

Loosening of a Preprophase Band of Microtubules in Onion (*Allium cepa* L.) Root Tip Cells by Kinase Inhibitors

Akiko Nogami and Yoshinobu Mineyuki*

Department of Biological Science, Faculty of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8526, Japan

ABSTRACT. Effects of kinase inhibitors on the preprophase band of microtubules in onion (*Allium cepa* L.) root tip cells were examined. Bundled microtubules in preprophase bands were dispersed on the cell cortex when onion seedlings were incubated with 2.5–5.0 mM 6-dimethylaminopurine. Fifteen min was enough for the bundled microtubules to disappear. Although many preprophase bands remained when the seedlings were incubated with 60 μ M staurosporin, these preprophase band microtubules were loosened and the width of the band became broad. These results suggest that some kinases are involved in the microtubule bundling in the preprophase band development.

Key words: *Allium cepa* (onion)/6-dimethylaminopurine/microtubules/preprophase band/root meristems/staurosporine

Introduction

The preprophase band (PPB) is a unique band of cortical microtubules (MTs) in higher plants that appears in premitotic cells. As the PPB is thought to play an essential role in the division site determination, to know the molecular mechanism of the MT organization during PPB development is important for the study of plant morphogenesis (15). The PPB first appears in the G2 phase as a broad MT band that is oriented parallel to the future division plane (8, 19). Then, MTs in the broad PPB gradually gather in G2 and prophase to form a narrow and bundled MT band that marks the ultimate cortical site where cell plate will fuse at the end of cytokinesis. The mature PPB disappears when the cell enters prometaphase (29–31). Although several molecules are known to be in the PPB, little is known of the role of these molecules (15).

Cyclin-dependent kinases (cdks) are kinases that bind to cyclins and regulate the progression of the eukaryotic cell cycle (21). Cdk homologues have been shown to be in the PPB by immunofluorescent micros-

copy (3, 4, 11, 14, 16, 20). The MPF, the complex of cdc2 (=cdk1) and cyclin B, is thought to be involved in the degradation of PPB MTs, because PPBs are rapidly broken down when prophase *Tradescantia* stamen hair cells are co-injected with active MPF and fluorescent tubulin (9). Accumulation of cells with a PPB in a synchronized tobacco suspension culture by a treatment of a kinase inhibitor in prophase (12) and the transient association of cdc2 and cyclin Ib with maize late PPBs just before their break down (14) support this idea. However, there have been several instances that suggest the existence of cdks on the developing PPBs. Although only 10% of prophase PPBs in maize root tips could be detected by an authentic anti-cdc2 antibody (4), the anti-PSTAIR antibody, an antibody that recognizes a large groups of cdks, recognizes most prophase PPBs and some interphase PPBs in onion root tips (20). In maize root tip cells, PPBs detected by the anti-maize cdc2 antibody were late PPBs (14). However, not only late PPBs but also developing PPBs were detectable by the anti-PSTAIR antibody (16) and anti-maize cyclin II also detects the developing PPBs (14).

If cdks that are expected to be in the developing PPBs have some role in the PPB development, there is a possibility that some kinase inhibitors affect MT behavior during PPB development. Our previous study showed that the width of PPB is broad in onion root tips treated with cycloheximide, which presumably inhibits cdk activity by inhibiting cyclin synthesis (24).

* To whom correspondence should be addressed: Department of Biological Science, Faculty of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8526, Japan.

Tel: +81-824-22-7111 (ext. 2836), Fax: +81-824-24-0734

E-mail: mineyuk@ipc.hiroshima-u.ac.jp

Abbreviations: cdk, cyclin-dependent kinase; 6-DMAP, 6-dimethylaminopurine; MT, microtubule; PPB, preprophase band.

This observation favours our idea. However, the inhibitor study reported previously did not pay attention to the width of the PPB (12). In the present study, we have examined the effect of 6-dimethylaminopurine (6-DMAP) and staurosporine on the PPB MT morphology and found that PPB MTs are loosened by these kinase inhibitors.

