Int. J. Dev. Biol. 38: 287-299 (1994)

Review

Mechanisms of the proliferation and differentiation of plant cells in cell culture systems

HIROO FUKUDA, MASAKI ITO[1], MUNETAKA SUGIYAMA and ATSUSHI KOMAMINE*

Biological Institute, Faculty of Science, Tohoku University, Sendai, Japan

ABSTRACT Plant cell functions have been investigated in various cell culture systems. In this review, we summarize results obtained from investigations of gene expression during the cell cycle in synchronized cultures of *Catharanthus roseus* during somatic embryogenesis in suspension cultures of *Daucus carota*, during organogenesis in tissue cultures of *Arabidopsis thaliana* and during the transdifferentiation of isolated mesophyll cells to tracheary elements in single-cell cultures of *Zinnia elegans*.

KEY WORDS: *cell cycle, organogenesis, plant cell culture, somatic embryogenesis, transdifferentiation*

Introduction

Plant cell cultures are useful tools for investigations of physiological phenomena such as cell proliferation and differentiation in plants. Experimental systems suitable for the analysis of cell functions or physiological phenomena are those in which a defined response, as an output, is induced in response to a defined input into target cells. In such experimental systems, the environment should be completely controllable and the population of target cells should, if possible, be homogeneous. These requirements are difficult to meet when intact plants are used as experimental systems. However, plant cells in culture can meet such requirements. A further requirement for an ideal experimental system is that it be a synchronized system in which responses occur at high frequency. If systems with low-frequency responses are used, cells engaged in specific events related to a certain physiological phenomenon or cell function are diluted by cells that are not engaged in such events. When we use asynchronous systems, measured parameters yield values that only provide an indication of the average responses of cells at different stages or phases. Thus, synchronized systems with high-frequency responses are required for investigations at the cellular and molecular level of physiological phenomena or cell functions in higher plants. We can establish experimental systems that meet such criteria by using cell culture techniques.

Our strategies for investigations of physiological phenomena in plants using cell culture systems have the following characteristics: 1) establishment of synchronous systems with high-frequency responses; 2) morphological and physiological analysis of the phenomena and 3) biochemical and molecular biological approaches to the characterization of mechanisms responsible for the phenomena.

In this review, we summarize our approaches to mechanisms of proliferation and differentiation of plant cells by application of the strategies outlined above.

Gene expression during the cell cycle in synchronized cultures of plant cells

Synchronous cell-division systems

To understand the cell cycle in plants, it is necessary to identify the biochemical and molecular biological events that are associated with the cell cycle and the timing of these events during the cell cycle. Analysis of the change in biochemical and molecular processes during the cell cycle requires a system in which progression of the cell cycle can be controlled. To this end, several systems for synchronizing the plant cell cycle have been developed. One system for synchronization of plant cells in suspension cultures involves inhibitors of DNA synthesis, such as aphidicolin (Nagata *et al.*, 1992).

In suspension cultures of periwinkle *(Catharanthus roseus)* cells, synchronized cell-division systems induced by two different methods have been established. In one system, synchrony is achieved by the double phosphate-starvation method (Amino *et al.*, 1983). In this system, cells are arrested at the G_1 phase of the cell cycle and readdition of phosphate induces the synchronous

Abbreviations used in this paper: ADS cells, actively DNA synthesizing cells; BA, benzyladenine; CAD, cinnamyl alcohol dehydrogenase; CIM, callus inducing medium; 4CL, 4-coumarate:CoA ligase; EMS, ethylmethane sulfonate; GUS, ß-glucuronidase; NAA, 1-naphthaleneacetic acid; NDS cells, none-DNA synthesizing cells; PAL, phenylalanine ammonia lyase; PCNA, proliferating cell nuclear antigen; SIM, shoot inducing medium.

***Address for reprints:** Laboratory of Plant Cellular and Molecular Biology, Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Mejiro, Tokyo 112, Japan. FAX: 3-3942.6189.

[1]Present address: Dept. of Biology, University of Tokyo, Hongo, Tokyo 113, Japan.

0214-6282/94/$03.00

progression of the cell cycle. In another system, cells are transferred to a medium without auxin and subsequent addition of auxin to the medium simulates progression of the cell cycle (Nishida *et al.*, 1992). In auxin-free medium, cells accumulate in the G_1 phase, and addition of auxin releases the G_1 arrest, cells enter the S phase and, thereafter, they divide in synchrony. The degree of synchrony achieved by these two methods is sufficiently high to allow an examination of changes in cellular components during the cell cycle.

Changes in gene expression during the cell cycle in synchronous cultures of periwinkle cells

The synchronous cell-division system induced by the double phosphate starvation method has been utilized for investigations of gene expression during the cell cycle. Cells at each phase of the cell cycle in synchronized cultures were harvested and poly(A)$^+$RNA was extracted. The products of *in vitro* translation of the poly(A)$^+$RNA were analyzed by two-dimensional gel electrophoresis. Among the approximately 500 translated products detected, three polypeptides appeared specifically in the S phase and one polypeptide was present specifically in the G_2 phase and during cytokinesis (Kodama *et al.*, 1989). This result demonstrated alterations in gene expression during the cell cycle. However, most genes were expressed throughout the cell cycle, suggesting that only a small number of cell cycle dependent genes play an important role in the progression of the cell cycle. Cell cycle dependent genes whose levels of expression fluctuate during the cell cycle, have been identified in yeast and animal systems. Among the cell cycle dependent genes identified to date, a good many appear to be essential for progression of the cell cycle (McKinney and Heintz, 1991).

Cell cycle dependent genes in higher plants

In yeasts and animal cells, various cell cycle dependent genes have been identified (McKinney and Heintz, 1991). By contrast, only limited information is available about cell cycle dependent genes in higher plants. We tried to examine the changes in the levels of mRNAs transcribed from genes that are expressed in a cell-cycle dependent manner and correspond to those identified in yeast or animal cells. Genes for proliferating-cell nuclear antigen (PCNA) and two different types of cyclin were analyzed in terms of their patterns of expression during the cell cycle in synchronous cultures of periwinkle cells established by the two different methods described above. The appearance of mRNA for PCNA is known as a molecular indicator of the S phase of the cell cycle. By contrast genes for cyclins are expressed in a cell cycle dependent manner with different temporal patterns of expression that depend on the particular type of cyclin.

PCNA

Proliferating-cell nuclear antigen (PCNA) has been shown to be an auxiliary protein of DNA polymerase-δ (Tan *et al.*, 1986; Bravo *et al.*, 1987). PCNA has an essential function in the replication of DNA in mammalian cells. When exponentially growing cells are exposed to antisense oligonucleotides complimentary to cDNA of PCNA, DNA replication is completely suppressed (Jaskulski *et al.*, 1988). Similarly, the *POL30* gene of yeast (*Saccharomyces cerevisiae*) that encodes PCNA has also been shown to be essential for DNA replication (Bauer and Burgers, 1990). In higher plants, a homolog of the gene for PCNA was identified initially in rice (Suzuka *et al.*, 1989). A rice genomic clone for rice PCNA was utilized as a probe to screen a periwinkle cDNA library. A periwinkle cDNA clone for PCNA was obtained, and it encoded a protein with a conserved primary structure relative to animal PCNA. The expression of the gene for PCNA was analyzed in synchronous cultures of periwinkle cells induced by the two different methods (Kodama *et al.*, 1991a). In both systems, similar patterns of fluctuation in the level of the transcript were observed. PCNA mRNA was absent from cells arrested in the G_1 phase. After stimulation of progression of the cell cycle by addition of phosphate or auxin, the PCNA mRNA remained undetectable during the G_1 phase and it began to increase at the G_1/S boundary, peaking in the S phase during the cell cycle. Thus, the transcriptional activity of the gene for PCNA fluctuates during the plant cell cycle, with preferential expression during the S phase.

Cyclins

Cyclin is a key regulatory component in the progression of the eukaryotic cell cycle, forming an active complex with $p34^{cdc2}$ kinase (Nurse, 1990).

Whereas expression of the $p34^{cdc2}$ kinase is relatively constant throughout the cell cycle, most cyclin proteins are only present at a particular point in the cell cycle. In animal cells, cyclin proteins have been classified as the A, B, C, D and E type on the basis of their primary structures. Different types of cyclin act essentially at different points in the cell cycle. In the case of B-type cyclins, biochemical and genetic analyses in a variety of organisms have uncovered a role for them in the control of entry into mitosis. A-Type cyclins appear to be involved in the regulation of the S phase in cultured animal cells (Pagano *et al.*, 1992; Zindy *et al.*, 1992; Cardoso *et al.*, 1993), although counter examples have been reported in *Drosophila* embryos (Lehner *et al.*, 1991) and *Xenopus* eggs (Fang and Newport, 1991). Cyclins C, D and E appear to be putative homologs of the product of the gene for G_1 cyclin of *S. cerevisiae*, *CLN*, which participates in G_1/S transition (Richardson *et al.*, 1989), since they can complement *CLN* mutants of *S. cerevisiae* (Lew *et al.*, 1991). Most of the genes for cyclins are regulated transcriptionally, showing periodic expression of their respective mRNA during progression of the cell cycle. An increase and decrease in the concentration of mRNAs for A-type cyclins precedes those of B-type cyclins. While mRNAs for B-type cyclins are specifically present at the G_2/M phase (Pines and Hunter, 1989), levels of mRNAs for A-type cyclins increase in the S phase but fall to zero at mitosis (Pines and Hunter, 1990). The presence of cyclin homologs in higher plants was demonstrated in carrot and soybean by isolation of cDNA clones (Hata *et al.*, 1991). Subsequently, cyclin cDNAs were isolated from *Arabidopsis* (Hemerly *et al.*, 1992) and alfalfa (Hirt *et al.*, 1992). Cyclin cDNAs from carrot and soybean were utilized for isolation of periwinkle homologs. From their primary structures, the cyclins of the carrot and soybean can be classified as A-type and B-type, respectively (Hirt *et al.*, 1992). Periwinkle homologs of genes for A-type carrot cyclin *(CYS)* and B-type soybean cyclin *(CYM)* have been isolated. Patterns of expression of *CYS* and *CYM* were examined in synchronous cultures of periwinkle cells induced by the auxin starvation method. Both of the genes were expressed periodically during the cell cycle. *CYM* mRNA appeared in the late G_2 phase and it was present during mitosis. The appearance of *CYS* mRNA preceded that of *CYM* mRNA. The level of *CYS* mRNA began to increase in the late S phase and reached a maximum at the G_2/M boundary. The fluctuations in levels of *CYS* and *CYM* mRNAs in the synchronous cultures correspond to typical patterns of expression of genes for A-type and B-type cyclins, respectively.

