Int. J. Dev. Biol. 38: 321-327 (1994)

Genes involved in the dedifferentiation of plant cells

TOSHIYUKI NAGATA*, SARAHMI ISHIDA, SEIICHIRO HASEZAWA and YOHSUKE TAKAHASHI

Department of Plant Sciences, Graduate School of Science, The University of Tokyo, Japan

ABSTRACT Since the initial process of culturing tobacco mesophyll protoplasts can be considered as a model system of dedifferentiation of higher plants, the mode of expression of genes induced by auxin, a key factor in inducing dedifferentiation, has been analyzed during the regaining of meristematic activity of quiescent and differentiated tobacco mesophyll. By differential screening we have isolated three auxin-regulated genes, which we named *parA, parB* and *parC. parA* and *parC,* which belong to the same gene family, were supposed to play a role in transcriptional regulation upon induction by auxin, while par*B* encoded glutathione *S*-transferase. Although it was supposed that the expression of these *par* genes should play a pivotal role in regaining the meristematic activity of the differentiated tobacco mesophyll cells, a possibility that other less abundantly expressing genes would have been neglected in these studies has not been excluded. On the other hand, the search for genes which would be involved in maintaining cell division activity in the dedifferentiated plant cells allowed us to isolate a few genes. One of these genes, designated *arcA,* belonged to a ß subunit-like protein of heterotrimeric G proteins. The significance of the involvement of this gene product in maintaining the meristematic activity of plant cells cultured *in vitro* has been discussed.

KEY WORDS: *auxin, dedifferentiation, tobacco BY-2 cell line, tobacco mesophyll protoplast, totipotency*

Introduction

From the point of view of developmental biology, plants are unique in that totipotency is widely recognized in various higher plants. When cells from differentiated and non-dividing tissues are cultured in appropriate conditions, they regain meristematic activity and divide to form colonies at high frequency. Subsequently whole plants can be regenerated from these colonies (Nagata and Takebe, 1971). Thus the totipotency is comprised of dedifferentiation and subsequent redifferentiation. Among other conditions, plant hormones have been shown to play a pivotal role during these processes. The molecular mechanism underlying the totipotency of plant cells is intriguing to understand the molecular sequence of differentiation, as a model system, in higher organisms. Since it is known that only a few factors of auxin and cytokinin as plant hormones play an important role, elucidation of the mode of expressions of genes induced by these plant hormones would give us clues to disclose the molecular mechanism of dedifferentiation.

As an experimental material, the culture system of tobacco mesophyll protoplasts has various advantages, since they are derived from typically differentiated tissues which have been actively involved in photosynthesis. Furthermore, the preparation of experimental materials has practically no limitation, as one can get more than 10^7 cells from 1 g of a leaf at an optimal condition. When they are cultured in a synthetic medium under appropriate conditions, they display the process of dedifferentiation in a synchronous manner (Nagata and Takebe, 1970). Thus we start to elucidate the molecular mechanism of dedifferentiation using tobacco mesophyll protoplasts. Thus far we have isolated 3 genes regulated by auxin from this system (Takahashi *et al.*, 1989; Takahashi and Nagata 1992a,b). The rationale for searching for such plant hormone-regulated genes is that one could look for molecular sequences upstream and downstream to these plant hormone-regulated genes. Thus in this article we shall try to unravel the molecular mode of dedifferentiation of plant cells based on the expression of these genes from our recent studies.

On the other hand, dedifferentiated stages of plant cells can be maintained in appropriate media mostly based on Murashige and Skoog medium (1962) or Linsmaier and Skoog medium (1966) containing auxin. Again the elucidation of the role of auxin in maintaining the dedifferentiated state would give us clues for understanding some facets of dedifferentiation. For this purpose, the cultured cells of tobacco BY-2 should be most suitable, since this cell line grows very fast and is composed of homogeneous cells and high synchrony in cell division can be induced (Nagata *et al.*,

Abbreviations used in this paper: ABP, auxin-binding protein; 2,4-D, 2,4-dichlorophenoxyacetic acid; GST, glutathione *S*-transferase; IAA, indole-3-acetic acid.

***Address for reprints:** Department of Plant Sciences, Graduate School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan. FAX: 81-3.56842063.