Materials and Methods

All the procedures of the culture method, inhibitor treatment and MT observation using immunofluorescence microscopy were done according to the methods described elsewhere (17). Shortly, onion (*Allium cepa* L. cv. Highgold Nigou, Sakata Seed Co., Yokohama, Japan) seeds were sown on a filter paper moistened with distilled water in a plastic container (124 mm wide \times 80 mm deep \times 19 mm high; Iuchi-Seieido Co., Osaka, Japan) and grown in a dark box at 25°C. For kinase inhibitor treatments, 4-d-old seedlings were transferred to a small plastic container (87 mm wide \times 57 mm deep \times 19 mm high; Iuchi-Seieido Co.) with 2.5 ml of inhibitor solutions. Staurosporine was purchased from Kyowa Medix Co. Ltd. (Tokyo, Japan) and 6-DMAP was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1 mM staurosporin dissolved in dimethyl sulfoxide or 30 mM 6-DMAP dissolved in distilled water was used as a stock solution, respectively. They were kept at -20°C and were diluted by distilled water just before use.

Two mm-long root tips of 4-d-old seedlings were fixed with 4% paraformaldehyde in PME buffer (50 mM Pipes, 5 mM EGTA, 1 mM MgSO₄·7H₂O, pH 6.8) overnight, washed with the buffer, and treated with a mixture of cellulase solution (20) for 15 min. After being washed with the buffer, root tips were squashed and air dried on the slide glass, then extracted with -20°C methanol for 10 min, washed with phosphate-buffered saline (PBS) for 10 min, and treated with a mouse monoclonal anti- β tubulin antibody (Amersham Japan Co., Tokyo, Japan) for 45 min. After washing with PBS for 15 min, cells were exposed to fluorescein isothiocyanate (FITC)-linked sheep anti-mouse immunoglobulin G (F(ab)₂ fragment, Sigma Chemical Co.) for 45 min, rinsed again with PBS for 10 min and covered with a solution containing 50% glycerol, 50 mM Tris buffer, pH 9.0 and 1 mg/ml *p*-phenylenediamine. Hoechst 33258 (10 mg/l) was included in the mounting medium to identify the nuclear stage. The width of a PPB was determined by an ocular micrometer in a Nikon microscope (\times 2 microscope equipped for epifluorescent illumination, Nikon Co., Tokyo, Japan).

Results

Dispersion of PPB MTs by 6-DMAP

Root tip cells treated with 0.15–5 mM 6-DMAP for 0–4 h were examined by fluorescent microscopy. The population of prophase cells increased remarkably by the 2.5 and 5 mM 6-DMAP treatment for 2 h and 4 h and the highest value of the population of prophase cells was about 1.5 times higher than that of the control root tips. In these conditions, just after cytokinesis some cells had two nuclei (Fig. 1). The population of prophase cells increased gradually until 4 h in the presence of 5 mM 6-DMAP, although the population of other mitotic cells (prometaphase, metaphase, anaphase and telophase) decreased to near 1% within 1 h (Fig. 2).

As prophase cells were accumulated by the treatment of 6-DMAP for 2 h, MT organization in prophase cells was examined in these conditions. In root tips without 6-DMAP, almost all of the prophase cells had a PPB and ca. 60% of them were bundled PPBs, i.e. the most compact configuration of PPBs (Fig. 3A). On the contrary, population of prophase cells with a PPB decreased and the bundled PPBs were not seen in prophase cells of root tips treated with 2.5 or 5 mM 6-DMAP for 2 h (Fig. 4). MT bands were loosened in these conditions, and individual MTs in PPBs were detectable by immunofluorescent microscopy. It was difficult to determine the boundary of PPB MTs in most prophase cells. MTs in some prophase cells dispersed on the cell surface as if they were interphase cortical MTs,