Isolation of cell cycle dependent genes from synchronous cultures of plant cells

In addition to characterization of the expression of genes that have been well studied in yeast or animal systems (for example, genes for PCNA or cyclins), an attempt was made to isolate cDNAs that are expressed periodically during the plant cell cycle in synchronous cultures. For this purpose, both systems for ensuring synchronous division of periwinkle cells were utilized. Using the system in which synchrony is induced by auxin starvation, we constructed a cDNA library from cells that had been cultured for two hours after addition of auxin. The cDNA library was screened differentially and several cDNAs (*cyc18, cyc19, cyc20* and *cyc21*) associated with alterations in levels of the corresponding mRNAs were isolated. From the nucleotide sequences of the cDNAs, some of them were found to be homologous to genes with known function. The expression of the *cyc18* gene was stimulated by addition of auxin and its transcript appeared transiently. The *cyc18* gene was found to be homologous to the *parA* gene isolated from tobacco mesophyll protoplast. The *parA* gene was isolated in a search for genes that were induced upon the addition of auxin. The expression of the *parA* gene was temporally correlated with the transition from the G_0 to the S phase (Takahashi *et al.*, 1989). Thus, as in the case of the *parA* gene, the pattern of expression of *cyc18* is associated with dedifferentiation and the start of cell proliferation rather than being dependent on the cell cycle of already cycling cells. The *cyc19* gene was revealed to encode heat shock protein 90 (HSP90) from the deduced amino acid sequence. The expression of *cyc19* was observed specifically in the S phase of the cell cycle in synchronous cultures. At present, it is difficult to explain why the gene for HSP90 is expressed specifically in the S phase. The *cyc20* and *cyc21* genes have similar patterns of expression during the cell cycle. Their mRNAs are absent in auxin-starved cells; the mRNAs increase gradually in level during the G_1 phase, with a peak during the S phase. Although the products of the these two genes are unknown, their importance in the cell cycle is suggested by their cell-cycle-related periodicity.

Similar studies were performed using the system of synchronous cell division induced by double phosphate-starvation (Kodama *et al.*, 1991b). Differential screening of a cDNA library, constructed from the cells in the S phase, resulted in isolation of several phase-specific cDNAs (*cyc02, cyc07, cyc15* and *cyc17*). Similar patterns of expression were observed for the *cyc02, cyc15* and *cyc17* genes. All were expressed in phosphate-starved cells but, after addition of phosphate, the levels of their mRNAs declined and then the mRNAs disappeared. Thereafter, the levels of the mRNAs increased to maximal levels during the S phase. The *cyc02* encodes a polypeptide constituted by 101 amino acids with a molecular mass of 16 kDa (Kodama *et al.*, 1991b). *Cyc02* encodes unidentified protein, *cyc15* and *cyc17* both encode a cell-wall protein, namely, extensin. *Cyc15* and cyc17 represent different genes on the periwinkle genome since they do not hybridize to each other. The fact that two cDNAs for extensin were isolated independently by differential screening suggests the physiological relevance of periodic expression of each gene during the cell cycle. Fluctuations in level of the expression of genes for extensin during the cell cycle may be related to the formation of the cell plate, which occurs during the M phase. The *cyc07* gene encodes a highly basic protein with a molecular mass of 35 kDa (Ito *et al.*, 1991). The encoded amino acid sequence is unrelated to those of any proteins reported previously. The *cyc07* gene is expressed specifically during the S phase of the cell cycle in the synchronous cultures (Kodama *et al.*, 1991b).

Expression and function of the cyc07 gene

The concentration of the *cyc07* mRNA in periwinkle cells fluctuates during the cell cycle, peaking during the S phase in two different systems for synchronous cell division (Ito *et al.*, 1991). The expression of the *cyc07* gene is completely suppressed by the addition of an inhibitor of DNA replication, namely, aphidicolin. The *cyc07* gene resembles genes for histones in terms of the pattern of changes in the level of mRNA during the cell cycle and the effect of an inhibitor of DNA synthesis on such expression. This tight coupling between expression of the *cyc07* gene and DNA synthesis suggests that the product of the *cyc07* gene participates in progression of the S phase. *cyc07* mRNA is present only in actively proliferating tissues in intact plantlets, for example, in root tips. Similarly, the gene is preferentially expressed in exponentially growing cells in tissue culture. The results of an analysis of the promoter of the *cyc07* gene is consistent with the distribution of the mRNA suggested by Northern blot analysis, as follows. The promoter of the *cyc07* gene was fused with a reporter gene, the gene for ß-glucuronidase (GUS). The promoter-GUS chimeric gene was introduced into *Arabidopsis*. In transgenic *Arabidopsis* plants, GUS-expressing cells were concentrated near the shoot apex, and their distribution was consistent with that of tissues expected to contain cycling cells, such as the shoot apex, root tips, the immature embryo, lateral root primordia and the axillary buds. Taken together, the results indicate that expression of the *cyc07* gene is closely correlated with cell proliferation, suggesting a specific function for the gene in progression of the cell cycle. Antibodies against the *cyc07*-encoded protein have been raised by injecting rabbits with a fusion protein that was expressed in bacteria. Immunostaining revealed the nuclear localization of this protein in periwinkle cells.

The presence of the *cyc07*-encoded protein is specific to actively cycling cells and the protein is absent from cells that have ceased to proliferate. Thus, the *cyc07* gene encodes a proliferating-cell-specific nuclear protein. This result, again, suggests that the function of the product of the *cyc07* gene is related to cell proliferation. Two closely related genes *(PLC1* and *PLC2)* from yeast were identified as a homologs of the cyc07 gene (Ito *et al.*, 1992). *PLC1* and *PLC2* encode nearly identical sequences of amino acids. The predicted amino acid sequences of *PLC1/2* are strikingly similar to that encoded by *cyc07*, clearly indicating that the *PLC1/2* genes are yeast homologs of *cyc07* from periwinkle. The extent of similarity is approximately 64%. The high degree of similarity in terms of primary structure between proteins from two evolutionarily distant species suggests an important function for the proteins in the maintenance of biological systems. In fact, *PLC1* and *PLC2* constitute a family of genes that are essential for proliferation of yeast cells. That is, inactivation of both *PLC1/2* genes simultaneously by site-directed disruption mutation results in the failure of cells to proliferate. Furthermore, haploid cells with a mutation in either one of these genes grow at a reduced rate (Ito *et al.*, 1992). Thus, the number of copies of the *PLC1/2* genes affects the rate of cell proliferation, leading us to hypothesize that the concentration of the proteins encoded by *PLC1/2* must exceed a threshold level for progression of the cell cycle and that the level of expression of this family of genes controls the progression of the cell cycle. The functions of both *PLC1* and *PLC2* can be replaced

by a functioning *cyc07* gene in yeast. Introduction of a plasmid that expressed *cyc07* cDNA reversed the reduction in growth rate caused by a disruption mutation in either *PLC1* or *PLC2*. Therefore, the *cyc07* gene is functionally, as well as structurally, homologous to *PLC1/2*. This observation suggests that these genes play the same role in biological systems and, perhaps, in a highly conserved aspect of cell proliferation. The expression pattern of *cyc07* in higher plant cells and the mode of action of the *PLC1/2* in yeast suggest together the importance of these genes in cell proliferation.

Mechanisms of somatic embryogenesis

Somatic embryogenesis is an ideal system for investigations of the entire process of differentiation of plants, as well as of the mechanisms of expression of totipotency in plant cells. This system has major advantages over zygotic embryos. For example, (a) the process of embryogenesis is easily observed; (b) external conditions that control embryogenesis can be manipulated, and (c) large numbers of embryos can easily be obtained. The aim of Haberlandt's first attempt in 1902 to establish plant tissue culture systems was to provide evidence for the totipotency of plant cells. The mechanism of somatic embryogenesis represents a fundamental problem in plant physiology. Recently, somatic embryogenesis has attracted the attention of plant biotechnologists, because it can serve as a useful system for production of transgenic cloned plants, as well as for obtaining a source of artificial seed materials.

In this section, physiological, biochemical and molecular biological aspects of somatic embryogenesis will be reviewed with emphasis on studies performed with carrot suspension cultures in our laboratory.

Establishment of high-frequency and synchronous systems of somatic embryogenesis

The first reports of somatic embryogenesis were published in 1958 by Steward *et al.* and Reinert. In the subsequent twenty year period, however, little progress was made in understanding the mechanisms of somatic embryogenesis because somatic embryogenesis could be induced *in vitro* only at low frequency and asynchronously in the available systems. In such systems, biochemical and molecular events specific for embryogenesis were masked by the activities of cells that were not engaged in embryogenesis. Furthermore, only average values for biochemical parameters related to various stages of embryogenesis could be determined when asynchronous embryogenesis systems were used. It was clear that high-frequency and synchronous systems for embryogenesis were required for investigations of mechanisms of somatic embryogenesis at the molecular level and it was for this reason that we established suitable systems using carrot suspension cultures (Fujimura and Komamine, 1979b). Embryogenic cell clusters were selected by sieving through a nylon screen and density gradient centrifugation in Ficoll. The clusters were transferred to medium that lacked auxin but contained zeatin at 10^{-7} M. Synchronized embryogenesis occurred from cell clusters at a frequency of about 90% in this system, which has proved useful for investigations of the process of embryogenesis from embryogenic cell clusters, which are designated State 1 cell clusters.