0214-6282/94/$03.00

Printed in Spain

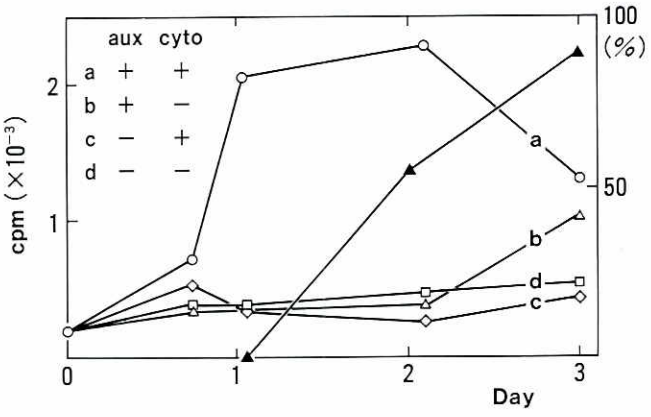

Fig. 1. Time course of DNA synthesis and cell division of tobacco mesophyll protoplasts cultured *in vitro*. *Tobacco mesophyll protoplasts were cultured in the medium of Nagata and Takebe (1970) supplemented with 2,4-D (1 mg/l) and 6-benzylaminopurine (1 mg/l)* **(a)**, *with 2,4-D* **(b)**, *with 6-benzylaminopurine* **(c)** *and without plant hormones* **(d)**. *DNA synthesis was assessed by the incorporation of 3H-thymidine into the acid-insoluble fractions. The triangle represents time course of the divided protoplasts cultured in the medium* **(a)** *assessed by staining with lactopropionic orcein. Without either plant hormone no cell division was observed.*

1992). When we looked for auxin-regulated genes in this experimental system, we isolated a few auxin-regulated genes and analyzed the mode of expression of one gene (Ishida *et al.*, 1993)

Thus in this study the dedifferentiation from differentiated and non-dividing cells and the maintenance of the dedifferentiated stages will be discussed with special reference to the role of auxin.

Results and Discussion

Time course of the culture of tobacco mesophyll protoplasts.

When we started the culture of tobacco mesophyll protoplasts in 1970, we learned the process of culturing the protoplasts was rather synchronous from morphological aspects (Nagata and Yamaki, 1973). We determined the time course of the DNA synthesis and cell division of mesophyll protoplasts which had been isolated from fully expanded tobacco leaves. Although the time course of DNA synthesis and cell division of tobacco mesophyll protoplasts had been previously reported by Zelcer and Galun (1976), we found that their results were rather different from what we had seen in our experiments. Reexamination of the time course of DNA synthesis assessed by ^{3}H-thymidine incorporation and cell division assessed by nuclear staining with lacto-propionic orcein revealed that the time course of the DNA synthesis and cell division was faster and more synchronous than that reported by Zelcer and Galun (1976) as shown in Fig. 1. DNA synthesis was initiated from 17 h and reached a maximum at 24 h, while cell division was observed later than 24 h and 90% of cells divided at least once until 3 days of culture. DNA synthesis was induced only in the presence of both auxin and cytokinin, and was not observed in the medium which lacked either of the two plant hormones. Only in the absence of cytokinin was some limited amount of 3H-thymidine incorporated into the acid-insoluble fraction after 3 days of culture. Although it is necessary whether this incorporation would reflect the actual DNA synthesis, this could reflect the cytokinin habituation as reported by Meins *et al.* (1980). Since the effect of the depletion of auxin from the medium was more drastic than that of cytokinin, we tried to examine the molecular changes induced by auxin first. In this trial we used the 7 mineral culture medium of Nagata and Takebe (1970), supplemented with thiamine, myo-inositol, 2,4-dichorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine, but not containing sucrose. We chose this medium because the viability of the cultured cells was maintained at a higher level than in more complex medium for at least 2-3 days of culture (unpublished results) and we could avoid the complexity arising from the interactions between components of more complex media. The induction of cell division under these conditions was more synchronous and rapid than other data (Fig. 1). However, later than 3 days of culture, cells proliferated more actively in the medium of Nagata and Takebe (1971). These results showed that within 24 h of culture tobacco mesophyll protoplasts displayed the progression of cell cycle from G_0 to S phase and suggested the prominent expression of some genes, which would be involved in the conversion of differentiated cells to actively dividing cells.

Search for auxin-regulated genes in tobacco mesophyll protoplasts

We looked for auxin-regulated genes after 1 day of culture by differential screening from the reason as described above. The other reason that we looked for auxin-regulated genes is that Meyer *et al.* (1981) demonstrated that auxin induced the appearance of two polypeptides, while cytokinin did not induce any new polypeptides in their analyses of 2-D gel electrophoresis.