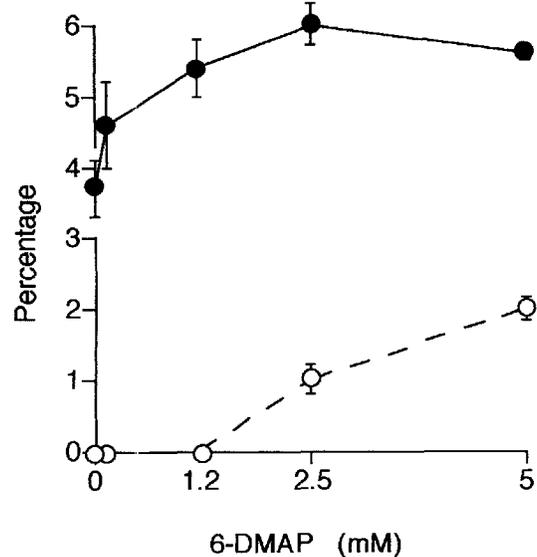


Fig. 1. Effect of 2 h treatments with various concentrations of 6-DMAP on the population of prophase cells (●) and that of cells with bi-nuclei (○) in onion root tip cells. Each point shows the mean \pm S.E.M. obtained from three different samples.

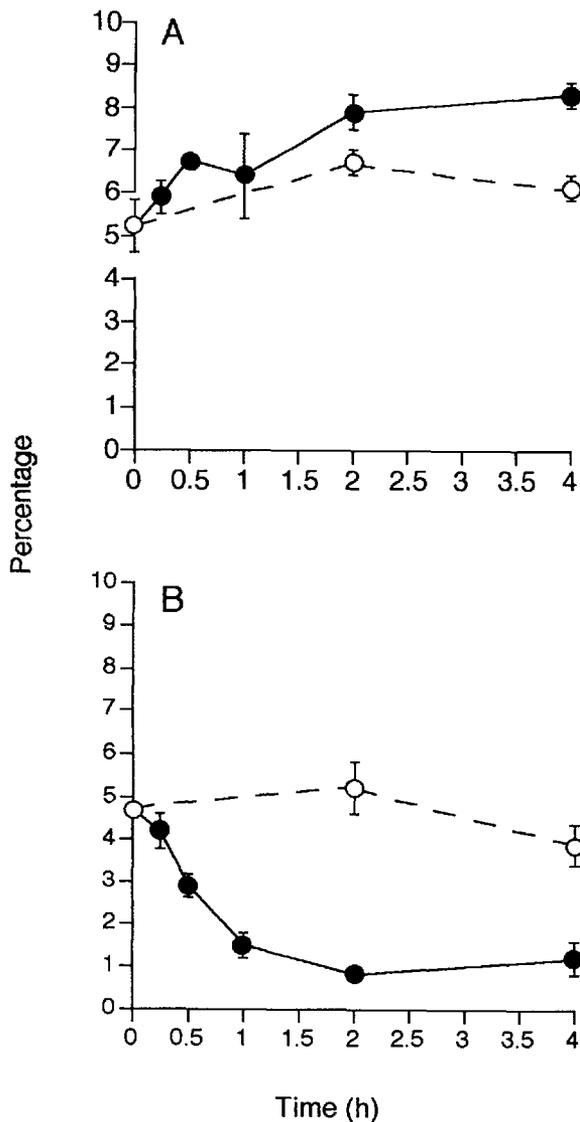


Fig. 2. Effect of duration of treatment without (○) or with (●) solution of 5 mM 6-DMAP on the population of prophase cells (A) and that of other mitotic (prometaphase, metaphase, anaphase and telophase) cells (B) in onion root tip cells. Each point shows the mean ± S.E.M. obtained from three different samples.

even in the late prophase stage and it was difficult to regard this as a PPB (Fig. 3C). Time course study showed that the bundled PPBs disappeared within 15 min of the 5 mM 6-DMAP treatment (Fig. 5).