Since State 1 cell clusters can differentiate to embryos in auxin-free medium without any trigger, embryogenesis can be considered to have already been determined in these State 1 cell clusters. Thus, the process of formation of State 1 cell clusters from single cells is also important in an analysis of the process of embryogenesis, and a system is required in which high-frequency embryogenesis occurs from single cells. We attempted to establish such a system (Nomura and Komamine, 1985). Competent single cells, which were small, round, rich in cytoplasm and designated State 0 cells, were collected by sieving of a culture through a nylon screen, density gradient centrifugation in Percoll and manual selection. When State 0 cells were pretreated with auxin (2,4-D at 5×10^{-8} M) for 6 days and then transferred to the auxin-free medium, embryos were formed at high frequency (85-90%). Pretreatment with auxin was essential and zeatin (10^{-6} M), mannitol (10^{-3} M) and a high concentration of oxygen (40%) had promotive effects. The single cells allow investigation of the entire process of development from somatic embryogenesis from single cells to whole plants. When State 0 cells were cultured directly in auxin-free medium, cells became elongated and they could not differentiate to embryos even if they were transferred to the medium containing auxin. Therefore, the culture of State 0 cells in auxin-free medium can be regarded as a process whereby totipotency is lost. By contrast, when State 0 cells are cultured in medium with auxin, transferred to auxin-free medium and then differentiate to embryos at high frequency, the entire process corresponds to the expression of totipotency. These two processes provide useful models to investigate what events occur during expression or loss of totipotency.

Phases in somatic embryogenesis

Detailed morphological observations have revealed that four phases, phases 0, 1, 2 and 3, can be recognized at the early stages of embryogenesis in the system described above (Fujimura and Komamine, 1980).

During phase 0, competent single cells (State 0) form embryogenic cell clusters (State 1) in the presence of auxin. At this time, the cell clusters formed from single cells gain the ability to develop into embryos when auxin is removed from the medium, giving rise to State 1 cell clusters. The subsequent phase, phase 1, is induced by transfer of State 1 cell clusters to auxin-free medium. During phase 1, cell clusters proliferate relatively slowly and apparently without any differentiation. After phase 1, rapid cell division occurs in certain parts of cell clusters, leading to the formation of globular embryos. This phase is designated phase 2. In the final phase, phase 3, plantlets develop from globular embryos via heart-shaped and torpedo-shaped embryos (Fig. 1).

Physiological factors affecting somatic embryogenesis

Auxin is the most important regulator of the induction and progression of embryogenesis and it has different effects during different phases of embryogenesis. The presence of 2,4-D or other auxins is required for the formation of embryogenic cell clusters

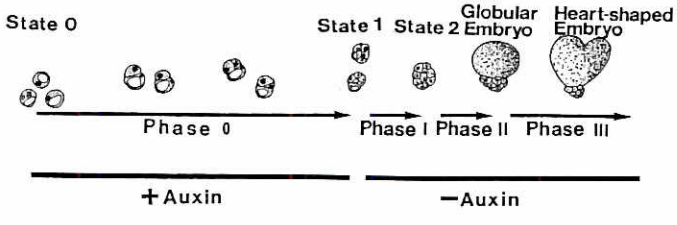

Fig. 1. Developmental phases of somatic embryogenesis in carrot suspension cultures.

(State 1) from single cells (State 0). This observation indicates that auxin is essential for induction of embryogenesis (phase 0). In other words, auxin is necessary if "competent" cells (State 0) are to express totipotency. However, auxin is inhibitory during phase 1 and in subsequent phases. The inhibitory effect of auxin is most obvious during phase 1. Since the original single cells cannot differentiate directly to form embryos in auxin-free medium, there are at least two stages in somatic embryogenesis: one is the stage requiring auxin and the other is that being inhibited by auxin. Antiauxins, 2, 4, 6-trichlorophenoxyacetic acid and p-chlorophenoxyisobutyric acid (PCIB) inhibit embryogenesis after phase 1 (Fujimura and Komamine, 1979a). Various findings suggest that auxin is required for induction of embryogenesis (the process whereby competent cells become embryogenic cell clusters), but it inhibits development of embryogenesis (the process whereby embryogenic cell clusters become plantlets).

A cytokinin, zeatin has promotive effects on embryogenesis at every phase, being most effective during phase 2, when active cell division occurs. Zeatin may be involved in the promotion of cell division. Other phytohormones, namely, gibberellins and abscisic acid, inhibit embryogenesis from cell clusters (Fujimura and Komamine, 1975). In addition to phytohormones, intercellular interactions are also important in somatic embryogenesis. A rather high cell density (10^5 cells/ml) is required for the formation of embryogenic cell clusters from single cells (Nomura and Komamine, 1985), whereas a lower cell density ($2x10^4$ cells/ml) favors the development of embryos from embryogenic cells (Fujimura and Komamine, 1979b).

Expression of polarities in early stages of somatic embryogenesis

As mentioned above, rapid cell division occurs in certain parts of cell clusters during phase 2, leading to the formation of globular embryos. The doubling time is 6.3 h during phase 2, while it is 51 h and 36 h in phase 1 and 3, respectively (Fujimura and Komamine, 1980). Polarity of DNA synthesis in cell clusters was confirmed during phases 1 and 2 by autoradiography with [^{3}H]-thymidine. However, polarity was eliminated when cell clusters were cultured under non-embryogenic conditions, i.e., in the presence of auxin. The polarized rapid division of cells or DNA synthesis is, therefore, considered to be specific to embryogenesis. It is important to investigate the mechanism of expression of the polarity of active DNA synthesis and rapid cell division if we are to understand embryogenesis.

We analyzed the polarity of DNA synthesis during phases 1 and 2 by computerized three-dimensional reconstruction from serial sections of cells that have been pulse-labeled with [^{3}H]-thymidine. The initial material was State 1 cell clusters. We confirmed that DNA-synthesis occurred randomly during the first three days of culture in the absence of auxin (phase 1), but polarized DNA synthesis was clearly observed between days 3 and 4 of culture (phase 2). Globular embryos were then formed, in which high DNA-synthetic activity was observed in the procambial and proepidermal cells, while no DNA-synthetic activity was detected in the suspensor-like structure.

In State 2 cell clusters, the polarized localization of cells that were actively synthesizing DNA was observed. We attempted to separate actively DNA synthesizing cells (ADS cells) from inactive cells (NDS cells) by density gradient centrifugation in Percoll after maceration to protoplasts, in an attempt to investigate differences at the molecular level between ADS and NDS cells. Proteins from both types of cells were compared by SDS-PAGE after labeling with [^{35}S]-methionine. Three proteins were detected in ADS cells that were not found in NDS cells. These proteins are candidates for markers of polarity of DNA synthesis specific for embryogenesis.

The polarized localization of poly(A)$^+$RNA was also detected in cell clusters at the end of phase 0 by *in situ* hybridization with [^{3}H]-poly(U). This polarity also disappeared when cell clusters were transferred to non-embryogenic conditions. Moreover, polarized localization of free calcium ions during embryogenesis was observed with a fluorescent indicator of calcium, namely, Fura 2.

Molecular aspects of somatic embryogenesis

Molecular markers of somatic embryogenesis

Many efforts have been made to identify molecular markers that are specific for somatic embryos (Sung and Okimoto, 1981, 1983; Chibbar *et al.*, 1989). Sung and Okimoto (1981) analyzed proteins that have been labeled *in vivo* by two-dimensional gel electrophoresis, and compared proteins synthesized in somatic embryos and in callus tissue. The patterns of protein synthesis were very similar except for the presence of two proteins, E1 and E2. These proteins were found in the somatic embryos but were barely detectable in the callus tissue.

We also found several molecular markers in our carrot embryogenesis system described above. Two-dimensional gel electrophoresis revealed the existence of three proteins, a, b and c, which could be detected throughout the process of expression of totipotency (phases 0-3) but disappeared during the process of loss of totipotency. Two mRNAs, 1 and 2, also showed the same pattern of appearance as proteins a, b and c. In addition, protein d appeared during Phase 0. In phases 1 and 2, more than 99% of polypeptides produced by in translation *in vitro* gave the same pattern when mRNAs from embryogenic and nonembryogenic cultures were translated. Two mRNAs that encoded polypeptides appeared exclusively in embryogenic cultures, while two others appeared in nonembryogenic cultures. These results indicate that perhaps only a few proteins may play important roles during embryogenesis and that changes in protein patterns are regulated at the transcriptional level.

Smith *et al.* (1988) described a monoclonal antibody designated 21D7 that reacted with a nuclear protein associated with cell division. We applied 21D7 to our system and, using Western blotting and immunocytochemical methods, we examined whether antigen 21D7 (21D7 protein) might be a candidate for a molecular marker of totipotency. The 21D7 protein was detected throughout the process of expression of totipotency, while it disappeared within 48 h during the process of loss of totipotency, that is, when State 0 single cells were cultured in the absence of auxin. Furthermore, when State 0 single cells were microinjected with the 21D7 monoclonal antibody, they elongated and failed to divide or differentiate even when cultured in the presence of auxin. These results indicate that expression of the 21D7 protein may be essential for the expression of totipotency.

Gene expression during somatic embryogenesis.

The most attractive approach to the elucidation of mechanisms of somatic embryogenesis involves the isolation of genes expressed specifically during embryogenesis and the characterization of their function. Many attempts have been made to pursue this approach (Choi *et al.*, 1987; Borkird *et al.*, 1988; Wilde *et al.*, 1988; Dure III *et al.*, 1989; Franz *et al.*, 1989; Aleith and Richter, 1990).

