Edited by
Ulrich Lüttge
Wolfram Beyschlag
Burkhard Büdel
Dennis Francis

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Editors

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Editors
Prof. Dr. Ulrich Lüttge
TU Darmstadt
Inst. Botanik
Schnittspahnstr. 3-5
64287 Darmstadt
Germany
luettge@bio.tu-darmstadt.de

Prof. Dr. Wolfram Beyschlag
Universität Bielefeld
Fakultät für Biologie
LS für Experimentelle Ökologie
33615 Bielefeld
Germany
w.beyschlag@uni-bielefeld.de

Prof. Dr. Burkhard Büdel
TU Kaiserslautern
FB Biologie
Abt. Allgemeine Botanik
Erwin-Schrödinger-Str.
67663 Kaiserslautern
Gebäude 13/2
Germany
buedel@rhrk.uni-kl.de

Dr. Dennis Francis
University of Cardiff
Cardiff School of Biosciences
Cardiff
United Kingdom CF10 3TL
francisd@cardiff.ac.uk

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Contents

Contributors

Peter W. Barlow
Department of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG, UK

Matthieu Bourdon
INRA (Institut National de la Recherche Agronomique), UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
matthieu.bourdon@bordeaux.inra.frr

J.A. Bryant
Department of Biological Sciences, University of Exeter, Perry Road, Exeter, EX4 4QG, UK

Catherine Cheniclet
INRA (Institut National de la Recherche Agronomique), UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
catherine.cheniclet@bordeaux.inra.fr

Christian Chevalier
INRA (Institut National de la Recherche Agronomique), UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
chevalie@bordeaux.inra.fr

Jack Donaldson
Living Biography, Orem, UT 84058, USA

David Dunigan
Department of Plant Pathology and Nebraska Center for Virology, University of Nebraska, Lincoln, NE 68583-0722, USA

Nathalie Frangne
Université de Bordeaux, UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
nathalie.frangne@bordeaux.inra.fr

Burkhard Frenzel
Institut für Botanik (210) der Universität Hohenheim, D-70593 Stuttgart, Germany
bfrenzel@uni-hohenheim.de

Dortje Golldack
Department of Biochemistry and Physiology of Plants, Faculty of Biology, University of Bielefeld, 33615 Bielefeld, Germany
dortje.golldack@uni-bielefeld.de

John Guyon
USDA Forest Service, Intermountain Region, Ogden, UT 84403, USA

Martin Heil
Dpto. de Ingeniería Genética. CINVESTAV - Irapuato, Km. 9.6 Libramiento Norte, CP 36821, Irapuato, Guanajuato, Mexico
mheil@ira.cinvestav.mx

Angela Hodge
Department of Biology, Area 14, PO Box 373, University of York, York YO10 5YW, UK
ah29@york.ac.uk

Jacqueline Lück
Chemin du Val d'Arenc 1226, Le Beausset, F 83330, France

Ines Lüking
Department of Biochemistry and Physiology of Plants, Faculty of Biology, University of Bielefeld, 33615, Bielefeld, Germany

Ulrike Mathesius
Department of Biochemistry and Molecular Biology, School of Biology, Australian National University and Australian Research Council Centre of Excellence for Integrative Legume Research, Linnaeus Way, Canberra ACT 0200, Australia
ulrike.mathesius@anu.edu.au

Elodie Mathieu-Rivet
INRA (Institut National de la Recherche Agronomique), UMR619 Biologie du Fruit, F-33883, Villenave d'Ornon, France
emathieu@bordeaux.inra.fr

Université de Bordeaux, UMR619 Biologie du Fruit, F-33883, Villenave d'Ornon, France
emathieu@bordeaux.inra.fr

Anna Moroni
Dipartimento di Biologia, Universitá di Milano, Italy

Mehdi Nafati
INRA (Institut National de la Recherche Agronomique), UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
mnafati@bordeaux.inra.fr

Université de Bordeaux, UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
mnafati@bordeaux.inra.fr

Toshiyuki Nagata
Faculty of Bioscience and Applied Chemistry, Hosei University, 3-7-2 Kajino-cho, Koganei-shi, Tokyo, 185-8584, Japan

The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan

Hans Pretzsch
Chair for Forest Growth and Yield Science, Technische Universität München, Am Hochanger 13, D-85354 Freising, Germany
H.Pretzsch@lrz.tum.de

Christian Printzen
Abteilung Botanik und Molekulare Evolutionsforschung, Senckenberg, Forschungsinstitut und Naturmuseum Frankfurt, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany
christian.printzen@senckenberg.de