Broadening of the width of PPB by staurosporin

In order to see whether staurosporin, another general kinase inhibitor, also affects the PPB morphology like 6-DMAP, root tip cells were treated with 2.0–80 μM staurosporin for 2 h. The population of the bundled PPB in prophase cells decreased to less than 20%

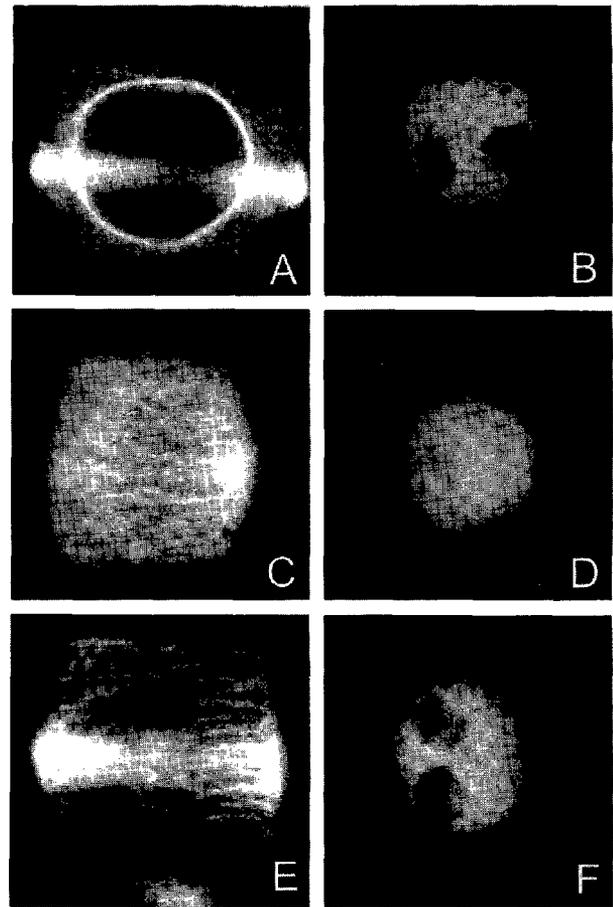


Fig. 3. Tubulin immunofluorescence (A, C and E) and nuclei stained with Hoechst 33258 (B, D and F) in prophase cells of onion root tips. A and B, C and D, E and F are the same cells, respectively. A; A bundled PPB without kinase inhibitor treatment. C; Dispersed cortical MTs treated with 5 mM 6-DMAP for 1 h. E; A broad PPB treated with 60 μM staurosporin for 2 h. × 1,150.

in the presence of 60 or 80 μM staurosporin and ca. 10% of prophase cells had no MT bands (Fig. 6). The morphology of MT bands was different from that induced by 6-DMAP. In staurosporin treated prophase cells, Figure 3C type MT distribution was rare and the majority were Figure 3E type broad MT bands. As we could measure the width of this broad MT band, we examined the effect of staurosporin on the width of MT band (Fig. 7). Results clearly showed that the width of MT bands became broad by the staurosporin treatment. The width of bundled PPBs in the presence of 2 μM staurosporin was already slightly wider than that of the control PPBs and the effect was prominent at the concentration of 60–80 μM.

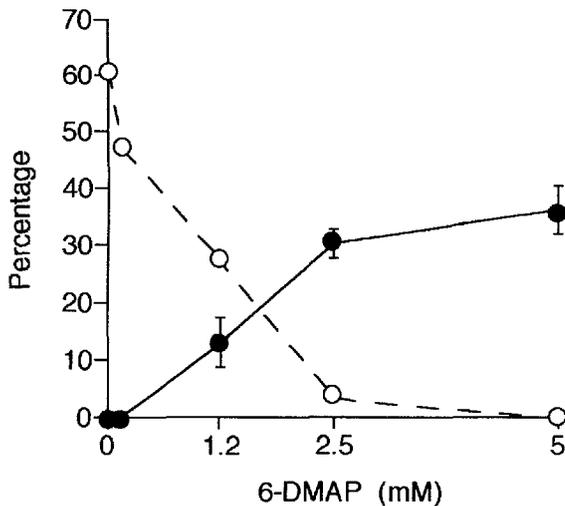


Fig. 4. Effect of 2 h treatments with various concentration of 6-DMAP on the population of cells with a bundled PPB (○) and that of cells without PPBs (●) in prophase cells of onion root tips. Most prophase cells without a PPB had an MT array of Figure 3C type. Each point shows the mean \pm S.E.M. obtained from three different samples.

