Materials and Methods

Plant materials and cell culture

Suspension culture of non-embryogenic cells of *Daucus carota* L. cv. US-Harumakigosun [Satoh et al., 1986], which had lost the ability to form somatic embryos, was maintained at two-week intervals in 100 ml of MS liquid medium [Murashige and Skoog, 1962] containing 4.5 x 10^{-6} M auxin (2,4-D, NAA and IAA, respectively) in 300-ml flasks. The flasks were placed on a gyratory shaker (75 rpm) at 25°C in the dark to allow cell growth.

PSK-α treatment

The PSK-α used for our bioassay experiments was chemically synthesized and sulfated as described previously [Matsubayashi and Sakagami, 1996]. It was dissolved in distilled water and used after sterilization by passage through a 0.45-μm filter (DISMIC 25cs; ADVANTEC, Tokyo). Various concentrations of PSK-α were added to the medium of different cultures at the start of culture.

Bioassay

Non-embryogenic cells were collected from two-week-old suspension culture and washed with excess phytohormone-free MS medium. The cells were cultured at 0.2 ml PCV/l in MS medium containing PSK-α (1 x 10^{-10} to 1 x 10^{-7} M) and auxin (2,4-D, NAA and IAA at 4.5 x

10^{-6} M, 4.5 x 10^{-7} M, 2.3 x 10^{-7} M). To entirely remove any auxin that remained in the cells, non-embryogenic cells were precultured in phytohormone-free MS medium for 14 days. The cells were collected and washed with MS medium five times, and then cultured at 0.2 ml PCV/l in MS medium containing PSK-α (1 x 10^{-10} to 1 x 10^{-7} M). Cell density (ml PCV/l) was defined as the packed cell volume (in ml) after centrifugation of one liter of culture at 100 x*g* for 5 min.

For counting, cultured cells were collected by centrifugation at 100 x *g* for 5 min after 14 days of culture, and precipitated cells were treated with an excess of maceration solution consisting of 10% HNO_3 and 10% CrO_3 for two days. Then the mixtures were centrifuged at 100 x*g* for 5 min and pellets were washed once with distilled water. After centrifugation at 100 x*g* for 5 min, each final cell pellet was suspended in distilled water, diluted to an appropriate cell density, and counted in a hemocytometer (Thoma; Erma, Tokyo).

In all experiments, non-embryogenic cells were cultured in 50-ml flasks containing 15 ml of test medium. All experiments with 3 replications were repeated at least twice and results are shown as mean ± standard deviation (SD).

ELISA sample preparation

Each CM aliquot (6 ml) was concentrated, lyophilized and dissolved

in 1.4 ml of distilled water. The solutions were passaged through a 0.45–μm filter, injected into a reverse-phase HPLC column (TSKgel ODS-80TsQA, TOSOH), and eluted with 10% acetonitrile containing 0.1% ammonium acetate. Fractions eluted at 6 to 8 min (corresponding to the PSK-α retention time) were collected in tubes and lyophilized.

Competitive ELISA system

The competitive ELISA procedure was described previously [Matsubayashi et al., 1999b]. ELISA was carried out on polystyrene 96-well plates (NUNC-Immuno Plate Maxisorp; NUNC Inc., Naperville, Ill., USA). The microtiter wells were coated with 100 μl of antigen (1.0 μg/ml) in PBS (10 nM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 150 mM NaCl) for 1 h at 37°C, washed three times with PBS, and blocked with 100 μl of 0.1% (w/v) bovine serum albumin in PBS for 0.5 h at 37°C. After three washes with PBST (PBS containing 0.1% (w/v) Tween 20), the plate was incubated with antibody and PSK-α standard or samples for 1 h at 37°C. During these periods, there was competition for the antibody between antigen bound to the plate and free PSK-α in the solution. The plates were then washed three times with PBST. Anti-rabbit IgG antibody conjugated with horseradish peroxidase was added at a 1:3000 dilution, and the plates were incubated for 1 h at 37°C. After three washes with PBST, orthophenylenediamine solution (0.4 mg/ml) in 100 mM citrate-NaOH

buffer (pH 5.0) containing 0.01% hydrogen peroxide was added, and the plates were incubated for 20 min at 37°C. The reaction was stopped with 2.5 N sulfuric acid, and optical density was recorded at a wavelength of 490 nm with a plate reader. All samples were assayed in triplicate.