Differential screening regarding auxin after 24 h of culture allowed us to isolate 3 auxin-regulated genes, which we named *par* (protoplast auxin-regulated)*A*, *parB* and *parC*. Next we describe the characteristics and mode of expressions of these genes induced by auxin. Northern hybridization revealed that these *par* genes were expressed as early as 10 min after the addition of auxin and reached a maximum after 4 h of culture, as shown in Fig. 2, in which the expression of *parB* was represented as the typical case among the auxin-regulated *par* genes. After 24 h of culture when active DNA synthesis was observed, the expression of *par* genes started to decrease. After 2 days of culture, when active cell division was observed, the expression of *par* genes was suppressed. From this evidence, we illustrated a schematic presentation of the relationship between the expression of *par* genes and the cell cycle progression (Fig. 3). It is concluded that the expression of *par* genes was induced immediately upon addition of auxin, but started to decrease after more than 24 h of culture. It was confirmed that the expression of *par* genes was closely linked with the entering of cells of G_0 phase into cell cycle, resulting in S phase. Apparently the expression of *par* was not dependent on any specific cell cycle, since it was evenly observed among the 4 cell cycle stages of G_1, S, G_2 and M prepared from highly synchronized tobacco BY-2 cells as described in Nagata *et al.* (1992) (unpublished results).

Subsequently we should ask what the functions of these *par* genes are. Since we learned that *parA* has homology to *parC*, we describe first the characteristics of *parA-related* genes and then describe the *parB*-related genes.

parA-related genes

The *parA* gene encodes a polypeptide of 220 amino acids, which was found to have homology to a protein having a molecular

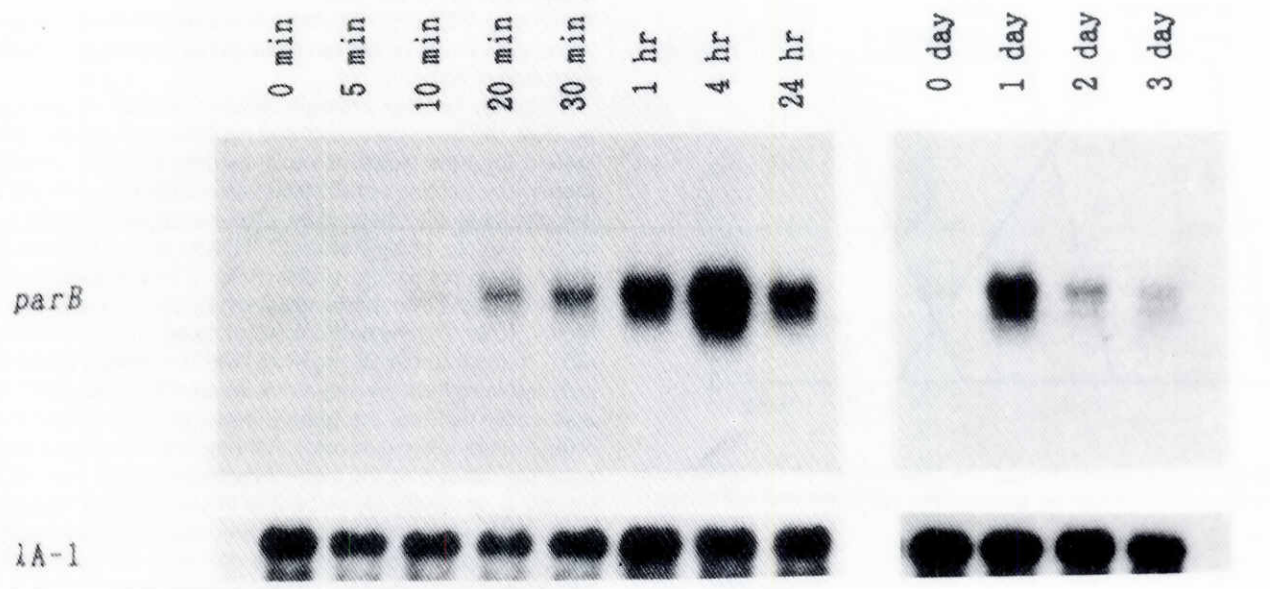

Fig. 2. Time course of the expression of *parB* gene. *Induction of kinetics of parB mRNA by the addition of 2,4-D was followed for 3 days by Northern hybridization using parB cDNA as a probe. λA-1 was used as a control whose expression was not dependent on auxin. The time course of the expression of parA and parC was essentially in the same line as that shown in this figure. RNA was extracted at the indicated times after the addition of 2,4-D. Each lane received 20 μg of total RNA.*