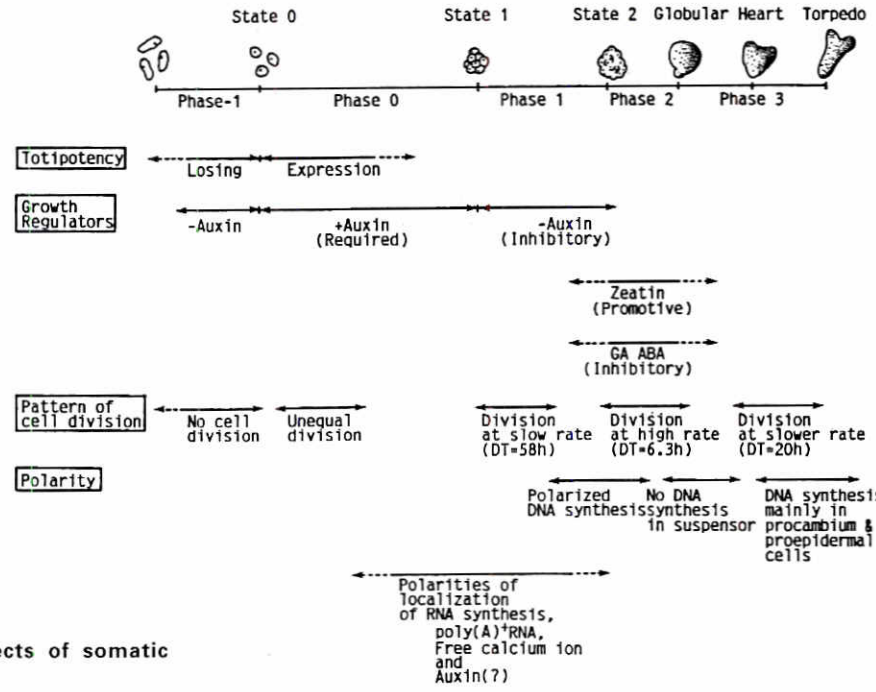

Fig. 2. Morphological and physiological aspects of somatic embryogenesis in carrot suspension cultures.

Choi *et al.* (1987) isolated several cDNA clones for mRNAs that were preferentially expressed during somatic embryogenesis in carrot by a combined immunoadsorption and epitope-selection method. Developmental regulation was analyzed in detail about two clones, and the expression of the genes corresponding to them was found to be associated with the heart-stage embryos (Borkird *et al.*, 1988). Sequence analysis revealed that one of the clones encoded an analog of LEA (late embryogenesis abundant) proteins (Dure III *et al.*, 1989). Aleith and Richter (1990) described cDNA clones isolated by differential screening from cDNA libraries established from mRNA extracted from somatic embryos of carrot. The expression of genes corresponding to some of the clones was roughly associated with the first morphogenetic or globular stage. Thus, many genes involved in embryogenesis have been isolated, but their functions remain unclear.

We constructed λgtII cDNA libraries from poly(A)$^+$RNA isolated from hypocotyls and roots of carrot seedlings and screened the cDNA libraries differentially to isolate the hypocotyl- or root-specific cDNAs. We isolated two cDNAs, CAR3 and CAR4, which corresponded to genes that were specifically expressed in hypocotyls, and another two cDNAs, CAR5 and CAR6, for genes which were specifically expressed in roots. Expression of these four genes was investigated during phases 1-3 by Northern hybridization. The level of expression of the genes that corresponded to CAR4 and 5 increased after globular embryos were formed, and that of the gene that corresponded to CAR6 increased a little sooner (after heart-shaped embryos had formed), while CAR3 mRNA was expressed earlier (before globular embryos had formed), i.e. during Phase 2. Expression of these mRNA was very limited in cells cultured in medium with 2,4-D, and it was strongly suppressed when 2,4-D was added to cultures of heart-shaped embryos. *In situ* hybridization revealed that CAR4 mRNA was expressed in the epidermis and in the regions around tracheary elements in torpedo-shaped embryos. The predicted amino acid sequence of the protein encoded by CAR4 was rich in proline (N-terminal region) and in leucine (C-terminal region) residues. A characteristic repeated motif was found in the proline-rich region, resembling repeated sequences found in proline-rich cell-wall proteins, such as p33 (carrot) (Chen and Varner, 1985) or PRP (soybean) (Hong *et al.*, 1987).

We also attempted to isolate genes specific for early stages of embryogenesis. We succeeded in the cloning of five cDNAs by differential screening between State 1 cell clusters, cultured in the absence of auxin for 5 days (preglobular embryos), and State 1 cell clusters cultured in the presence of auxin for 5 days. These clones were designated CEM1, 2, 3, 4 and 5. One of the them, CEM1, was revealed by Northern hybridization to be expressed preferentially prior to and after the globular stage of embryogenesis. We determined the nucleotide sequence of CEM1. The predicted amino acid sequence of the protein encoded by CEM1 was found to exhibit high homology to elongation factor (EF-1α) of eukaryotic cells. The extent of homology was 76.4% for human EF-1α, 76.8% for *Xenopus*, 73.1% for yeast, 81.0% for *Euglena* and 94.2% for *Arabidopsis*. EF-1α is an essential for elongation of the peptide chain during protein synthesis on the ribosome. We investigated the distribution of CEM1 mRNA during somatic embryogenesis in carrot cells by *in situ* hybridization. Accumulation of the specific mRNA was observed in the spherical regions of the globular embryos and in the meristematic regions of heart- and torpedo-stage embryos. It seemed that CEM1 mRNA was expressed in close association with cell-division activity during embryogenesis. The results described here are summarized in Figs. 2 and 3.

Genetic analysis of organogenesis in *Arabidopsis thaliana* in tissue culture

During organogenesis in primary cultures, plant cells are reactivated so that they acquire organogenic competence, and then

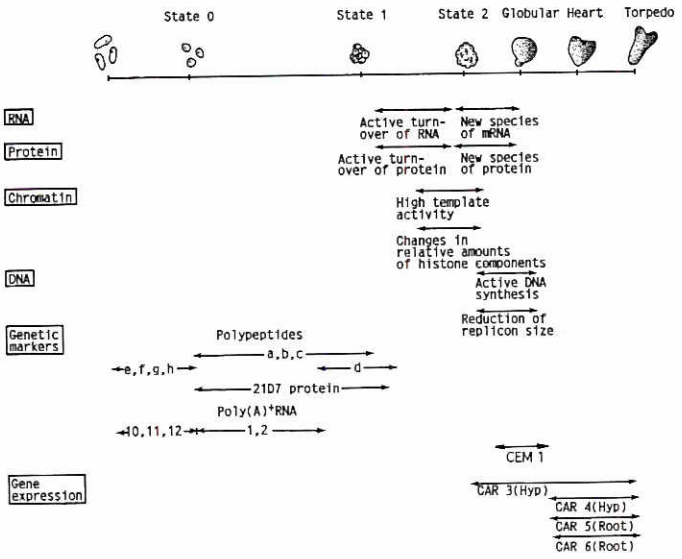

Fig. 3. Molecular aspects of somatic embryogenesis in carrot suspension cultures.

they are induced to form shoot or root meristem under the control of phytohormones. Although this process has been studied by many researchers, the key to organogenic competence and the molecular mechanisms of organogenesis remain unknown. Genetic analysis, which has not been exploited extensively in this field, can be expected to improve the present situation.

Organogenic responses in tissue culture often vary, depending on the genotype of donor plants. The genetic basis of such variations has been studied in several crops. For example, a locus, designated *Rg-1*, that controls shoot regeneration in tomato was recently identified and mapped (Koornneef *et al.*, 1993). Information obtained from these studies is very important for biotechnologic manipulation of crops but is far from providing adequate clues to the nature of the entire process of organogenesis. The systematic isolation and utilization of mutants exhibiting defects at various stages of organogenesis is necessary for the genetic dissection of organogenesis.

Arabidopsis thaliana is a model plant suitable for molecular and classical genetic studies (Meyerowitz, 1989). A procedure for the induction of the efficient and rapid redifferentiation of shoots from root explants of this plant has been developed (Valvekens *et al.*, 1988) making the *Arabidopsis* system amenable to genetic analysis of organogenesis. In this procedure, excised root segments are pre-cultured for several days on callus-inducing medium (CIM) that contains 2,4-D and kinetin, and then they are cultured on shoot-inducing medium (SIM) that contains N^6-(2-isopentenyl) adenine and IAA.

Preculture of root segments on CIM is essential for the induction of shoot redifferentiation, and explants seem to acquire organogenic competence for shoot redifferentiation during this preculture period. Only tissues competent for shoot redifferentiation can respond to the transfer to SIM by forming shoots. For the genetic dissection of organogenesis, it is also very convenient that the experimental system is composed of two kinds of tissue cultures which may be induced at different stages of the organogenic process.

As a first step towards a genetic analysis of organogenesis, we screened descendants of EMS-mutagenized *Arabidopsis* plants and isolated mutants that were temperature-sensitive for the redifferentiation of shoots from root explants. A comparative examination of shoot redifferentiation at 22°C (the permissive temperature) and 27°C (the restrictive temperature) led to the identification of three temperature-sensitive mutants (Yasutani *et al.*, 1994). Genetic tests of these mutants indicated that the temperature-sensitive redifferentiation of shoots resulted from single, nuclear, recessive mutations in three different genes, which were designated *SRD1, SRD2, SRD3,* respectively. Since none of the mutations caused temperature-sensitive growth of callus or of redifferentiated shoots, SRD genes seem to play a role not in the fundamental events that are essential for cell proliferation but in more specific aspects of organogenesis.