Synchronization of cell cycle

Non-embryogenic cells cultured for 7 days were treated to synchronize their cell cycles. In the propyzamide block-release experiment, the two-step blocking method was used. The suspension culture was first transferred to fresh MS medium supplemented with 4.5×10^{-6} M 2,4-D and 20 mg/ml aphidicolin (Wako Pure Chemical). The cells were incubated for 72 h, washed ten times with 3% (w/v) sucrose, and then incubated for 8 h in 100 ml of fresh MS medium supplemented with 4.5×10^{-6} M 2,4-D. Following the addition of 100 μl of 6 mM propyzamide (Wako Pure Chemical), the cells were incubated for an additional 8 h. They were then washed thoroughly with 3% sucrose and re-suspended in 100 ml of fresh MS medium containing a low concentration of 2,4-D (4.5×10^{-7} M) with or without PSK- α (1×10^{-7} M), or a high concentration of 2,4-D (4.5×10^{-6} M) without PSK-α. Samples were collected at the time points indicated in Figure 5. For the propyzamide addition experiment, cells were blocked in the S phase by treatment with aphidicolin, as described above. Simultaneously with the release from the aphidicolin, propyzamide was

added, which triggered cells to accumulate in the G2/M phase. Samples were collected at the time points indicated in Figure 10.

Flow cytometry (FCM)

For FCM, a Partec PA cytometer (Partec, Münster, Germany), equipped with a mercury lamp and combinations of KG1, BG38, UG1, TK420, and GG435 filters, was used. To isolate nuclei, a portion (3 ml) of the suspension culture was collected after release from cell cycle blocking as described above. The collected cells were washed with 3% sucrose and treated for 30 min with an enzyme mixture: 2% cellulase, 1% pectinase, 0.05% macerozyme, and 0.4 M mannitol. Nuclei were isolated using 0.5 ml Galbraith buffer [Galbraith et al., 1983]. The isolated nuclei solution was filtered through 70-μm and 30-μm nylon mesh, and at least 1.5 ml of a staining solution, composed of 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w:v) PVP, 0.1% (v:v) Triton X-100, and 2.5 mg/l 4',6-diamidino-2-phenylindole (DAPI), pH 7.5, was added to the filtered nuclei solution. After 5-10 min of staining, the stained nuclei were subjected to FCM analysis for determination of the relative nuclear DNA content on a log or linear scale histogram. At least 7,000 nuclei were counted for each sample. Results were analyzed using FlowJo software (Tree Star, Inc.). All experiments were performed in duplicate.

Mitogenic activity

Non-embryogenic cells were prepared as described above for the propyzamide block-release experiment. The initial cell density was adjusted to 0.2 ml PCV/l. The cultured cells were collected on alternate days for four days and treated with an excess of a maceration solution, consisting of 10% HNO_3 and 10% CrO_3, for two days. The mixtures were then centrifuged at 100 xg for 5 min and the pellets were washed once with distilled water. Following centrifugation at 100 xg for 5 min, each pellet of cells was suspended in distilled water at an appropriate cell density. Finally, the cells were counted in a hemocytometer (Thoma; Erma, Tokyo).

In all experiments, non-embryogenic cells were cultured in 50-ml flasks containing 15 ml medium. Each experiment was performed in duplicate and averages of results are shown along with their standard deviations.

Isolation of carrot cyclin D gene

A cDNA library was constructed from RNA of carrot non-embryogenic cells using an RT-PCR High kit (TOYOBO). A degenerate oligonucleotide primer, CD1, designed based on a highly conserved region of plant D-type cyclins, and the oligo$(dT)_{20}$-P7 primer included in the RT-PCR kit were used to amplify carrot D-type cyclin transcripts from reverse-transcribed carrot non-embryogenic cell RNA. The CD1 degenerate primer,