mass of 24 kDa, which was induced in *Escherichia coli* upon culture in nutritionally starved conditions (Takahashi *et al.*, 1991). Since the bacterial protein bound equimolar to RNA polymerase and is suggested to regulate transcription, it is supposed that the *parA* gene product would have a similar function in higher plants as well. This supposition is based on the fact that RNA polymerase is structurally well preserved from prokaryotes to eukaryotes (Sweeter *et al.*, 1987). If the *parA* gene product has a role in regulating transcription, the subcellular localization of their gene products should be in the nuclei of eukaryotic cells. In fact, the immunostaining of tobacco mesophyll protoplasts cultured for 6 h in the presence of auxin with an anti-*parA* antibody showed unambiguous localization of *parA* gene product in the nucleus, while freshly prepared protoplasts did not show such a staining pattern (unpublished results). The functional proof of the *parA* gene product in transcriptional regulation, however, has not been elucidated at the molecular level.

parC, which had been isolated as one of auxin-regulated genes from the cDNA library of cultured tobacco mesophyll protoplasts, was found to have homology with *parA* (Takahashi and Nagata, 1992b). This fact prompted us to look for other genes which are related to *parA* in the cDNA library. The search for such genes in the cDNA library using *parA* gene as a probe enabled us to isolate another related gene of *C-7* under a less stringent hybridization condition. *C-7* did not respond to auxin at all, but rather the expression of *C-7* was predominantly observed in the differentiated tissues such as leaves and stems. The evidence that the *C-7* gene was under the regulation of developmental stages promoted us to compare the 5' non-coding regions among these genes, since there should be some regulatory elements for directing the expression and repression of these genes responding to external stimuli and internal signals (work in progress).

On the other hand, it should be intriguing to look for other *parA*-related genes in other culture conditions than tobacco mesophyll protoplasts and in other plants. The search for *parA*-related genes in GenBank revealed that there have been reported several genes as shown in Table 1. Although the most common function was their response to auxin, some other responses were observed. Although a cDNA clone of pLS216 was initially isolated as a cytokinin-responding clone, the clone was finally found to respond to auxin as well (Dominov *et al.*, 1992). *Gmhsp 26-A* (pGH2-4) was induced by various kinds of stress conditions including heat shock and heavy metal treatment (Czarnecka *et al.*, 1988). Although *prp1* from potato was originally reported to respond to elicitors, it was also found to respond to auxin (Strittmatter, personal communication). Since all *parA*-related genes have homology to the bacterial 24 kDa protein described above, they should have a function in regulating transcriptional regulation.

parB-related genes

The sequence determination of *parB* gene showed that it encodes a polypeptide of 213 amino acids and has homology with a maize glutathione *S*-transferase III (GST; RX: glutathione R-transferase, EC 2.5.1.18) (Grove *et al.*, 1988). To examine whether the gene product of *parB* has in fact an enzymatic activity of GST, we inserted the coding sequence of *parB* into a bacterial expression vector, pKK233-2. When *E. coli* harboring this plasmid was induced with isopropyl β-D-thiogalactopyranoside, the bacteria produced a fusion protein, which showed high activity of GST. Thus it was confirmed that auxin-regulated *parB* gene encodes GST. A further question is what is the function of the GST during the cell cycle progression from G_0 to *S* phase, although thus far functions of GST have been generally supposed to be involved in detoxification of xenobiotics (Rushmore *et al.*, 1990). Alternatively, since

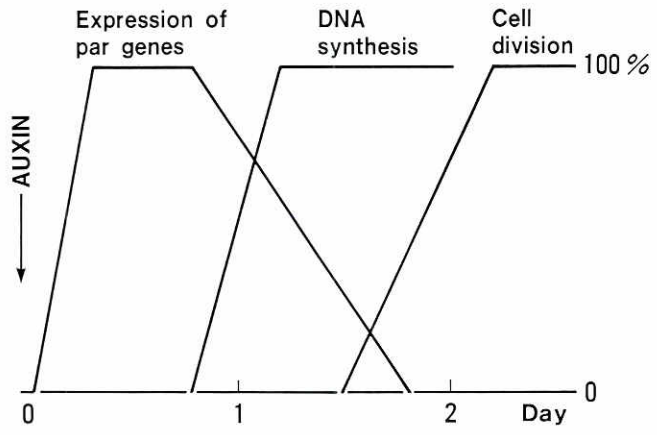

Fig. 3. Schematic illustration of the time course of the addition of auxin, the expression of *par* genes, DNA synthesis and cell division

