when cells were treated with PSK-α alone, demonstrating that auxin is essential for PSK-α promotion of cell proliferation. In humans, multiple lines of evidence implicate cross talk between steroid hormone and growth factor as a modulator of endocrine response in breast cancer. Based on studies of human breast cancer both *in vitro* and *in vivo* [Gee et al., 1996; Nicholson and Gee, 1996], it is increasingly evident that estrogens promote the autocrine expression of growth factor signaling pathway components in estrogen-responsive and estrogen-dependent human breast cancer cell lines, notably transforming growth factor α (TGF α) [Bates et al., 1988], insulin-like growth factor-II (IGF-II) [Brunner et al., 1993], growth factor receptors [Bertois et al., 1989; Chrysogelos et al., 1994], and IGF-I receptor [Freiss et al., 1990].

If so, does auxin also require PSK-α for the cell proliferation? In rice, when anti-sense *OsPSK* gene was introduced into the Oc cell, the rate of cell growth was decreased as compared with no transformed Oc cell under the condition of 2,4-D containing medium [Yang et al., 1999b], suggesting that only auxin is insufficient for the cell proliferation.

PSK- α promotes progression of the G1 cell cycle

How PSK- α stimulates the cell proliferation by inducing intracellular signal response? In animal, growth factors are not regulated to be present throughout the life-time of a cell in order to exert their effect, but specifically in terms of the induction of DNA synthesis, growth factors are required for a defined period in G1 after which further progress through the cell cycle becomes independent of the exogenous signal [Pardee, 1974; Rossow et al., 1979]. To clarify the physiological role of PSK- α in the cell cycle, I investigated reciprocal action between PSK- α and cell cycle on the cell proliferation.

From the propyzamide block release experiments, the G1 cell cycle that progresses slowly in low auxin conditions was stimulated by treatment with PSK-α to the levels seen in rapidly cycling cells growing in the presence of high concentrations of auxin (Fig. 5), suggesting that PSK-α is an extracellular signal involved in G1 cell cycle progression. However, cell cycle progression of only a portion of the cells was stimulated by PSK-α. Carrot non-embryogenic cells are difficult to synchronize to the high degree achieved in tobacco BY-2 cell line that is widely used in cell cycle studies [Nagata et al., 1992; Samuels et al., 1998]. However, the proliferation of tobacco BY-2 cell was not affected by PSK-α in any conditions examined (data not shown). Thus, I tried to make a condition for partial synchronization of cell cycle in carrot non-embryogenic-cells using

cell cycle arresting reagents. Under the condition I analyzed the cell cycle progression. This was the best way at the time I had started the work.

The flow cytometry results were supported by RT-PCR analysis of the cell cycle-related genes *DcCycD2, DcH4* and *DcCycB1;1* (Figs. 8, 9 and 10). These genes were more quickly activated in the presence of PSK-α than in medium lacking PSK-α. Also, these expression patterns were identical to the cell cycle expected from the FCM results. However, it is unknown why the expression of *DcCycB1;1* rapidly decreased at 6 h in low 2,4-D medium with PSK-α. In plants, both shoot and root apical meristems are mosaics of quickly and slowly cycling cells. The main difference between these two populations is the length of G1 phase [Lyndon, 1973], suggesting that this phase is the most responsive to signals that change cell-cycle length. The significance of G1 controls in commitment to the cell cycle has been shown in yeast, flies and mammals [Neufeld et al., 1998]. In human cells, D-type cyclins act as growth factor sensors, with their expression depending more on extracellular stimuli than on the position of the cell cycle [Sherr, 1993; Sherr, 1996]. In transgenic tobacco plants, overexpression of *CycD2* produced a reduction in the length of the cell cycle G1 phase and quicker cell cycling [Cockcroft et al., 2000]. It is conceivable that D-type cyclins act as sensors for PSK-α in plants.