Jean-Pierre Renaudin
Université de Bordeaux, UMR619 Biologie du Fruit, F-33883 Villenave d'Ornon, France
jean-pierre.renaudin@bordeaux.inra.fr

M. Sarala
University of Oulu, Department of Biology, PO Box 3000, FIN-90014, Oulu, Finland
marian.sarala@oulu.fi

Samuel B. St. Clair
Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT 84602, USA
stclair@byu.edu

Ulrike Süthoff
Department of Biochemistry and Physiology of Plants, Faculty of Biology, University of Bielefeld, 33615 Bielefeld, Germany

K. Taulavuori
University of Oulu, Department of Biology, PO Box 3000, FIN-90014, Oulu, Finland
kari.taulavuori@oulu.fi

E. Taulavuori
University of Oulu, Department of Biology, PO Box 3000, FIN-90014, Oulu, Finland
erja.taulavuori@oulu.fi

Gerhard Thiel
Institute of Botany, Technische Universität Darmstadt, D-64287, Darmstadt, Germany
thiel@bio.tu-darmstadt.de

Jurriaan Ton
Department of Biological Chemistry, Rothamsted Research. Harpenden, Herts, AL5 2JQ, United Kingdom
jurriaan.ton@bbsrc.ac.uk

Norbert Uehlein
Institute of Botany, Technische Universität Darmstadt, D-64287, Darmstadt, Germany
uehlein@bio.tu-darmstadt.de

James L. Van Etten
Department of Plant Pathology and Nebraska Center for Virology, University of Nebraska, Lincoln, NE 68583-0722, USA

Review

Curriculum Vitae

Toshiyuki Nagata, born on January 9, 1945, Nagano Prefecture, Japan

Education

1968	B.Sc. University of Tokyo
1973	Ph.D. University of Tokyo

Professional experience

1973–1979	University of Tokyo
1979–1983	Nagoya University
1983–1990	Associate Professor, National Institute of Basic Biology
1990–2007	Professor, University of Tokyo
2007	Professor Emeritus, University of Tokyo
2007	Professor, Hosei University
2008 to date	Professor and Dean, Faculty of Bioscience and Applied Chemistry, Hosei University
1974–1975, 1976, 1983	Alexander von Humboldt Fellow, Max-Planck-Institut für Biologie, Tübingen, Germany
1980–1982	Member of US-Japan Scientific Collaboration Program on Plant Biotechnology
1995–2001, 2005–2007	Director of the Botanical Gardens, University of Tokyo

Honors

1997	Alexander von Humboldt Research Award
1998	Associate Member of the EMBO
2005	Research Award of Botanical Society of Japan
2006	Research Award of Japanese Society of Plant Cell and Molecular Biology

A Journey with Plant Cell Division: Reflection at My Halfway Stop

Toshiyuki Nagata

Contents

Abstract I have studied various aspects of plant cell division for more than 40 years, and here I try to illustrate some foci among them. First, establishment of the induction of cell division in freshly isolated protoplasts from leaves is described, as this is important not only for biotechnology, but also for the cell cycle transition from G_0 to S phase, whose importance has not been fully understood yet. The role of plant hormones, auxin and cytokinin, on this process has not been fully elucidated. Secondly, establishment of a model plant cell system of tobacco BY-2 cells and the high level of cell cycle synchrony achieved using this system is also an important theme for me. However, I will not go into details, but instead refer to the two

T. Nagata
Faculty of Bioscience and Applied Chemistry, Hosei University, 3-7-2 Kajino-cho, Koganei-shi, Tokyo, 185-8584, Japan
Professor Emeritus, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan

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recently published monographs edited by myself (Nagata T, Hasezawa S, Inzé D, Biotechnology in agriculture and forestry, vol. 53, Tobacco BY-2 Cells. Springer, Berlin, 2004; Nagata T, Matsuoka K, Inzé D, Biotechnology in agriculture and forestry, vol. 58. Tobacco BY-2 cells. From cellular dynamics to omics, Springer, Berlin, 2006). Further, habituation, discovered by Gautheret (Bull Soc Chim Biol 24:13–47, 1942), will be described, and I will describe how recently we have identified key molecules that may play an important role in this process. Finally, I will try to illustrate aspects of heterophylly from our studies on *Ludwigia arcuata* (Onagraceae). Although heterophylly is thought to be mostly related to cell elongation in leaves and induced by environmental cues, we found that in *L. arcuata,* it is closely associated with cell division. This system offers an opportunity to understand the role of cell division upon leaf shape determination as affected by environmental cues. Thus, as my scientific issues are always associated with cell division, their aspects and background stories will be described in this chapter.