there is some specific homology of *parB*-GST with placental GSTs among various GSTs, there should be a specific functional link between *parB*-GST and placental GSTs. The placental GSTs have been shown to be closely related with tumorigenesis and are assigned to be a marker enzyme of *in vitro* carcinogenesis (Satoh *et al.*, 1985). For understanding the actual function it is necessary to carry out more molecular biological and biochemical examinations. By the way, regarding the search for the clue to find the function of GST, we examined intracellular localization of the *parB* gene products by immunostaining using an antibody against a *parB* gene product. Such examination showed that the *parB* gene products were accumulated in tobacco mesophyll protoplasts during culture in the medium containing auxin (unpublished results). The *parB* gene products were localized in the cytoplasm, but were detected in the nuclei. It was conspicuous that chloroplasts were surrounded with these antigens. Thus the intracellular localization of *parB* gene products was significantly different from that of *parA* gene products. It is possible that the GST regulates the level of glutathione (GSH) resulting in the transdifferentiation of differentiated tobacco mesophylls, as Earnshaw and Johnson (1988) suggested that the concentration of glutathione as a substrate of GST would regulate the intracellular pH, resulting in development of embryogenesis of carrot cells cultured *in vitro*.

Regarding the *parB* gene product as GST, Droog *et al.* (1993) claimed that *parA*-gene and *parB*-gene families should be unified as a GST family because of very weak homology between these two gene families. This claim is solely based on the fact that a gene *103*, which has 47% identity to *parA* at the protein level, showed an enzymatic activity of GST, although it was relatively weak. However, this evidence does not necessarily support their claim, because as described above, *parA* gene products were localized in the nucleus, while *parB* gene products were localized primarily in the cytoplasm. It is improbable that two proteins which had intracellularly different locations would have the same function. Since the *parA* gene product had only a residual level of GST activity (unpublished results), it is more reasonable to interpret that *parA* and *parB* genes had a common ancestor and evolved in different directions. Such an example was reported by Tomarev and Zinovieva (1988) that lens crystallins in squid are composed of a protein having an enzymatic activity of GST, but having no function as GSTs. In this case the protein which shares a common ancestor with GSTs has been utilized as lens crystallins during the evolution of squid.

Recently another possible function of GST as the *parB* gene product has been demonstrated by Zettl *et al.* (1994). When they looked for new types of auxin-binding proteins (ABPs) in the membrane fractions of *Arabidopsis thaliana*, they found one protein of a molecular mass of 24 kDa which bound specifically azide-indole-3-acetic acid (5-azide-[7-^{3}H]IAA), a photoaffinity analogue of IAA, upon illumination under UV light. The sequence determination of a cDNA corresponding to this protein showed that it has 57% identity to *parB* gene product and showed an enzymatic activity of GST. Independently Bilang *et al.* (1993) showed that an ABP from cultured cells from *Hyoscyamus muticus* encodes GST. We have also confirmed that the gene product of *parB* bound 5-azide-[7-^{3}H]IAA (unpublished results). Although the binding of IAA to GST could be related to the detoxification of IAA in tissues, another intriguing possibility would be that this binding that occurred in the association with plasma membranes would regulate the intermediate signal transduction pathway downstream from linoleic acid, resulting in the modulation of the level of jasmonic acid, a new type of plant regulators (Farmer and Ryan, 1992). At present, however, some ambiguity remains, since the binding site of IAA to the GST has not been determined whether it binds to a substrate site or non-substrate site of GST. When this binding is clarified, the discussion regarding this issue will become clearer. Anyway the evidence that auxin-regulated *parB*-GST binds auxin should reflect some intriguing harmony in the signal transduction chain having a close link with the regulation of plant growth and development.

Thus far, no molecular analysis has been reported regarding the role of cytokinin during the process of dedifferentiation, and we have not examined this process. Only one notice is that a cDNA clone of pLS217 that responded to auxin was originally isolated as a cytokinin-responsive gene (Dominov *et al.*, 1992). According to