Temperature-shift experiments were performed with the temperature-sensitive mutants to estimate the times at which products of the *SRD* genes expressed their functions during the redifferentiation of shoots from root explants (Ozawa, Yasutani, Sugiyama and Komamine, unpublished). The results suggested

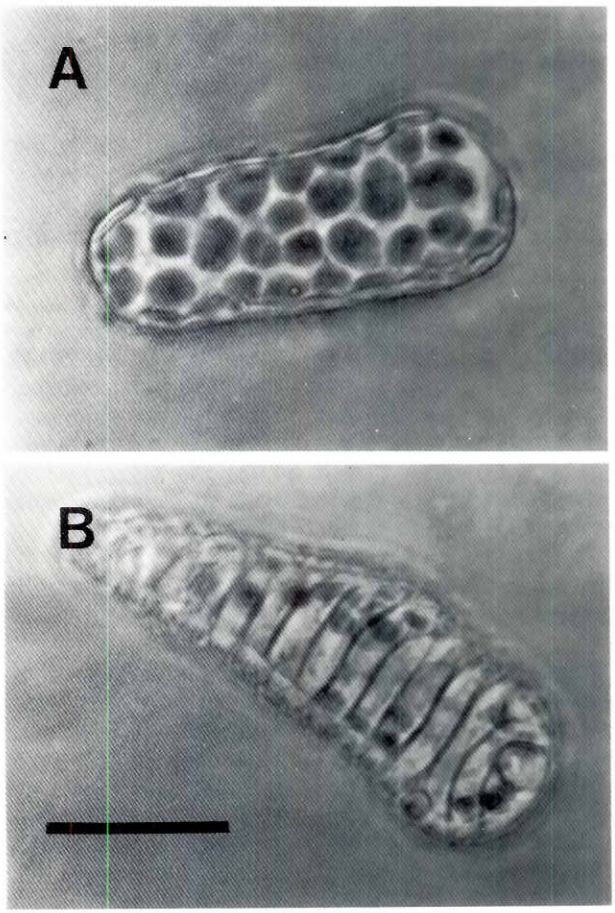

Fig. 4. Transdifferentiation of a single mesophyll cell into a tracheary element. (A) *A single mesophyll cell.* **(B)** *A tracheary element formed after 58 h in culture without any intervening cell division. Bar, 25 μm. (From Fukuda and Kobayashi, 1989).*

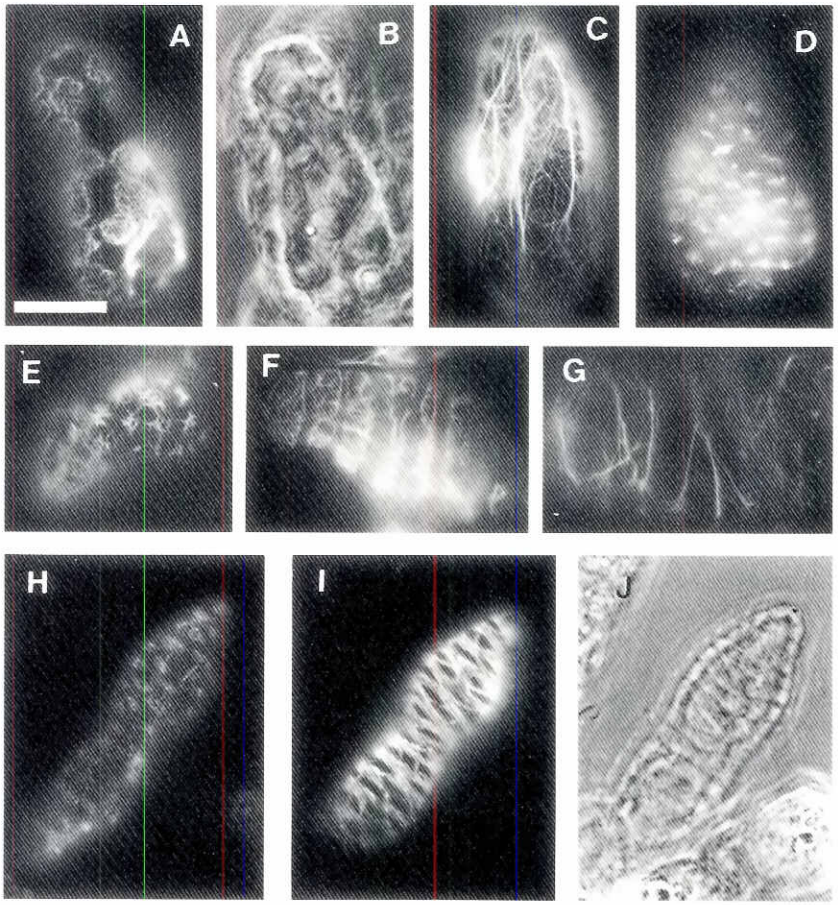

Fig. 5. Changes in the organization of actin filaments during differentiation of tracheary element from mesophyll cells of *Zinnia elegans*. **(A)** *Actin filaments in a freshly isolated cell;* **(B)** *phase-contrast image of the cell shown in A;* **(C)** *actin filaments in a cell at 48 h;* **(D,E,F)** *actin filaments in a cell at 60 h;* **(G)** *actin filaments in a cell at 75 h;* **(H)** *actin filaments in a cell at 66 h;* **(I)** *microtubules in the cell shown in H;* **(J)** *phase-contrast image of the cell shown in H. Bar, 20 μm. (From Kobayashi et al., 1987, 1988).*

that *SRD3* is involved in the acquisition of organogenic competence during preculture on CIM, while *SRD1* and *SRD2* are involved in later stages of the redifferentiation of shoots induced by transfer to SIM. We anticipate that the temperature-sensitive mutants, *srd1*, *srd2*, and *srd3*, which exhibit defects at different stages of shoot redifferentiation, will be powerful tools not only for the genetic dissection of organogenesis but also for identification of genes that are essential for organogenesis.

Direct transdifferentiation of isolated mesophyll cells into tracheary elements

Differentiation of plant cells is plastic. Differentiated cells can transdifferentiate *in vitro* to other types of cell. Differentiation of parenchyma cells into tracheary elements is an excellent example of transdifferentiation that occurs at the cellular level in higher plants (Fukuda, 1992). *In situ,* vessels and tracheids are formed from cells that are referred to as tracheary elements. The tracheary elements are differentiated from cells of the procambium of the root and shoot in primary xylem or from cells produced by the vascular cambium in the secondary xylem (Torrey *et al.*, 1971; Roberts *et al.*, 1988). *In vitro*, tracheary elements can be induced from various types of cell, such as parenchyma and epidermal cells, by wounding and/or treatment with phytohormones (Fukuda, 1992). Tracheary elements are characterized by the formation of a secondary cell wall with annular, spiral, reticulate or pitted wall thickenings. At maturity, fully differentiated tracheary elements lose their nuclei and cell contents, forming a hollow, tubular system.

We have established an experimental system *in vitro* as a useful model for the study of the differentiation of tracheary element. Single mesophyll cells isolated from *Zinnia* leaves can transdifferentiate directly into tracheary elements without cell division, synchronously and at high frequency after wounding and exposure to a combination of auxin and cytokinin (Fig. 4; Fukuda and Komamine, 1980a). In this section we shall summarize our recent molecular and biochemical studies of the differentiation of *Zinnia* mesophyll cells into tracheary elements. More detailed information can be found in our recent review articles (Fukuda, 1989a, 1992, 1994; Sugiyama and Komamine, 1990; Fukuda *et al.*, 1993).

Experimental system

Single mesophyll cells are isolated mechanically from the first true leaves of 14-day-old seedlings of *Zinnia elegans*. Isolated cells were cultured in a liquid medium with 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 1 mg/l benzyladenine (BA). As a result, 30-60% of isolated cells differentiate into tracheary elements synchronously after 60 to 80 h of culture (Fukuda and Komamine, 1980a, 1982). More than 60% of the tracheary elements formed were differentiated directly, without intervening the S phase or the M phase in the cell cycle, from mesophyll cells which arrested at the G_0 or G_1 phase (Fukuda and Komamine, 1980b, 1981).

Initiation

Both auxin and cytokinin are prerequired for the induction of transdifferentiation of *Zinnia* mesophyll cells (Fukuda and Komamine, 1980a). Differentiation starts when both hormones are present simultaneously (Fukuda and Komamine, 1985). In addition, wounding is also necessary for the initiation of differentiation (Church and Galston, 1989). Transdifferentiation into tracheary elements is not restricted to mesophyll cells and can even be induced in epidermal cells.

Events occurring during differentiation

Cytoskeleton

Microtubules in differentiating tracheary elements are localized in bands over the thickenings of secondary walls. Disruption of microtubules by treatment with drugs such as colchicine causes the formation of unlocalized secondary wall thickenings. These observations have led to the hypothesis that microtubules determine the wall pattern by defining the position and orientation of secondary walls. Kobayashi *et al.* (1987, 1988) were the first to note that, in addition to microtubules, actin filaments are involved in the regulation of the development of localized thickenings of secondary walls during the differentiation of tracheary elements in *Zinnia* cells (Fig. 5). They presented a coordinated mechanism whereby actin filaments are involved in the reorganization of microtubules which, in turn, regulate the spatial disposition of secondary walls (Fukuda and Kobayashi, 1989).

The dynamic organization of microtubules in differentiating *Zinnia* cells is accompanied by an increase in the number of microtubules (Fukuda, 1987). The increase was found to be due to *de novo* synthesis of tubulin that contained both α and β subunits. Recently, three different cDNA clones for ß-tubulin were isolated from differentiating *Zinnia* cells (Yoshimura, Demura and Fukuda unpublished data). These three cDNAs shared 80-81% identity at the nucleotide level and 91-96% identity at the predicted amino-acid level. Detailed analysis of the expression of the three corresponding genes for ß-tubulin (*ZTUB*1, 2, and 3) with 3'-non-translated regions as probes indicated that the three genes are expressed differentially during the differentiation of tracheary elements in *Zinnia* cells. The expression of *ZTUB1* and *ZTUB3* was not observed in freshly isolated *Zinnia* mesophyll cells but the levels of their transcripts increased rapidly between 24 and 48 h of culture prior to secondary wall formation. The expression of *ZTUB1* precedes that of *ZTUB3*. In contrast, *ZTUB2* showed weak but continuous expression through the culture period. The rapid increase in the expression of *ZTUB1* and *ZTUB3* at the early stage would allow formation of large amounts of tubulin, which is necessary for the dynamic changes in the organization of microtubules that are associated with secondary wall formation.

In addition to regulation at the synthetic level, levels of tubulin are also regulated at the degradative level in *Zinnia* cells in culture (Fukuda, 1989b). Degradation was most active after 48 h of culture when synthesis of tubulin was still occurring. These observations suggest the rapid turnover of tubulin protein at 48 h of culture, namely, at the time when microtubules change dynamically prior to alterations in cell morphology.

Synthesis of lignin

Lignification occurs specifically on the secondary wall thickenings and is a characteristic biochemical marker of tracheary elements. The biosynthesis of lignin involves many enzymes, which include phenylalanine ammonialyase (PAL) at the first step and peroxidase at the final step.

