Results

Effect of PSK- α on cell proliferation in the presence of 2,4-D

Non-embryogenic cells proliferate and are universally maintained in medium containing a high 2,4-D concentration (4.5 x 10^{-6} M). To investigate the relationship between 2,4-D and PSK-α in cell proliferation, I cultured cells in medium that contained different combinations and concentrations of both 2,4-D and PSK-α. After 14 days of culture, the number of cells in each medium was counted. When cells were cultured in medium that contained a high 2,4-D concentration (4.5 x 10^{-6} M), addition of PSK-α did not stimulate proliferation compared to the control (Fig. 1A). In contrast, when cells were cultured in medium containing a low concentration of 2,4-D (4.5 x 10^{-7} M), cell numbers increased depending on the concentration of PSK-α added (Fig. 1B), reaching the same level as medium containing a high concentration of 2,4-D in cultures with a PSK-α concentration of 1 x 10^{-7} M.

Effect of PSK- α on cell proliferation in the presence of IAA or NAA

To investigate whether the stimulation of cell proliferation by PSK-α is specific to 2,4-D or a general effect of auxin, I tested two other auxins, NAA and IAA. Non-embryogenic cells were cultured in medium containing different combinations and concentrations of IAA or NAA and PSK-α. After a 14-day culture, cells in each medium were counted. Cell proliferation was not notably promoted by PSK-α addition, neither at 4.5 x

10^{-6} M nor 4.5 x 10^{-7} M concentrations of IAA or NAA (Figs. 2A-a, b and 2B-a, b). When non-embryogenic cells were cultured in medium containing even lower concentrations of IAA or NAA (2.3 x 10^{-8} M) and PSK-α, cell proliferation was stimulated, depending on the PSK-α concentration (Figs. 2A-c, 2B-c).

Effect of 2,4-D, IAA and NAA on PSK-α production

Although it is well known that auxin in combination with cytokinin stimulates generally cell division in tissue culture, the addition of auxin to basal medium can be sufficient to initiate and sustain callus growth in carrot. However, there is no information on auxin involvement in PSK-α production. Therefore, I determined the quantity of secreted PSK-α in medium derived from non-embryogenic cell culture with high and low concentrations of auxin (2,4-D, IAA and NAA). CM was collected two days after culture, then concentrated, purified by reverse-phase HPLC, and measured by competition ELISA with anti-PSK-α antibody. The quantified PSK-α contained not only full-length PSK-α, but also its C-truncated tetrapeptide, PSK-β, since PSK-β is the enzymatic degradation product by PSK-α. In culture media containing a high concentration of each of the auxins, 2,4-D, NAA, and IAA, the amounts of secreted PSKs were 1.61 x 10^{-9} M, 1.49 x 10^{-9} M, and 1.99 x 10^{-9} M, respectively. For low concentrations of 2,4-D, NAA and IAA, the amounts were 0.2 x 10^{-9} M,

0.84 x 10^{-9} M and 0.95 x 10^{-9} M, respectively (Fig. 3). This indicates that auxin is involved in PSK production.

Independent effect of PSK-α on the promotion of cell proliferation

I further investigated whether cell proliferation are stimulated by adding PSK-α alone. Cells were precultured in medium without 2,4-D for 14 days to entirely remove any remaining 2,4-D in the cells. The precultured cells were then cultured in phytohormone-free medium containing PSK-α. After 14 days, cell numbers were no higher than in the absence of PSK-α (Fig. 4).

Effect of PSK-α on the progression of G1 cell cycle

In carrot non-embryogenic cell cultures, maximum cell proliferation occurred in high 2,4-D medium without exogenous PSK-α. PSK- α did not show any additional stimulatory effect under these conditions (Fig. 1). Therefore, the effect of PSK- α on the cell cycle of cells growing in low 2,4-D medium was examined using ploidy analysis of the nuclear content by means of flow cytometry. In order to determine whether PSK- α promotes progression of the G1 phase, non-embryogenic cells were synchronized at the M phase using propyzamide treatment. After release from propyzamide blocking, the ploidy distribution of cells in low 2,4-D medium with or without PSK- α (Figs. 5A, 5B) was investigated. In low

2,4-D medium without PSK- α, the number of G1 cells at 60 h after release from M phase arrest decreased, and simultaneously the number of G2/M cells increased (arrows in Figs. 5A and 5D). However, when PSK- α was added to low 2,4-D medium, the decrease in G1 cells and increase in G2/M cells were first observed 36 h after the release from propyzamide blocking (arrows in Figs. 5B and 5E). As a positive control, I also examined nuclei from cells grown in high 2,4-D medium without PSK- α (Figs. 5C and 5F). The results were consistent with those seen in cells from low 2,4-D medium with PSK- α. This indicates that the length of the G1 cell cycle of some non-embryogenic cells is shortened by about 24 h by the addition of PSK- α to cells in low 2,4-D medium, as compared to cells in medium without PSK- α.