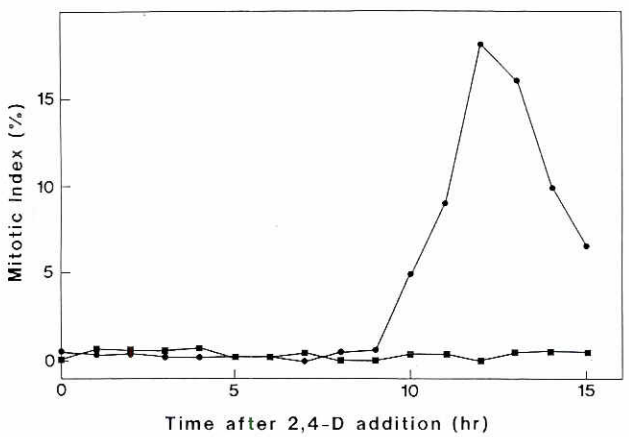

Fig. 4. Induction kinetics of the cell division of tobacco BY-2 cells induced by the addition of auxin. *2,4-D (0.2 mg/l) was added to the cells which had been starved for auxin for 3 days. Mitotic indices were assessed under fluorescence microscopy after staining with 2',6-diamidino-2-phenylindole.*

TABLE 1

***parA*-RELATED GENES**

	parA	*parC*	*C-7*	103	107	*prp1*	pLS216	Gmhsp26-A	*Bz-2*
Plant materials	tobacco mesophyll protoplast	tobacco mesophyll protoplast	tobacco mesophyll protoplast	tobacco cell suspension	tobacco cell suspension	potato leaves	tobacco cell suspension	soybean seedlings	maize
Inducers	auxin	auxin	—	auxin	auxin	elicitor (auxin)	auxin cytokinin	stresses	ND
Tissue specific expressions	root (root)	all organs (root)	all organs tip	root	—	ND	ND	ND	tassel
ATTTA*	3	1	0	1	0	0	2	1	1
Homology to *parA*	100%	68%	65%	42%	67%	51%	92%	37%	31%
References	Takahashi *et al.* (1989)	Takahashi and Nagata (1992b)	Takahashi and Nagata (1992b)	van der Zaal *et al.* (1991)	van der Zaal *et al.* (1991)	Taylor *et al.* (1990)	Dominov *et al.* (1992)	Czarnecka *et al.* (1988)	Nash *et al.* (1990)

ND, not determined. *, sequence that gives the instability of mRNA (Takahashi *et al.*, 1991).

their examination, cytokinin-responsive and auxin-responsive pathways were mutually interacted and protein phosphorylations were involved in this process. However, the role of cytokinin in the dedifferentiation process remains for further studies.

Dedifferentiation

As already described above, the expression of three auxin-regulated *parA, parB* and *parC* was in parallel with the progression of dedifferentiation of differentiated and non-dividing mesophyll cells. These three genes seemed to be expressed so abundantly that they were isolated by differential screening. This does not exclude the possibility that other less abundantly expressing genes are involved in this process. In fact, Hayashi *et al.* (1992) isolated an auxin-regulated gene, whose function has not been identified yet, from tobacco mesophyll protoplasts by T-DNA gene tagging. This gene should play some important role, as overexpression of this gene resulted in the auxin-autotrophic growth of protoplasts cultured *in vitro.* Thus information on the molecular mechanism of dedifferentiation is gradually accumulating.

On the other hand, there are some researchers who have discussed the involvement of several genes during the "dedifferentiation" of differentiated cells. However, their terminology is apparently different from what we have defined as dedifferentiation. Jamet *et al.* (1990) claimed that the dedifferentiation of tobacco mesophyll protoplasts would be induced immediately after the isolation of protoplasts irrespective of the presence of plant hormones. This is difficult to reconcile with the common definition of the term "dedifferentiation", which should include the induction of active cell division. A more reasonable interpretation would be that the definition of dedifferentiation by Jamet *et al.* (1990) should be one of subsidiarily required factors for dedifferentiation, but not main factors which are absolutely required for dedifferentiation, since auxin and cytokinin were not indispensable for inducing meristematic activity of differentiated cells.

Maintenance of the dedifferentiated state of plant cells by auxin

Dedifferentiated state of plant cells can be maintained in the medium containing auxin. Although this phenomenon has been known since the beginning of plant tissue culture (Skoog and Miller, 1958) and is used for the main strategy of plant biotechnology, the molecular basis of the role of auxin in this process has not been elucidated yet. Recently Ishida *et al.* (1993) demonstrated that there is a clear causal relationship between the addition of auxin and the induction of cell division (Fig. 4), when auxin was added to tobacco BY-2 cells that had been cultured in auxin-starved conditions. The search for auxin-regulated genes 4 h after the addition of auxin made it possible to isolate one auxin-regulated gene, which was named *arcA*. The sequence determination of *arcA* revealed that it has homology to a β subunit of heterotrimeric G proteins and has a structure of amino acid repeats designated WD 40 repeats. This is the first finding of the involvement of G proteins in plant hormone-regulated phenomena. Regarding the role of the *arcA* gene product, involvement in the signal transduction chain of heterotrimeric G proteins has not been excluded. However, some deviation of its structure from the typical G β subunit suggests that the *arcA* product would not act as a G ß of heterotrimeric G proteins, but rather would have a protein-protein interaction with another protein having a TPR motif which is composed of repeated units of 34 amino acids (Goebel and Yanagita, 1991), resulting ultimately in the induction of cell division. Thus far the signal transduction chain upstream and downstream to *arcA* has not been demonstrated and it is urgently required for such a study. The expression of *parA* and *parB,* which was expressed during the induction of meristematic activity, has been shown to be involved in the