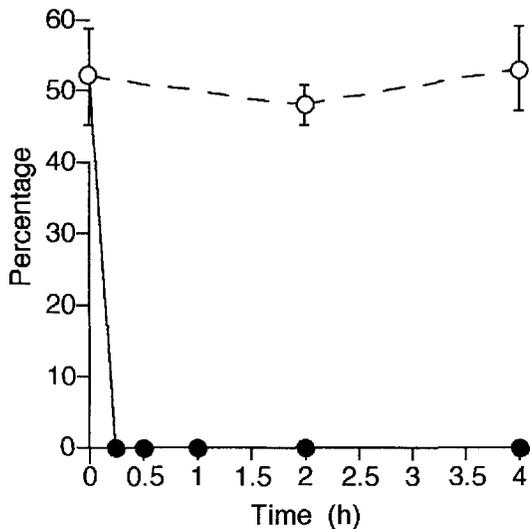


Fig. 5. Effect of duration of treatments without (○) or with (●) solution of 5 mM 6-DMAP on the population of cells with a bundled PPB in prophase cells of onion root tips. Each point shows the mean \pm S.E.M. obtained from three different samples.

Discussion

The present study clearly shows that PPBs could not be kept as a narrow, bundled band in the presence of 6-DMAP or staurosporin. This observation may be the first example of the alteration of PPB MTs by kinase inhibitors. In 6-DMAP treated cells, the bundled MTs

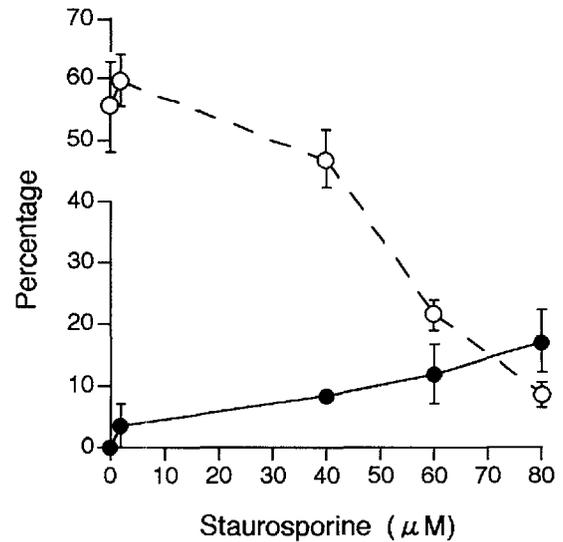


Fig. 6. Effect of 2 h treatments with various concentrations of staurosporine on the population of cells with a bundled PPB (○) and that of cells without PPBs (●) in prophase cells of onion root tips. Each point shows the mean \pm S.E.M. obtained from three different samples.

disappeared and individual MTs were dispersed on the cell cortex (Fig. 3C). The dispersion of MTs on the cell cortex occurs within 15 min of 6-DMAP application. This means that 6-DMAP induces dispersion of bundled narrow PPB MTs. In staurosporine treated cells, many PPBs were broad MT bands (Fig. 3E). The broad PPBs are seen in the early PPBs in control root tips that are going to bundle (15) and are also seen in prophase cells treated with cycloheximide (24) or those treated with cytochalasins (5, 18). The distance between adjacent MTs becomes less as the MT bundling progresses in control cells, but the distance between adjacent MTs cannot be shortened in broad PPBs induced by cycloheximide (24). There is a possibility that the process of this MT bundling is also inhibited by staurosporine. It is too early for us to conclude whether 6-DMAP and staurosporine attack the same site of PPB development or not, but these MT arrays can be explained by the loosening of MT bands. As MTs remain in the cell cortex in the presence of these inhibitors, these inhibitors may not attack the connection between the plasma-membrane and MTs.