Effects of PSK- α on mitogenic activity

To verify that PSK- α stimulates cell proliferation by promotion of cell cycle progression, I examined the mitogenic activity of PSK- α. After non-embryogenic cells were released from propyzamide blocking at the M phase, cells were counted using a hemocytometer (Fig. 6). In low 2,4-D medium, cell numbers were higher at two days after propyzamide release in the presence of PSK- α, as compared to those grown in medium lacking PSK- α. In high 2,4-D medium without PSK- α, mitogenic activity was identical to that in low 2,4-D medium with PSK- α. These results indicate

that PSK- α stimulates mitogenic activity in low 2,4-D medium.

Expression changes in cell cycle-related genes by PSK- α treatment

The above ploidy analysis indicated that progression of the cell cycle in non-embryogenic cells is promoted by PSK- α. To verify these results, I further performed RT-PCR analysis using carrot cell cycle-related genes as marker genes.

First, I isolated a full-length carrot cyclin D cDNA clone, designating it *Dauca;CycD2* (involves in later G1 phase, here *DcCycD2*) (Fig. 7). The nucleotide sequence and deduced amino acid sequence of carrot *DcCycD2* are shown in Fig. 7. This gene contains an open reading frame of 1146 bp encoding a protein of 382 amino acids, with a conserved region of approximately 100 amino acid residues known as the cyclin box, which is involved in cdk binding [Lee and Harlow, 1993; Jeffrey et al., 1995; Renaudin et al., 1998]. An Rb interaction LxCxE motif, previously observed in the cycD cyclins, is present near the N-terminus.

Two other carrot cell cycle-related genes, *DcH4* (involves in S phase) and *DcCycB1;1* (involves in G2/M phase), were used along with *DcCycD2* for RT-PCR. Transcript levels were investigated at various time points after releasing the cells from propyzamide blocking. In low 2,4-D medium, the increase of the transcript level of *DcCycD2* was observed at 30 h after propyzamide release. However, in low 2,4-D medium with addition of

PSK-α or in high 2,4-D medium without PSK-α as positive control was observed at 18 h after propyzamide release (Fig. 8). These increased time roughly corresponded to the later G1 phase when compared to the cell cycle expected from FCM analysis of Fig. 5. Also, in low 2,4-D medium, the increase of the transcript level of *DcH4* and *DcCycB1;1* was higher with addition of PSK- α as compared to samples from medium without PSK- α (Figs. 9 and 10). In the positive control culture of cells in high 2,4-D medium without PSK- α, the level of *DcH4* and *DcCycB1;1* transcripts increased to the same extent as in low 2,4-D medium with PSK- α. These increased time also roughly corresponded to the S or G2/M phase when compared to the cell cycle expected from FCM analysis of Fig. 5.

Effect of PSK-α on the progression of S-G2 cell cycle

I also investigated the effect of PSK- α on the cell cycle outside the G1 phase. Initially, non-embryogenic cells were blocked at the S phase with the addition of aphidicolin. Simultaneously with the release from aphidicolin, propyzamide was added to the synchronized cells to block them at the M phase. Fig. 11 represents the time course of the percentage of the cells accumulated in the G2/M phase(s) after addition of propyzamide. In low 2,4-D medium with PSK- α, the percentage of cells blocked in the G2/M phase(s) eight hours after propyzamide addition was higher than in cells growing in low 2,4-D medium without PSK- α. In high 2,4-D medium

without PSK- α, the percentage was identical to that in low 2,4-D medium with PSK- α. This result shows that PSK- α is involved in cell cycle progression at S-G2 phases other than the G1 phase.

Effect of PSK- α on re-entry into cell cycle from quiescent state

When non-embryogenic cells were cultured in phytohormone-free MS medium, they did not divide. These arrested cells can then be triggered to divide with supplementation of auxin. Nuclear DNA contents were determined for carrot non-embryogenic cells starved of phytohormones in MS medium for 7 days, using flow cytometry (Fig. 12). Populations of 2C and 4C cells were observed in the arrested cells, suggesting that in non-embryogenic cells the arrest phase of the cell cycle is the G1 and G2 phases (called the G0 and G2q phases). To confirm whether PSK- α influences cells to re-enter the cell cycle from the quiescent state, I carried out RT-PCR analysis of cell cycle-related genes using quiescent non-embryogenic cells. During the whole culture periods, transcripts of *DcCdc2* (involves in G1/G2 phase) and *DcCycB1;1* accumulated more strongly with addition of PSK- α than in low 2,4-D medium lacking PSK- α. In high 2,4-D medium as positive control, its transcripts also accumulated more than in low 2,4-D medium without PSK-α (Fig. 13, 16). Then, transcripts of *DcH4* accumulated earlier with addition of PSK- α than in low 2,4-D medium lacking PSK- α, and in high 2,4-D medium without PSK- α the transcripts

were comparable to that in low 2,4-D medium with PSK-α (Fig. 15). However, the transcript level of *DcCycD2* was weak and showed fluctuation (Fig. 14).