maintenance of the meristematic activity (unpublished results). Since the involvement of another gene during this process was suggested (unpublished results), it is intriguing to disclose other genes committed in this process. Then the understanding of the induction of cell division in plant cells would be more clearly demonstrated at the molecular level.

Redifferentiation

When we discuss the molecular mechanism of totipotency, it is necessary to include a description of the process of redifferentiation of plants from dedifferentiated cells. Although these phenomena were well-known and used for an important technique for plant biotechnology, thus far no systematic study has been carried out. Thus we cannot enter into any detailed discussion on this subject at this moment. We would like to note simply that as an experimental system the redifferentiation from small colonies starting from mesophyll protoplasts would be suitable, since the redifferentiation process of these colonies is synchronous and some preliminary analyses have been initiated (work in progress).

We have discussed the molecular mechanism of dedifferentiation and the maintenance of the meristematic state of plant cells. Since these processes were primarily regulated by auxin, the elucidation of the expression of genes induced by auxin has been conducted. Several genes isolated from these processes allowed us to elucidate characteristics of totipotency of plant cells to some extent, but we need much more extensive studies to understand an essential frame of this phenomenon. Nevertheless, the experimental system that has been conducted thus far has demonstrated that molecular studies are feasible with plant materials.

Materials and Methods

The process of dedifferentiation was examined with tobacco mesophyll protoplasts prepared from fully expanded leaves of tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) which were grown in a greenhouse. The tobacco mesophyll protoplasts were isolated essentially according to the two step procedure and cultured in the medium as described by Nagata and Takebe (1970). The effects of plant hormones were examined as described by Nagata and Takebe (1970). Colony formation and subsequent regeneration of whole plants was carried out as described in Nagata and Takebe (1971).

The maintenance of the dedifferentiated stages of tobacco cells was examined with a rapidly growing tobacco BY-2 cell line derived from *N. tabacum* L. cv. Bright Yellow-2 (Nagata *et al.*, 1992). The 8-day-old tobacco cells were washed thoroughly with the medium without 2,4-D and cultured further in the same medium without 2,4-D for 3 days. The 3-day-old cells that became elongated were designated auxin-starved cells. Upon addition of auxin to these cells, cell division was induced only in the medium to which auxin was supplemented.

Molecular biological procedures including differential screening regarding plant hormones were carried out as described by Takahashi *et al.* (1989).

Acknowledgments

This work was supported in part by grants from the Mitsubishi Foundation and from the Japanese Ministry of Agriculture, Forestry and Fisheries of Japan.

References

BILANG, J., MacDONALD, H., KIND, P.J. and STURM, A. (1993). A soluble auxin-binding protein from *Hyoscyamus muticus is* a glutathione S-tranferase. *Plant Physiol. 102:* 29-34.

DOMINOV, J.A., STENZLER, K., LEE, S., SCHWARZ, J.J., LEISNER, S. and HOWELL, S.H. (1992). Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. *Plant Cell 4:* 451-461.

DROOG, F.N.J., HOOYKAAS, P.J.J., LIBBENGA, K.R. and van der ZAAL, E.J. (1993). Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione *S*-transferase and one member indeed show *in vitro* GST activity. *Plant Mol. Biol. 21:* 965-972.

EARNSHAW, B.A. AND JOHNSON, M.A. (1988). The effect of glutathione on development in wild carrot suspension cultures. *Biochem. Biophys. Res. Commun. 133:* 988-993.

FARMER, E. and RYAN, C. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell 4:* 129-134.

GOEBEL, M. and YANAGIDA M. (1991). The TPR snap helix: a novel protein repeat from mitosis to transcription. *Trends Biochem. Sci. 16:* 173-177.

GROVE, G., ZARLENGO, R.P., TIMMERMAN, K.P., LI, N.Q., TAM, M.F. and TU, C.P.D. (1988). Characterization and heterospecific expression of cDNA clones in the maize GSH *S*-transferase multigene family. *Nucleic Acids Res. 16:* 425-438.