1 Introduction

The title of this chapter may sound a bit strange. It originates from the following fact: I retired from the University of Tokyo in the spring of 2007 at the age of 62 and became a professor emeritus, as the age limitation of Japanese national universities is rather strict! So, when I was asked to write a review reflecting my past, I thought this may be a good time to reflect what I have tried to do in research. However, I am newly appointed as a full professor at Hosei University, a kind of ivy league university in Tokyo and further, I am appointed as Dean of the Faculty of Bioscience and Applied Chemistry, which started in the Spring of 2008. Although currently, I am strongly involved in administration, I wish to extend what I have done before. So, I remain in science and to this aim, I am preparing for this new start!

When I was first asked to write this chapter, the editor simply asked me to write a kind of reflective account of my work with tobacco BY-2 cells that spans more than 27 years of research. However, I have published two monographs on this subject recently (Nagata et al. 2004, 2006) and many colleagues contributed to these two volumes, so I did not think that reiteration of those reviews would be very worthwhile. Subsequently, the editor told me that this chapter could be redirected towards some specific aspect of plant genetics. One of the distinguished contributors to Progress in Botany's special reviews was Professor Diter von Wettstein (2006), whom I know very well. Hence, I was particularly honored to be given the opportunity of contributing in the same way. I decided to write this review with greater emphasis placed on my studies of tobacco mesophyll protoplasts, as there are a certain numbers of "hidden stories" on this topic.

I have sought answers to the problem of cell division events throughout my career. However, I have to confess I feel uneasy when challenged by new problems and, as such, this review mainly reflects how I have been pulled towards the control of cell division in plants; like a magnet.

2 Attempts to Culture Protoplasts

I like to start with culture of tobacco mesophyll protoplasts. This is partly because this relates to my first published study but this is also related to an important issue of cell cycle transition, from G_0 to S phase, in which there are still many unresolved issues. In fact, my academic career started with culturing tobacco mesophyll protoplasts, which became major parts of my Ph.D. thesis at the University of Tokyo. Perhaps, I should tell what was behind the start of this work. When I graduated from the University of Tokyo in 1968, it coincided with a year of massive student discontent and social unrest in Japan; I refer to it as "the university struggle." Actually the university graduation ceremony for the academic year of 1968 was canceled because of this struggle. Then, I was enrolled in the graduate school there in the Spring of 1968. Although I captured basic techniques on how to handle plant cells according to plant cell and tissue techniques under the supervision of Professor Toshio Yamaki, there was much less enthusiasm on how these techniques could be directed to key scientific issues. Nonetheless, I established a partial cell synchrony system using the tobacco XD6 cell line. After auxin-starvation of the cells in auxin-free medium, I could see that the addition of auxin to these auxin-depleted cells induced a partial cell synchrony. Although I never published this result, this experience was useful for inducing auxin-induced cell synchrony in the auxin-starved tobacco BY-2 cells more than 20 years later (Ishida et al. 1993).

Because of the chaotic situation on the Tokyo campus, I sought some sanctuary. So I asked Dr. Itaru Takebe, Governmental Institute for Plant Virus Research, Chiba, to join his group as a kind of voluntary student. They had just published a methodology for the preparation of protoplasts from tobacco leaves (Takebe et al. 1968) and their successful infection with tobacco mosaic virus (TMV) (Takebe and Otsuki 1969). Although I had several speculative ideas, one of my initial intentions was to isolate subcellular fractions and to examine the effects of plant hormones on these components. Needless to say, protoplasts are single naked cells in the strict sense and it was intriguing to work with this novel material. When I visited Dr. Takebe, he told me that a reliable technique for making protoplasts from leaves had not been established in any cultural conditions and that this was an urgent task to be resolved. This sounded a reasonable challenge for a young student, so I decided immediately to establish suitable culture conditions for preparing protoplasts from tobacco mesophyll. It took a month or so to establish conditions for preparing enzymes that could function effectively in aseptic conditions, as at that time, such techniques had not been well established. Further, most of the works had to be done in a normal laboratory condition that lacked a laminar flow chamber. Thus, most work had to be done on a Saturday, as it was quiet with nobody around to infect the cultures! Within a month, I established protoplasts from tobacco mesophyll aseptically but they died quickly. Most of possible media were tried in which some protoplasts survived for a week or so but no cell division was observed apart from sporadic endomitosis. This situation lasted for 3–4 months.