used for 3'-RACE, was as follows: 5'-AARTTYGTITTYGARGCIAARACIA-3'. Amplified PCR fragments were subcloned into pGEM-T vectors (Promega) and subsequently transformed into DH5a using the freeze/thaw method. A fragment that contained a partial carrot cyclin D sequence, containing the C-terminal end of the protein ending with a stop codon, was amplified. This information was used to design gene-specific primers to amplify the rest of the carrot cyclin D sequence from an adaptor-ligated cDNA library constructed from carrot non-embryogenic cell RNA using a Marathon™ cDNA kit (CLONTECH). The gene-specific primer CDC1 (5'-CTCTTGTTACACAAATTGCCACAGC-3') and the AP1 primer (CLONTECH) were used to amplify the 5' end of the carrot cyclin D transcript. Amplified PCR fragments were subcloned into pGEM-T vectors (Promega) and subsequently transformed as described above. Analysis of the nucleotide sequence showed that the fragment contained the initiation codon for the carrot cyclin D protein. The primers CDC5 (5'-CCGGGCAGGTGGAGACACATATAA-3') and CDC3 (5'-TGCTCAAGGCACAAGATTCTCTCTC-3') were used to amplify a full-length carrot cyclin D fragment.

DNA sequencing

All pGEM vectors containing the cDNA inserts of interest were

purified using the QIAGEN Plasmid Purification kit according to the manufacturer's protocol. Sequencing was conducted using the Dye Terminator Primer Sequencing kit (Applied Biosystems) on an ABI 373A DNA sequencer (Applied Biosystems). Homology searches were conducted using the non-redundant combined databases of the National Center of Biotechnology Information (NCBI) using the BLAST network search. DNA and protein alignments were performed using Genetyx software.

RNA extraction and RT-PCR

Carrot non-embryogenic cells were synchronized at the M phase using propyzamide. After release from propyzamide treatment, cells were collected at different time points (0-60 h) in medium with a low 2,4-D concentration (4.5 x 10^{-7} M) with or without PSK-α (1 x 10^{-7} M), and in medium with a high 2,4-D concentration (4.5 x10^{-6} M) without PSK-α. In addition, non-embryogenic cells were starved for seven days in phytohormone-free MS medium. The starved cells were cultured under the conditions described above and collected at different time points (0-48 h).

Total RNAs were extracted from the collected cells using a phenol/SDS and LiCl method as described by Shirzadegan et al. (1991). To eliminate contaminating DNA, RNA samples were treated for 1 h at 37°C with RNase-free DNase (Promega) in the presence of ribonuclease inhibitor (RNasin, Promega). One mg of the total RNA was reverse-transcribed

using 25 pmol oligo(dT)20-P7 and MMLV reverse transcriptase (RNaseH-) (RT-PCR High Kit, TOYOBO) according to the manufacturer's protocol. PCR was carried out on a 1/100 diluted sample of the RT products. PCR reaction conditions were as follows: 94°C, 9 min (94°C for 30 s, 56°C for 30 s, 72°C for 1 min) 30 times; final extension at 72°C for 5 min. The amplification was within the logarithmic range. Primers were designed from *Dauca;CycD2* (here *DcCycD2*) and from other cell cycle-related genes, such as *Dauca;Cdc2* (here *DcCdc2*), *Dauca;CycB1;1* (here *DcCycB1;1*), and *DcH4,* which was previously isolated from carrot (unpublished data), as well as carrot ubiquitin as an internal standard. The sense-strand primers were DcCycD2F (5'-CCGTCCCATTACAGAGAGAT GAAG-3'), DcCdc2F (5'-GGCTACAAGTGTTGTGCATAGCG-3'), DcCycB1;1F (5'-TACAAGATGTACAGAGAGATG-3'), DcH4F (5'-GCGT AGACGACATCCATAGCAGTG-3') and UbiquitinF (5'-AGAACCCTAGC CGACTACAACATCC-3'). The antisense-strand primers were DcCycD2R (5'-GCAGGCTTGGATTCTCCATTTC-3'), DcCdc2R (5'-CATCAACTGG TGTGGAGTAGTGGC-3'), DcCycB1;1R (5'-GATTCTCCAAGTAGTGG C–3'), DcH4F (5'-GGCGGTGTCAAGAGGATTTCAG-3') and UbiquitinR (5'–CACTTTGCCACTCTCATCCACCTT-3'). PCR products were analyzed by Northern hybridization.

Northern blot analysis

RT-PCR products were electrophoresed on 1.5% agarose gels, blotted

onto nylon filters (BIODYNE B) using standard procedures [Shirzadegan et al., 1991] and hybridized to carrot *DcCycD2, DcCdc2, DcCycB1;1*, *DcH4*, and *Ubiquitin* probes. Probes were labeled using a Random Primed DNA Labeling kit and [^{32}P] ATP. Hybridization was performed overnight at 65°C.