HAYASHI, H., SCHELL, J. and WALDEN, R. (1992). Activation of a plant gene by T-DNA tagging: auxin-independent growth *in vitro. Science 258:* 1350-1353.

ISHIDA, S., TAKAHASHI, Y. and NAGATA, T. (1993). Isolation of cDNA of an auxin-regulated gene encoding a G protein b subunit-like protein from tobacco BY-2 cells. *Proc. Natl. Acad. Sci. USA 90:* 11152-11156.

JAMET, E., PARMENTIER, Y., DURR, A., CRIQUI, M.C. and FLECK, J. (1990). Is ubiquitin involved in the dedifferentiation of higher plants? *Cell Differ. Dev. 29:* 37-46.

LINSMAIER, E.M. and SKOOG, F. (1966). Organic growth factor requirements of tobacco tissue culture. *Physiol. Plant. 18:* 100-127.

MEINS, F., Jr., LUTZ, J. and FORSTER, R. (1980). Factors influencing the incidence of habituation for cytokinin of tobacco pith tissue culture. *Planta 150:* 264-268.

MEYER, Y., ASPART, L. and CHATIER, Y. (1981). Hormonal control of mitotic development in tobacco protoplasts. Two dimensional distribution of newly-synthesized proteins. *Plant Physiol. 75:* 1027-1033.

MURASHIGE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant. 15:* 473-497.

NAGATA, T. and TAKEBE, I. (1970). Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. *Planta 92:* 301-308.

NAGATA, T. and TAKEBE, I. (1971). Plating of isolated tobacco mesophyll protoplasts on agar medium. *Planta 99:* 12-20.

NAGATA, T. and YAMAKI, T. (1973). Electron microscopy of isolated tobacco mesophyll protoplasts cultured *in vitro. Z. Pflanzenphysiol. 70:* 452-459.

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.

NASH, J., LUEHRSEN, K.R. and WALBOT, V. (1990). *Broze-2* gene of maize: reconstruction of a wild-type allele and analysis of transcription and splicing. *Plant Cell 2:* 1039-1049.

RUSHMORE, T.H., KING, R.G., PAULSON, K.E. and PICKETT, C.B. (1990). Regulation of glutathione *S*-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by lanar aromatic compounds. *Proc. Natl. Acad. Sci. USA 87:* 3826-3830.

SATOH, K., KITAHARA, A., SOMA, Y., INABA, Y., HATAYAMA, I. and SATO, K. (1985). Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA 82*: 3964-3968.

SKOOG, F. and MILLER, C.O. (1958). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro. Soc. Exp. Biol. Symp. 11:* 118-131.

SWEETER, D., MONET, M. and YOUNG, R.A. (1987). Prokaryotic and eukaryotic RNA polymerase have homologous core subunits. *Proc. Natl. Acad. Sci. USA 84:* 1192-1196.

TAKAHASHI, Y. and NAGATA, T. (1992a). *parB:* An auxin-regulated gene encoding glutathione *S*-transferase. *Proc. Natl. Acad. Sci. USA 89:* 56-59.

TAKAHASHI, T. and NAGATA, T. (1992b). Differential expression of an auxin-regulated gene, *parC*, and a novel related gene, C-7, from tobacco mesophyll protoplasts in response to external stimuli and in plant tissues. *Plant Cell Physiol. 33:* 779-787.

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.

TAKAHASHI, Y., KUSABA, M., HIRAOKA, Y. and NAGATA, T. (1991). Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. *Plant J. 1:* 327-332.

TOMAREV, S.I. and ZINOVIEVA, R.D. (1988). Squid major lens polypeptides are homologous to glutathione *S*-transferase subunits. *Nature 336:* 86-88.

van der ZAAL, E.J., DROOG, F.N.J., BOOT, C.J.M., HENSGENS, L.AM., HOGE, J.H.C., SCHILPEROORT, R.A. and LIBBENGA, K.R. (1991). Promoters of auxin-induced genes from tobacco can lead to auxininducible and root tip-specific expression. *Plant Mol. Biol. 16:* 983-998.

ZELCER, A. and GALUN, E. (1976). Culture of newly isolated tobacco protoplasts: precursor incorporation into protein, RNA and DNA. *Plant Sci. Lett. 7:* 331-336.

ZETTL, R., SCHELL, J. and PALME, K. (1994). Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azide-[7-3H]-indole-3-acetic acid: identification of a glutathione *S*-transferase. *Proc. Natl. Acad. Sci. USA 91:* 689-693.