Culture of somatic cells including leaf cells was first proposed by Gottlieb Haberlandt (1902), but the real start of plant cell and tissue culture was much later. After the discovery of the plant hormone, auxin, basic disciplines of plant tissue culture started through studies of Philip R. White (1939) and Roger Gautheret (1939). However, culture of cells directly prepared from leaves was far behind. By 1968, there were two cases, in which cell division was observed in mesophyll cells directly isolated from leaves. Kohlenbach (1960) reported cell division in mesophyll cells of *Macleaya cordata* (Papaveraceae), while Joshi and Noggle (1967) reported successful culture of isolated leaf cells of peanut (*Arachis hypogaea*). Generally speaking, however, leaf cells were not considered to be suitable for culturing. This is well reflected in a rather comprehensive study by Joshi and Ball (1968), in which they described mechanical separation of mesophyll cells from several species and their trials to culture them. They concluded that primarily, mechanical isolation of mesophyll cells from leaves was very difficult even if their division was observed in culture. Such a case was peanut, where cell division was observed only in adjacent cells and but never in freely suspended cells. Referring to a rather negative description for the culture of leaf cells even in the second edition of the standard monograph of "The cultivation of animal and plant cells" by White (1964), they also discussed difficulties in culturing mesophyll cells, such as palisade and spongy parenchyma cells. They concluded that failure to culture mesophyll cells from leaves was because of the complete differentiated state of these cells and that meristematic cells may offer the only feasible start point for leaf cell culture in vitro. Thus, the consensus view was that it was impossible! However, I persisted with this problem and I recall a laboratory members' excursion to Oku-Nikko, ca. 100 km from the city of Tokyo on 2 November 1969. Just before the trip, I made a fresh preparation of tobacco mesophyll protoplasts. When I returned on November 5, I suddenly observed the first induced cell division. It was not sporadic, but most cells divided in a week or so, which was reproducible. What was the difference between this successful case and previous unsuccessful ones? Previously our material was tobacco cultivar Bright Yellow, a common cultivar in Japan. After unsuccessful experiments with this material, I tried to make protoplasts from another cultivar Xanthi nc, a model system for studying TMV infection and local lesion assays. Such a simple change of cultivar choice was a complete surprise. However, the more I thought about it, it became increasingly clear that the quality of the starting material and the need for it to be fresh and grown under optimal conditions became increasingly important criteria for guaranteeing the success of protoplast culture. This success countered the worries expressed by Joshi and Ball (1968) that isolation of mesophyll cells was very difficult. Indeed my results gave me confidence that leaf protoplast culture could be applied more widely.

In addition to emphasizing these critical preparative points, I dared to add another seemingly unusual observation. Good protoplasts which have mitotic competence and well-able to form colonies always have a tendency to show clear "blinking" or "sparkling" of grana in their chloroplasts when viewed under blight field illumination. If blinking of grana is weak, healthy protoplasts cannot be

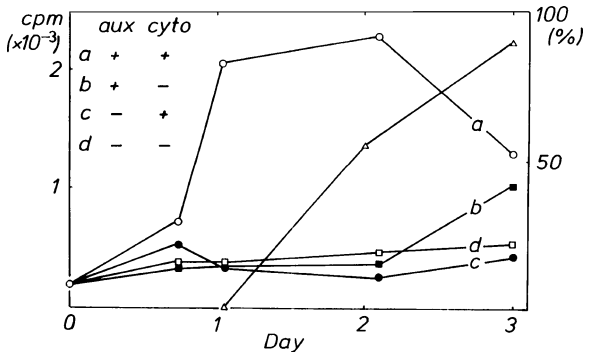

Fig. 1 Time course of DNA synthesis in cultured tobacco mesophyll protoplasts. Cell division was observed only in both presence of auxin and cytokinin. Although DNA synthesis was observed in the sole presence of cytokinin, this DNA synthesis did not result in the completion of cell cycle. DNA synthesis was measured by the incorporation of ^{3}H-thymidine into acid-insoluble fraction; however, this DNA synthesis was also confirmed by the incorporation of BrdU into nucleus, which was examined by staining with an antibody against BrdU (Shimizu and Nagata, unpublished observations)

obtained. Although I tried to explain this on numerous occasions and indeed demonstrated to others in the laboratory, it seems to have gone unnoticed in more recent protocols for protoplast preparation. So we published our findings, but we did not realize at the time that Nagata and Takebe (1970) would be listed as Citation Classics by the Institute for Scientific Information (ISI) in 1988.