PAL activity increases in a differentiation-specific manner in differentiating *Zinnia* cells (Fukuda and Komamine, 1982). In differentiating *Zinnia* cells, at least four different isotypes of the gene for PAL are expressed at 48 h of culture (Fukuda and Demura, unpublished data). We have constructed a binary vector that contains a fragment of DNA from the gene for PAL in an antisense orientation. This vector was introduced into segments of *Zinnia* leaves from which roots were induced. In these transformed roots, the antisense DNA suppressed not only the endogenous PAL activity but also the normal development of the xylem (Tateishi and Fukuda, unpublished data). This observation suggests that PAL plays a critical role in formation of the xylem. Analysis of the expression of these genes for PAL should provide additional information about the regulation of gene expression during differentiation.

In addition to PAL activity, the activities of many other enzymes involved in lignin synthesis, such as shikimate dehydrogenase, cinnamate hydroxylase, *O*-methyltransferases, 4-coumarate:CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD) and wall-bound peroxidases, have been reported to increase in association with the differentiation of tracheary elements (Fukuda *et al.*, 1993).

Cinnamyl alcohols are delivered to the cell walls, where they are polymerized into lignin by peroxidases in a free-radical reaction (Lewis and Yamamoto, 1990). In differentiating *Zinnia* cells, increases in the activities of two types of wall-bound peroxidase, which are ionically bound to cell walls and tightly bound to cell walls, respectively, are coupled with the synthesis of the lignin (Fukuda and Komamine, 1982). The suppression of the deposition of lignin by specific inhibitors of PAL, such as L-α-aminooxy-ß-phenylpropionic acid, causes an increase in the activity of the ionically bound peroxidase fraction, a result that suggests that ionically bound peroxidase is fixed into the secondary walls, where it becomes tightly bound peroxidase, with the progression of lignin deposition (Sato *et al.*, 1993). The ionically bound peroxidase fraction contains at least five cationic isozymes (Masuda *et al.*, 1983; Sato *et al.*, 1993). Although the activities of all the cationic isozymes increase with the age of the culture, only the activities of isozymes designated P4 and P5 increase in a differentiation-specific manner, with the increase in P5 activity preceding that in P4. Cells cultured for 72 h were separated into four fractions by Percoll density gradient centrifugation. The mature tracheary elements were concentrated in the >20% Percoll fraction. P5, a differentiation-specific isozyme, was a major component of the ionically bound peroxidases that were extracted from the fraction rich in mature tracheary elements, and P1 and P2 were also found in this fraction. The results suggest that, although P5 is essential for lignification, other peroxidases may also play roles in this process.

Autolysis

The first visible sign of autolysis during the differentiation of tracheary elements is the disruption of the tonoplast. In differentiating *Zinnia* cells, the tonoplast is disrupted several hours after the formation of visible secondary walls, with the loss of cell contents following several hours later. This process is irreversible and is a typical example of programmed cell death. During this process, many hydrolytic enzymes can be expected to appear for the rapid degradation of macromolecules. We observe that a specific protease was induced at the late stage of differentiation of tracheary ele-

TABLE 1

CHARACTERISTICS OF TED cDNAs

cDNA	(bp)	Amino acid	Characteristics	Homology	*In situ* expression
TED1	(1700)			mitochondria F1-ATPase α-subunit	
TED2	1183	325	hydrophobic regions	ζ-crystallin of guinea pig (NADPH:quinone oxidoreductase)	future xylem and/or phloem cells procambial cells
TED3	1435	319	(NGY) motifs repeated amino acid sequences		differentiating or future vessel cells
TED4	535	95	transit peptide metal-binding "finger"	aleurone-specific-protein (B11E) of barley NS-lipid transfer protein	differentiating or future xylem cells

ments with a rapid decrease in its activity after the completion of differentiation (Minami and Fukuda, unpublished data). Partial purification of the protease indicated that it was a cysteine protease with a molecular mass of approximately 30 kDa. Recently, Ye and Varner (1993) isolated a cDNA clone for a protease similar to papain, a typical cysteine protease, as a differentiation-specific clone from *Zinnia* cells. This clone may encode the same protease as the one that we identified.

Thelen and Northcote (1989) indicated that nucleolytic activities are also induced transiently at the late stage of the differentiation of tracheary elements in *Zinnia* cells. Several nucleases appear 12 h prior to formation of visible secondary walls and their levels increase rapidly during the maturation phase of differentiation. At this late stage, induction of many other hydrolytic enzymes can be expected. The induction of hydrolytic enzymes should be very strictly regulated to ensure the rapid and ordered degradation of various macromolecules. A more detailed analysis of hydrolytic enzymes is urgently required if we are to understand the programmed cell death that occurs during the late stage of differentiation of tracheary elements.

Expression of some newly isolated genes related to differentiation

About 12 h before the start of the secondary walls deposition, minor but differentiation-specific changes can be observed on two-dimensional polypeptide maps of *Zinnia* cells (Fukuda and Komamine, 1983). Two newly synthesized polypeptides appear in cells cultured in the medium that induces formation of tracheary elements, but not in cells cultured in a control medium, and the synthesis of these polypeptides continues at least until the time at which the secondary wall begins to form.

Differential screening of a cDNA library yielded four clones that contained cDNA inserts whose corresponding mRNAs were expressed preferentially in cells in differentiation-induced culture (Table 1; Demura and Fukuda, 1993). The cDNAs were designated *TEDs* (for Tracheary Element Differentiation-related genes) 1 to 4, respectively. *TED1* cDNA corresponded to the *mt atpA* gene (mitochondrial gene for the α-subunit for F1-ATPase) in *Zinnia*. A homology search revealed significant similarity between TED2 and the gene for zeta-crystallin, which was recently demonstrated to function as an NADPH: quinone oxidoreductase, from the guinea-pig lens (REF). The polypeptide sequences deduced from *TED3* cDNA indicate that *TED3* encodes a novel hydrophilic protein with an Asp-Gly-Tyr motif that is repeated fifteen times. The polypeptide encoded by *TED3* may be a protein that is located in the secondary walls of tracheary elements. *TED4* cDNA encodes a polypeptide of 10 kDa with a transit peptide at the N-terminus. A homology search with the nucleotide and deduced amino-acid sequences of *TED4* revealed significant similarity to those of the barley aleurone-specific clone, B11E. Each *TED* gene was expressed preferentially in cells in differentiation-induced culture, from 24 h before the start of formation of secondary walls.

In situ hybridization using probes derived from the above-mentioned cDNAs in various tissues from young *Zinnia* seedlings indicated that the expression of the corresponding genes is restricted to cells that are involved in the process of vascular differentiation during development in intact plants (Table1, Demura and Fukuda, 1994). *TED3* was expressed specifically in differentiating vessel elements or in the cells that were expected soon to differentiate into vessel elements, in all organs tested. The expression of *TED4* was also restricted to vascular or future vascular cells, in particular, xylem cells. *In situ* hybridization with sections of cotyledons revealed that *TED4* was expressed at an earlier stage of vessel differentiation in the main leaf vein than was *TED3*. In contrast to *TED3* and *TED4*, *TED2* was not expressed in the xylem or the phloem of the main leaf vein. Instead, it was expressed in two small areas wherein parenchyma cells might differentiate into vascular cells to form new veins. In root tips, *TED2*-specific transcripts were observed in restricted procambial regions that were predicted to form phloem and xylem. These results suggest that *TED2* transcripts accumulate at the very early stage of the differentiation of meristematic cells into vessel cells. *In situ*, the genes corresponding to the *TED* cDNAs seem to be expressed in the following order: *TED2*, *TED4* and *TED3*. Cells expressing *TED2*-specific mRNA seem to be procambium-like cells and have the capacity to become future phloem and xylem cells, and cells expressing *TED3*-specific mRNA are differentiating vessel cells or cells that have the capacity to become vessel cells. Thus, during differentiation from meristematic cells to vessel cells, the capacity for differentiation may be switched from pluripotency to single potency, whereby cells can become vessel cells exclusively.

An overview of differentiation to tracheary elements

Figure 6 provides a summary of various events that occur during transdifferentiation of isolated mesophyll cells of *Zinnia elegans*