6-DMAP and staurosporine are known to inhibit MPF activity and block the cell cycle progression in animal cells (1, 7, 10, 22, 23, 26, 27, 28). Although the concentration of 6-DMAP used in the present study is slightly higher than that used in animal cell cycle studies, the accumulation of prophase cells and the formation of cells with bi-nuclei (Fig. 1) can be explained if 6-DMAP inhibits the transition from pro-

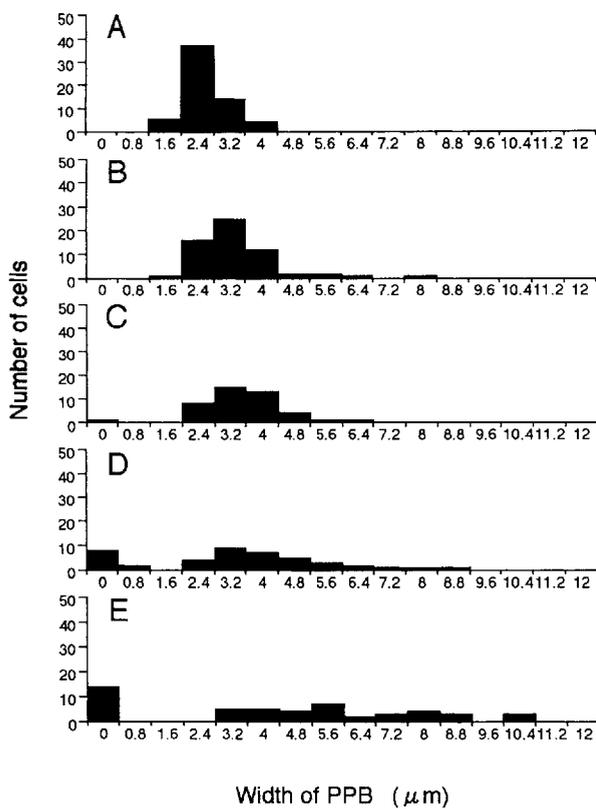


Fig. 7. Width of PPB MTs in prophase cells of onion root tips treated without (A) or with 2.0 μM (B), 40 μM (C), 60 μM (D) and 80 μM (E) staurosporine for 2 h. Width 0 means cells without PPBs. Although many cells in this category had dispersed cortical MTs, cells without any MTs were also included.

phase to prometaphase and some processes of cytokinesis as reported in animal cells (10, 22, 23, 26, 27, 28). Katsuta and Shibaoka (12) reported that 20 μM or higher of staurosporine is necessary to accumulate PPBs in tobacco cells, although 0.2 μM of staurosporine blocks the cell cycle at late G2 in animal cells (1). The effective concentration of staurosporine for the induction of malformed PPBs in the present study is also well above the concentration used to inhibit MPF activity in animal cells (7). The width of broad PPBs induced by the 60 or 80 μM staurosporine is wider than that induced by cycloheximide (24). As the slightly wide PPBs induced by 2 μM staurosporine (Fig. 7B) resembles broad PPBs induced by cycloheximide in terms of the width of MT bands, there is a possibility that kinases attacked by staurosporine are different at low concentrations and at high concentrations in onion root tip cells.

As 6-DMAP and staurosporine inhibit not only cdk but also other kinases, it is difficult to know whether the loosening of the PPB MTs was induced by cdk or other kinases. Recently some cdk specific inhibitors are

known (13) and some of them are shown to block plant cells at G1/S and G2/M transition points (6, 25) or to induce abnormal spindle formation (2). These chemicals may be useful for the further studies on the mechanism of MT bundling during PPB development.

Acknowledgments. We thank Mr. Masaki Shimamura for his technical assistance. This work was supported in part by a research grant from the Ministry of Education, Science and Culture in Japan, the Ito Science Foundation, and the Uragami Foundation to YM.