PSK-α promotes progression of the S-G2 phase outside the G1 phase

I have shown that PSK-α is also involved in the S-G2 phase outside the G1 phase. From aphidicolin-release-propyzamide-addition experiment, the percentage of cells accumulating at the G2/M phase(s) was higher in the presence of PSK-α than in cultures without PSK-α (Fig. 11). In the plant cell cycle, both the G2 and G1 phases respond to extracellular signals such as plant growth regulators (PGRs). As mitotic activators, auxin, cytokinin and GA regulate the G2-M transition by activating the production of CDKs and A- and B-type cyclins [Hilde and Dirk, 2001]. Auxin enhances both *CDKA:1* and mitotic cyclin mRNA levels in roots in conjunction with the induction of cell division [Hemerly et al., 1993; Doerner et al., 1996]. It has also been reported that auxin analogues influence a restriction point in the G2 phase of cell cycles in tobacco LT cells [Koens et al., 1995]. PSK-α may regulate the S-G2-M transition of the cell cycle through a signal transduction pathway, just as the known PGRs do. However, it seems that the activation of G1 cell cycle progression by PSK-α, rather than other cell cycle phases, is the main influence on the stimulation of cell proliferation. The importance of the G1 phase is not necessarily due to its absolute duration, but rather to the fact that it is a period when there is considerable variation between populations of cells multiplying at different rates [Heath, 1993].

PSK-α enhances re-entry into the cell cycle from the quiescent state

When non-embryogenic cells were cultured in phytohormone-free medium, the cells were arrested mainly in the G1 phase, although arrested cells in the G2 phase were also observed. To date, most data show G0 (non-cycling G1) as the resting place for the majority of non-cycling cells in both plants and animals. My data indicate that PSK-α has a role in the re-entry of quiescent cells into the cell cycle. From RT-PCR analysis of cells grown in starvation conditions, cell cycle-related genes (*DcCdc2*, *DcH4* and *DcCycB1;1*) were remarkably activated by addition of PSK- α as compared to cells in medium lacking PSK- α (Figs. 13, 15 and 16). The stimulated expression by PSK- α was similar to that of rapidly dividing cells in auxin-supplemented medium. In mammalian cell cultures, the transcription of D-type cyclin genes was highly growth-factor-dependent [Matsushime et al., 1991; Baldin et al., 1993; Sewing et al., 1993]. Growth-factor-starved cells or quiescent cells show low levels of Cyclin D mRNA, and upon addition of growth factor, they show rapidly increasing expression levels that reach a maximum after 10 h of serum stimulation and 8 h before the initiation of DNA synthesis [Sewing et al., 1993]. In plants, CycD cyclins are regulated in response to extracellular signals known to affect plant cell growth. It is unknown the reason why the expression of *DcCycD2* fluctuated in all cases and decreased strongly at 24 h in low 2,4-D medium with PSK- α (Fig. 14), but the expression of *DcCdc2* and

DcCycB1;1 was affected by PSK- α (Figs. 13, 15 and 16). Cytokinin treatment can cause cells to undergo the G0-to-G1 transition [Nougarede et al., 1996]. Cytokinin also increases the proportion of rapidly cycling cells in meristems by inducing G0 cells to enter the cell cycle [Gonthier et al., 1987]. *Nicotiana plumbaginifolia* suspension-cultured cells arrested in G2 by cytokinin starvation exhibit high levels of inactive Cdc2 phosphorylated at Tyr15 [Zhang et al., 1996]. One immediate consequence of cytokinin-induced entry into mitosis is dephosphorylation of Cdc2. As in tobacco suspension-culture cells, carrot non-embryogenic cells showed low levels of *DcCdc2* mRNA in media with low concentrations of auxin, but transcription of this gene conspicuously increased with supplementation of PSK- α or high levels of auxin (Fig. 13). It is possible to say that PSK- α and auxin may also be involved in the dephosphorylation of *DcCdc2* for re-entry into mitosis, although no direct evidence for this is shown in this study.

Conclusions

In this study, I indicated following results; 1) PSK-α promotes the cell proliferation under the low auxin condition in which mitotic activity decreased, 2) auxin involves in the production of PSK-α, 3) PSK-α requires auxin for the stimulation of cell proliferation, 4) PSK-α promotes the cell proliferation by activating the cell cycle progression, and 5) PSK-α

enhances re-entry into the cell cycle from the quiescent state. Fig 17 shows a schematic model for the promotion of cell proliferation by PSK-α. In brief, auxin involves in the production of PSK-α and the PSK-α stimulates the cell proliferation by activating the cell cycle induced by auxin.

This study is the first investigation on the mechanism of cell proliferation by peptidyl plant growth factor PSK-α. Although further researches are needed to demonstrate the physiological and molecular biological mechanisms of PSK-α, this and the further studies will contribute for understanding more the action of the known plant growth regulators.