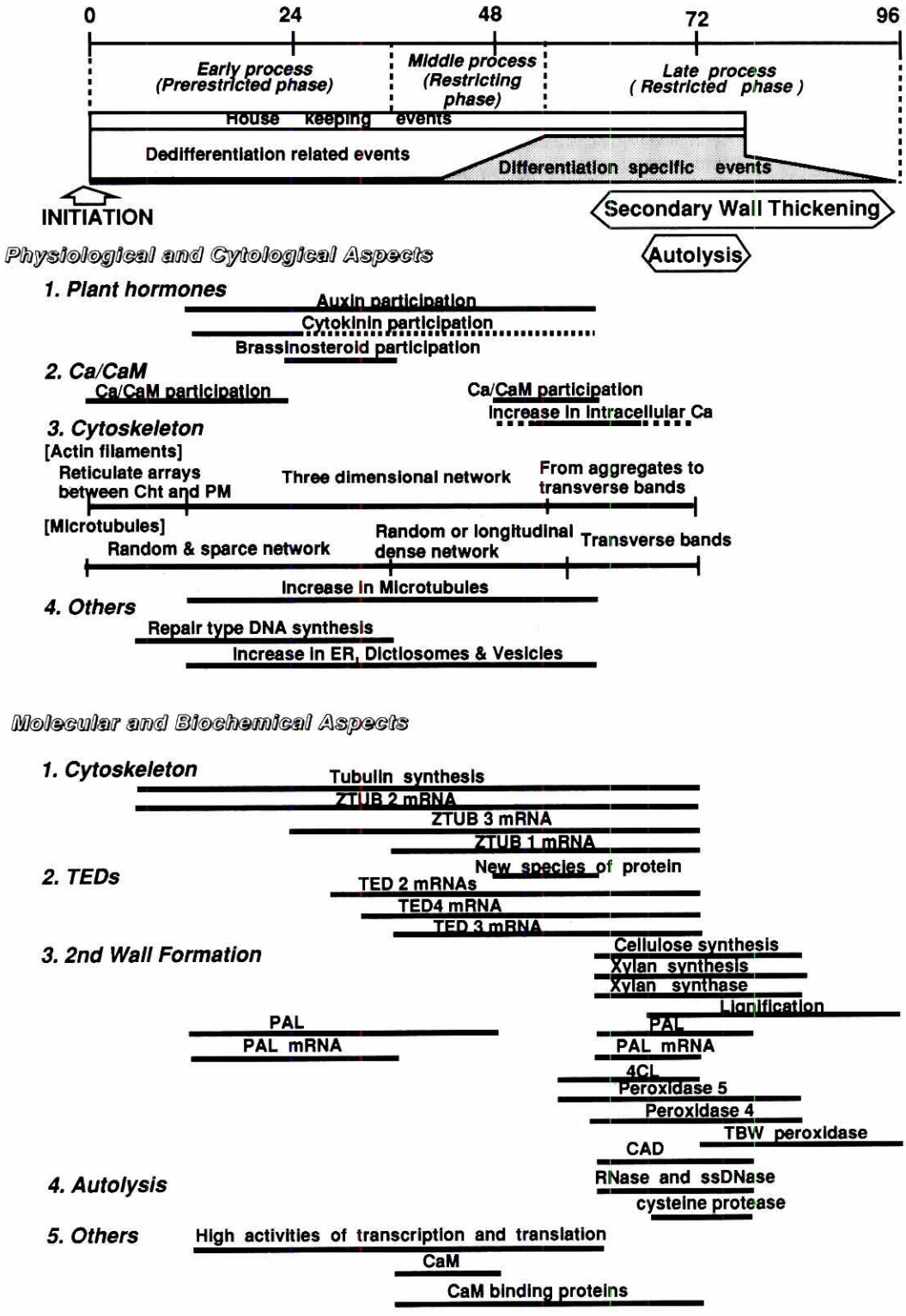

Fig. 6. Sequential events during the differentiation of single mesophyll cells of *Zinnia elegans* into tracheary elements. (A) *Physiological and cytological events.* **(B)** *Molecular and biochemical events. CaM, calmodulin; Cht, chloroplast; PM, plasma membrane; PAL, phenylalanine ammonialyase; 4CL, 4-coumarate: CoA ligase; TBW, tightly bound to the cell walls; CAD, cinnamyl alcohol dehydrogenase.*

into tracheary elements (Fukuda, 1992, 1994). The initiation of differentiation occurs upon wounding and exposure to phytohormones, isolated mesophyll cells differentiate into tracheary elements. The process of differentiation can divided into early, middle and late processes, which can be defined as prerestricted, restricting and restricted processes, respectively.

The early process is thought to be complex, involving a variety of events that can themselves be classified into three groups, namely, housekeeping events, dedifferentiation-related events and differentiation-specific events. Most of the events in this early process occur in almost all cultured cells, i.e., in both differentiating and non-differentiating cells, and, therefore, they are not specific to

differentiation. However, these events are necessary for the progression of differentiation. For example, the expression of genes for tubulin proteins during the early process is observed both in the differentiation-induced and control cultures. However, synthesis of tubulin is essential for the construction of new arrays of microtubules during the late process of differentiation, which controls the ordered thickenings of secondary walls. Such events may be involved in the dedifferentiation process, during which isolated mesophyll cells lose their potential to function as photosynthetic cells and acquire the ability to grow and differentiate in the new environment. Unfortunately, no differentiation-specific events at the early stage have yet been defined.

In the middle process, the capacity for differentiation of cells that have dedifferentiated from mesophyll cells is becoming restricted from pluripotency that would allow differentiation into xylem and/or phloem cells to single potency for differentiation to tracheary elements. This process seems to correspond to the process *in vivo* whereby meristematic cells change to vessel cells via procambium.

The late process involves a variety of events specific to the formation of tracheary elements, most of which seem to be associated with thickening of the secondary wall and autolysis. In this process, cells are engaged, probably irreversibly, in the formation of tracheary elements. Although much information has been accumulated on formation of secondary wall, little information is available about autolysis. The analysis of the mechanism of autolysis is of considerable interest because this process is considered to be a typical example of programmed cell death. Further extensive studies of this process are now necessary.

References

ALEITH, F. and RICHTER, G. (1990). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. *Planta 183:* 17-24.

AMINO, S., FUJIMURA, T. and KOMAMINE, A. (1983). Synchrony induced by double phosphate starvation in suspension cultures of *Catharanthus roseus*. *Physiol. Plant. 59:* 393-396.

BAUER, G.A. and BURGERS, M.J. (1990). Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene. *Nucleic Acids Res. 18:* 261-265.

BORKIRD, C., CHOI, J.H., JIN, Z., FRANZ, G., HATZOPOULOS, P., CHORNEAU, R., BONAS, R., PELEGRI, F. and SUNG, Z.R. (1988). Developmental regulation of embryonic genes in plants. *Proc. Natl. Acad. Sci. USA 85:* 6399-6403.

BRAVO, R., FRANK, R., BLUNDELL, P.A. and MacDONALD-BRAVO, H. (1987). Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. *Nature 326:* 515-517.

CARDOSO, M.C., LEONHARDT, H. and NADAL-GINARD, B. (1993). Reversal of terminal differentiation and control of DNA replication: cyclin A and cdk2 specifically localized at subnuclear sites of DNA replication. *Cell 74:* 979-992.

CHEN, J. and VARNER, J.E. (1985). Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. *Proc. Natl. Acad. Sci. USA 82:* 4399-4403.

CHIBBAR, R.N., POLOWICK, P.L., NEWSTED, W.J., SHYLUK, J. and GEORGES, F. (1989). Identification and isolation of a unique esterase from the medium of non-embryogenic cell line of cultured carrot cells. *Plant Cell Tissue Org. 18:* 47-53.

CHOI, J.H., LIU, L.S., BORKIRD, C. and SUNG, Z.R. (1987). Isolation of cDNA clones for rare embryo specific antigens in carrot cell cultures. *Proc. Natl. Acad. Sci. USA 84:* 1906-1910.

CHURCH, D.L. and GALSTON, A.W. (1989). Hormonal induction of vascular differentiation in cultured *Zinnia* leaf disks. *Plant Cell Physiol. 30:* 73-78.

DEMURA, T. and FUKUDA, H. (1993). Molecular cloning and characterization of cDNAs associated with tracheary element differentiation in cultured *Zinnia* cells. *Plant Physiol. 103:* 815-821.

DEMURA, T. and FUKUDA, H. (1994). Novel vascular-cell-specific genes whose expression is regulated temporally and spatially during vascular system development. *Plant Cell.* (In press).

DURE, L. III, CROUCH, M., HARADA, J., HO, T.D., MUNDY, J., RALPH, Q., THOMAS, T. and SUNG, Z.R. (1989). Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol. Biol. 12:* 475-486.

FANG, F. and NEWPORT, J.W. (1991). Evidence that the G_1-S and the G_2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell 66:* 731-742.

FRANZ, G., HATZOPOULOS, P., JONES, T.J., KRAUSS, M. and SUNG, Z.R. (1989). Molecular and genetic analysis of an embryogenic gene, DC 8, from *Daucus carota* L. *Mol. Gen. Genet. 218:* 143-151.

FUJIMURA, T. and KOMAMINE, A. (1975). Effects of growth regulators on embryogenesis in a carrot suspension culture. *Plant Sci. Lett. 5:* 359-364.

FUJIMURA, T. and KOMAMINE, A. (1979a). Involvement of endogenous-auxin in somatic embryogenesis in a carrot cell suspension culture. *Z. Pflanzenphysiol. 95:* 13-19.

FUJIMURA, T. and KOMAMINE, A. (1979b). Synchronization of somatic embryogenesis in a carrot cell suspension culture. *Plant Physiol. 64:* 162-164.

FUJIMURA, T. and KOMAMINE, A. (1980). The serial observation of embryogenesis in a carrot cell suspension culture. *New Phytol. 86:* 213-218.

FUKUDA, H. (1987). Change in tubulin synthesis in the process of tracheary element differentiation and cell division of isolated *Zinnia* mesophyll cells. *Plant Cell Physiol. 28:* 517-528.

FUKUDA, H. (1989a). Cytodifferentiation in isolated single cells. *Bot. Mag. Tokyo 102:* 492-501.

FUKUDA, H. (1989b). Regulation of tubulin degradation in isolated *Zinnia* mesophyll cells in culture. *Plant Cell Physiol. 30:* 243-252.

FUKUDA, H. (1992). Tracheary element differentiation as a model system of cell differentiation. *Int. Rev. Cytol. 136:* 289-332.

FUKUDA, H. (1994). Redifferentiation of single mesophyll cells into tracheary elements. *Int. J. Plant Sci.* (In press).

FUKUDA, H. and KOBAYASHI. H. (1989). Dynamic organization of the cytoskeleton during tracheary element differentiation. *Dev. Growth Differ. 31:* 9-16.

FUKUDA, H. and KOMAMINE. A. (1980a). Establishment of an experimental system for tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans* . *Plant Physiol. 65:* 57-60.

FUKUDA, H. and KOMAMINE. A. (1980b). Direct evidence for cytodifferentiation to tracheary elements without intervening mitosis in a culture of single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol. 65:* 61-64.

FUKUDA, H. and KOMAMINE. A. (1981). Relationship between tracheary element differentiation and the cell cycle in single cells isolated from the mesophyll of *Zinnia elegans*. *Physiol. Plant. 52:* 423-430.

FUKUDA, H. and KOMAMINE. A. (1982). Lignin synthesis and its related enzymes as markers of tracheary-element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Planta 155:* 423-430.

FUKUDA, H. and KOMAMINE. A. (1983). Changes in the synthesis of RNA and protein during tracheary element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Cell Physiol. 24:* 603-614.

FUKUDA, H. and KOMAMINE. A. (1985). Cytodifferentiation. In *Cell Culture and Somatic Cell Genetics of Plants* (Ed. K. Vasil), Vol. 2. Academic Press, Orlando, pp. 149-212.