References

1. ABE, K., YOSHIDA, M., USUI, T., HORINOCHI, S., and BEPPU, T. 1991. Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. *Exp. Cell Res.*, **192**: 122–127.
2. BINAROVÁ, P., DOLEŽEL, J., DRABER, P., HEBERLE-BORS, E., STRNAD, M., and BÖGRE, L. 1998. Treatment of *Vicia faba* root tip cells with specific inhibitors to cyclin-dependent kinases leads to abnormal spindle formation. *Plant J.*, **16**: 697–707.
3. BÖGRE, L., ZWERGER, K., MESKIENE, I., BINAROVÁ, P., CSIZMADIA, V., PLANCK, C., WAGNER, E., HIRT, H., and HEBERLE-BORS, E. 1997. The cdc2Ms kinase is differently regulated in the cytoplasm and in the nucleus. *Plant Physiol.*, **113**: 841–852.
4. COLASANTI, J., CHO, S.-O., WICK, S., and SUNDARESAN, V. 1993. Localization of the functional p34^{cdc2} homolog of maize in root tip and stomatal complex cells: association with predicted division sites. *Plant Cell*, **5**: 1101–1111.
5. ELEFThERIOU, E.P. and PALEVITZ, B.A. 1992. The effect of cytochalasin D on preprophase band organization in root tip cells of *Allium*. *J. Cell Sci.*, **103**: 989–998.
6. GLAB, N., LABIDI, B., QIN, L.-X., TREHIN, C., BERGOUNIX, C., and MEIJER, L. 1994. Olomoucine, an inhibitor of the cdc2/Cdk2 kinases activity, blocks plant cells at G1 to S and G2 to M cell cycle transitions. *FEBS Lett.*, **353**: 207–211.
7. GOTOH, Y., MORIYAMA, K., MATSUDA, S., OKUMURA, E., KISHIMOTO, T., KAWASAKI, H., SUZUKI, K., YAHARA, I., SAKAI, H., and NISHIDA, E. 1991. *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. *EMBO J.*, **10**: 2661–2668.
8. GUNNING, B.E.S. and SAMMUT, M. 1990. Rearrangements of microtubules involved in establishing cell division planes start immediately after DNA synthesis and are completed just before mitosis. *Plant Cell*, **2**: 1273–1282.
9. HUSH, J.M., WU, L., JOHN, P.C.L., HEPLER, L.H., and HEPLER, P.K. 1996. Plant mitosis promoting factor disassembles the microtubule preprophase band and accelerates prophase progression in *Tradescantia*. *Cell Biol. Inter.*, **20**: 275–287.
10. JESSUS, C., RIME, H., HACCARD, O., VAN LINT, J., GORIS, J., MERLEVERDE, W., and OZON, R. 1991. Tyrosine phosphorylation of p34^{cdc2} and p42 during maturation of *Xenopus* oocyte. Antagonistic action of okadaic acid and 6-DMAP. *Development*, **111**: 813–820.
11. JOHN, P.C.L., ZHANG, K., and DONG, C. 1993. A p34^{cdc2}-based cell cycle: its significance in monocotyledonous, dicotyledonous and unicellular plants. In *Molecular and Cell Biology of the Plant Cell Cycle* (J.C. Ormrod, and D. Francis, eds.).

- Kluwer Academic Publishers, Dordrecht, pp.9–34.
12. KATSUTA, J. and SHIBAOKA, H. 1992. Inhibition by kinase inhibitors of the development and the disappearance of the preprophase band of microtubules in tobacco BY-2 cells. *J. Cell Sci.*, **103**: 397–405.
 13. MEIJER, L. 1996. Chemical inhibitors of cyclin-dependent kinases. *Trends Cell Biol.*, **6**: 393–397.
 14. MEWS, M., SEK, F.J., MOORE, R., VOLKMAN, D., GUNNING, B.E.S., and JOHN, P.C.L. 1997. Mitotic cyclin distribution during maize division: implications for the sequence diversity and function of cyclins in plants. *Protoplasma*, **200**: 128–145.
 15. MINEYUKI, Y. 1999. The preprophase band of microtubules: its function as a cytotkinetic apparatus in higher plants. *Inter. Rev. Cytol.*, **187**: 1–49.
 16. MINEYUKI, Y., AIOI, H., YAMASHITA, M., and NAGAHAMA, Y. 1996. A comparative study on stainability of preprophase bands by the PSTAIR antibody. *J. Plant Res.*, **109**: 185–192.
 17. MINEYUKI, Y., IIDA, H., and ANRAKU, Y. 1994. Loss of microtubules in the interphase cells of onion (*Allium cepa* L.) root tips from the cell cortex and their appearance in the cytoplasm after treatment with cycloheximide. *Plant Physiol.*, **104**: 281–284.
 18. MINEYUKI, Y. and PALEVITZ, B.A. 1990. Relationship between preprophase band organization, F-actin and the division site in *Allium*. Fluorescence and morphometric studies on cytochalasin-treated cells. *J. Cell Sci.*, **97**: 283–295.
 19. MINEYUKI, Y., WICK, S.M., and GUNNING, B.E.S. 1988. Preprophase bands of microtubules and the cell cycle: kinetics and experimental uncoupling of their formation from the nuclear cycle in onion root-tip cells. *Planta*, **174**: 518–526.
 20. MINEYUKI, Y., YAMASHITA, M., and NAGAHAMA, Y. 1991. p34^{cdc2} kinase homologue in the preprophase band. *Protoplasma*, **162**: 182–186.
 21. MORGAN, D.O. 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu. Rev. Cell Dev. Biol.*, **13**: 261–291.
 22. NÉANT, I. and GUERRIER, P. 1988. 6-dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. *Exp. Cell Res.*, **176**: 68–79.
 23. NÉANT, I. and GUERRIER, P. 1988. Meiosis reinitiation in the mollusc *Patella vulgata*. Regulation of MPF, CSF and chromosome condensation activity by intracellular pH, protein synthesis and phosphorylation. *Development*, **102**: 505–516.
 24. NOGAMI, A., SUZAKI, T., SHIGENAKA, Y., NAGAHAMA, Y., and MINEYUKI, Y. 1996. Effects of cycloheximide on preprophase bands and prophase spindles in onion (*Allium cepa* L.) root tip cells. *Protoplasma*, **192**: 109–121.
 25. PLANCHAIS, S., GLAB, N., TRÉHIN, C., PERENNES, C., BUREAU, J.-M., MEIJER, L., and BERGOUNIX, C. 1997. Roscovitine, a novel cyclin-dependent kinase inhibitor, characterizes restriction point and G2/M transition in tobacco BY-2 cell suspension. *Plant J.*, **12**: 191–202.
 26. REBHUN, L.I., WHITE, D., SANDER, G., and IVY, N. 1973. Cleavage inhibition in marine eggs by puromycin and 6-dimethylaminopurine. *Exp. Cell Res.*, **77**: 312–318.
 27. RIME, H., HUCHON, D., JESSUS, C., GORIS, J., MERLEVEDE, W., and OZON, R. 1990. Characterization of MPF activation by okadaic acid in *Xenopus* oocyte. *Cell Differ. Dev.*, **29**: 47–58.
 28. RIME, H., NÉANT, I., GUERRIER, P., and OZON, R. 1989. 6-Dimethylaminopurine (6-DMAP), a reversible inhibitor of the transition to metaphase during the first meiotic cell division of the mouse oocyte. *Dev. Biol.*, **133**: 164–179.
 29. WICK, S.M. and DUNIEC, J. 1983. Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. I. Pre-prophase band development and concomitant appearance of nuclear envelope-associated tubulin. *J. Cell Biol.*, **97**: 235–243.
 30. WICK, S.M. and DUNIEC, J. 1984. Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. II. Transition between the pre-prophase band and the mitotic spindle. *Protoplasma*, **122**: 45–55.
 31. WICK, S.M., SEAGULL, R.W., OSBORN, M., WEBER, K., and GUNNING, B.E.S. 1981. Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristematic plant cells. *J. Cell Biol.*, **89**: 685–690.