FUKUDA, H., YOSHIMURA, T., SATO, Y. and DEMURA, T. (1993). Molecular mechanism of xylem differentiation. *J. Plant Res. 3:* 93-107.

HATA, S., KOUCHI, H., SUZUKA, I. and ISHII, T. (1991). Isolation and characterization of cDNA clones for plant cyclins. *EMBO J. 10:* 2681-2688.

HEMERLY, A., BERGOUNIOUX, C., VAN MONTAGU, M., INZE, D. and FERREIRA, P. (1992). Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA 89:* 3295-3299.

HIRT, H., MINK, M., PFOSSER, M., BOGRE, L., GYORGYEY, J., JONAK, C., GARTNER, A., DUDITS, D. and HEBERLE-BORS, E. (1992). Alfalfa cyclins: differential expression during the cell cycle and in plant organs. *Plant Cell 4:* 1531-1538.

HONG, J.C., NAGAO, R.T. and KEY, J.L. (1987). Characterization and sequence analysis of a developmentally regulated putative cell wall protein gene isolated from soybean. *J. Biol. Chem. 262:* 8367-8376.

ITO, M., KODAMA, H. and KOMAMINE, A. (1991). Identification of a novel S-phase specific gene during the cell cycle in synchronous cultures of *Catharanthus roseus* cells. *Plant J. 1:* 141-148.

ITO, M., YASUI, A. and KOMAMINE, A. (1992). A gene family homologous to the S-phase specific gene in higher plants is essential for cell proliferation in *Saccharomyces cerevisiae*. *FEBS Lett. 301:* 29-33.

JASKULSKI, D., DERIEL, J.K., MERCER, W.E., CALABERETTA, B. and BASERGA, R. (1988). Inhibition of cellular proliferation by antisense oligonucleotides to PCNA cyclin. *Science 240:* 1544-1546.

KOBAYASHI, H., FUKUDA, H. and SHIBAOKA, H. (1987). Reorganization of actin filament associated with the differentiation of tracheary elements in *Zinnia* mesophyll cells. *Protoplasma 138:* 69-71.

KOBAYASHI, H., FUKUDA, H. and SHIBAOKA, H. (1988). Interrelationship between the spatial disposition of actin filaments and microtubules during the differentiation of tracheary elements in cultured *Zinnia* cells. *Protoplasma 143:* 29-37.

KODAMA, H., ITO, M., OHNISHI, N., SUZUKA, I. and KOMAMINE, A. (1991a). Molecular cloning of the gene for plant proliferating-cell nuclear antigen and expression of this gene during the cell cycle in synchronized cultures of *Catharanthus roseus* cells. *Eur. J. Biochem. 197:* 495-503.

KODAMA, H., KAWAKAMI, N., WATANABE, A. and KOMAMINE, A. (1989). Phase-specific polypeptides and poly(A)$^+$ RNAs during the cell cycle in synchronous cultures of *Catharanthus roseus* cells. *Plant Physiol. 89:* 910-917.

KODAMA, M., ITO, M., HATTORI, T., NAKAMURA, K.and KOMAMINE, A. (1991b). Isolation of genes that are preferentially expressed at the G_1/S boundary during the cell cycle in synchronized cultures of *Catharanthus roseus* cells. *Plant Physiol. 95:* 406-411.

KOORNNEEF, M., BRADE, J., HANHART, C., HORSMAN, K., SCHEL, J., SOPPE, W., VERKKERK, R. and ZABEL, P. (1993). Characterization and mapping of a gene controlling shoot regeneration in tomato. *Plant J. 3:* 131-141.

LEHNER, C.F., YAKUBOVICH, N. and O'FARREL, P.H. (1991). Exploring the role of *Drosophila* cyclin A in the regulation of S phase. *Cold Spring Harbor Symp. Quant. Biol. 56:* 465-475.

LEW, D.J., DULIC, V. and REED, S.I. (1991). Isolation of three novel human cyclins by rescue of G_1 cyclin (Cln) function in yeast. *Cell 66:* 1197-1206.

LEWIS, N.G., and YAMAMOTO, E. (1990). Lignin: occurrence, biogenesis and biodegradation. *Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:* 455-496.

MASUDA, H., FUKUDA, H. and KOMAMINE, A. (1983). Changes in peroxidase isoenzyme pattern during tracheary element differentiation in culture of single cells isolated from the mesophyll of *Zinnia elegans*. *Z. Pflanzenphysiol. 112:* 417-426.

McKINNEY, J.D. and HEINTZ, N. (1991). Transcriptional regulation in the eukaryotic cell cycle. *TIBS 16:* 430-435.

MEYEROWITZ, E.M. (1989). *Arabidopsis*, a useful weed. *Cell 56:* 263-269.

NAGATA, T., NEMOTO, Y. and HASEZAWA, S. (1992). Tobacco BY-2 cell line as the HeLa cell in the cell biology of higher plants. *Int. Rev. Cytol. 132:* 1-30.

NISHIDA, T., OHNISHI, N., KODAMA, H. and KOMAMINE, A. (1992). Establishment of synchrony by starvation and readdition of auxin in suspension cultures of *Catharanthus roseus* cells. *Plant Cell Tissue Org. 28:* 37-43.

NOMURA, K. and KOMAMINE, A. (1985). Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. *Plant Physiol. 79:* 988-991.

NURSE, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature 344:* 503-507.

PAGANO, M., PEPPERKOK, R., VERDE, F., ANSORGE, W. and DRAETTA, G. (1992). Cyclin A is required at two points in the human cell cycle. *EMBO J. 11:* 961-971.

PINES, J. and HUNTER, T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with P34^{cdc2}. *Cell. 58:* 833-846.

PINES, J. and HUNTER, T. (1990). Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature 346:* 760-763.

REINERT, J. (1958). Untersuchungen über die Morphogenese an Gewebekulturen. *Ber. Deut. Bot. Ges. 71:* 15.

RICHARDSON, H.E., WITTENBERG, C., CROSS, F. and REED, S.I. (1989). An essential G_1 function for cyclin-like proteins in yeast. *Cell 59:* 1127-1133.

ROBERTS, L.W., GAHAN, P.B. and ALONI, R. (1988). *Vascular Differentiation and Plant Growth Regulators*. Springer-Verlag, Berlin, pp. 154.

SATO, Y., SUGIYAMA, M., GORECKI, R.J., FUKUDA, H. and KOMAMINE, A. (1993). Interrelationship between lignin deposition and the activities of peroxidase isozymes in differentiating tracheary elements of *Zinnia*: analysis with L-α-aminooxy-ß-phenylpropionic acid and 2-aminoindan-2-phosphonic acid. *Planta 189:* 584-589.

SMITH, J.A., KRAUSS, M.R., BORKIRD, C. and SUNG, Z.R. (1988). A nuclear protein associated with cell divisions in plants. *Planta.* 174: 462-472.

STEWARD, F.C., MAPES, M.O. and MEARS, K. (1958). Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Am. J. Bot. 45:* 705-708.

SUGIYAMA, M. and KOMAMINE, A. (1990). Transdifferentiation of quiescent parenchymatous cells into tracheary elements. *Cell Differ. Dev. 31:* 77-87.

SUNG, Z.R. and OKIMOTO, R. (1981). Embryonic proteins in somatic embryos of carrot. *Proc Natl. Acad. Sci. USA. 78:* 3683-3687.

SUNG, Z.R. and OKIMOTO, R. (1983). Coordinate gene expression during somatic embryogenesis in carrot. *Proc. Natl. Acad. Sci. USA. 80:* 2661-2665.

SUZUKA, I., DAIDOJI, H., MATSUOKA, M., KADOWAKI, K., TAKASAKI, Y., NAKANE, P.K. and MORIUCHI, T. (1989). Gene for proliferating-cell nuclear antigen (DNA polymerase auxiliary protein) is present in both mammalian and higher plant genomes. *Proc. Natl. Acad. Sci. USA 86:* 3189-3193.

TAKAHASHI, Y., KURODA, H., TANAKA, T., MACHIDA, Y., TAKEBE, I. and NAGATA, T. (1989). Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts. *Proc. Natl. Acad. Sci. USA 86:* 9279-9283.

TAN, C., CASTILLO, C., SO, A.G. and DOWNEY, K.M. (1986). An auxiliary protein for DNA polymerase- from fetal calf thymus. *J. Biol. Chem. 261:* 12310-12316.

THELEN, M.P., and NORTHCOTE, D.H. (1989). Identification and purification of a nuclease from *Zinnia elegans* L.: a potential molecular marker for xylogenesis. *Planta 179:* 181-195.

TORREY, J.G., FOSKET, D.E. and HEPLER, P.K. (1971). Xylem formation: a paradigm of cytodifferentiation in higher plants. *Am. Sci. 59:* 338-352.

VALVEKENS, D., VAN MONTAGU, M. and LIJSEBETTENS, M. (1988). Agrobacterium tumefaciens-mediated transformation of Arabidopsis thalianaroot explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA.* 85: 5536-5540.

WILDE, H.D., NELSON, W.S., BOOIJ H., DE VRIES, S.C. and THOMAS, T.L. (1988). Gene-expression programs in embryogenic and non-embryogenic carrot cultures. *Planta. 176:* 205-211.

YASUTANI, I., OZAWA, S., NISHIDA, T., SUGIYA, M. and KOMAMINE, A. (1994). Isolation of temperature-sensitive mutants of *Arabidopsis thaliana* that are defective in the redifferentiation of shoots. *Plant Physiol.* (In press).

YE, Z-H. and VARNER, J.E. (1993). Gene expression patterns associated with *in vitro* tracheary element formation from isolated mesophyll cells of *Zinnia elegans*. *Plant Physiol. 103:* 805-813.

ZINDY, F., LAMAS, E., CHENIVESSE, X., SOBCZAK, J., WANG, J., FESQUET, D., HENGLEIN, B. AND BRECHOT, C. (1992). Cyclin A is required in S phase in normal epithelial cells. *Biochem. Biophys. Res. Commun. 182:* 1144-1154.