In our work, high frequency of cell division was induced in tobacco mesophyll protoplasts in a rather simple medium supplemented with auxin and cytokinin under white light illumination (Nagata and Takebe 1970); this straightforward approach is not considered too much by others working in this field. In fact, DNA synthesis was detected as early as 12 h after protoplast isolation and the first cell division was detected by the second day of culture under optimal condition (Fig. 1). In a week, most protoplasts divided at least once. Regarding this issue, there have been published several related published articles (Zelcer and Galun 1976; Meyer et al. 1984). However, in other studies, start of DNA synthesis and cell division was much slower and nonsynchronous. Only according to our original protocol, can such rapid induction of DNA synthesis and cell division be observed. For sustained cell division, further development of our techniques was required, which will be described below.

Immediately after that, regeneration of whole plants from single protoplasts of tobacco mesophyll was shown using a revised medium in which high frequency of the induction of cell division in respective leaf protoplasts was shown (Nagata and Takebe 1971). In particular, when protoplasts were embedded in an agar medium, a high frequency of colony formation was confirmed unambiguously. Although we did not emphasize this point in writing the paper, another interesting phenomenon was observed. It was clear that colony formation was completely dependent on cell density (ca. 1,000 ml^{-1}) that was a threshold marker for forming colonies. This is

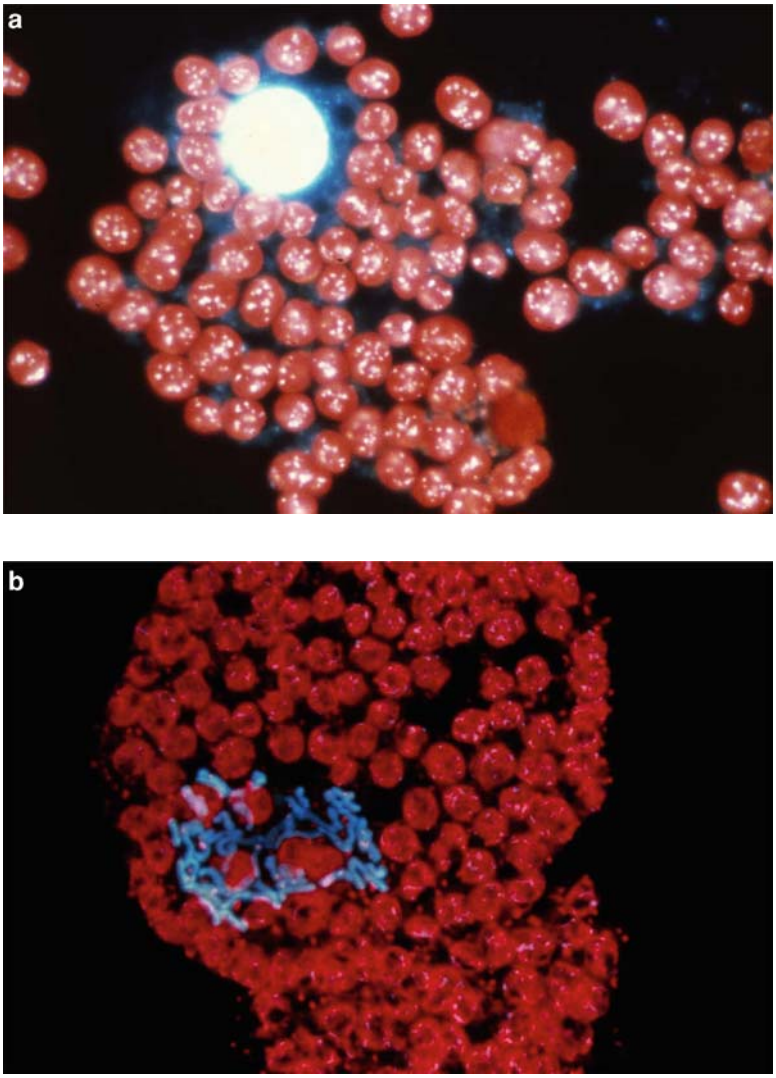

Fig. 2 Strict dependency of colony formation of plated tobacco mesophyll protoplasts on cell density. (**a**) When tobacco mesophyll protoplasts were embedded in the agar medium, colony formation was observed at higher than 1,000 per ml. (**b**) Embedding of the protoplasts in the agar medium was carried out on the agar medium of the same composition (bottom layer). When the thickness of embedded agar medium was made in a gradient from left to right, colony formation was only observed in the left half of the plated medium. Although in the right half, cells were embedded in the agar medium, no colony was observed. This may be explained by the leakage of the factors that were produced by cells to the bottom layer so that level of the factors necessary for colony formation became lower, resulting in the abortion of colony formation. This factor may be something-like Phytosulfokine as described by Matsubayashi and Sakagami (1996)

reflected in Fig. 2, as colony formation was observed above a threshold density, but never below it. We thought that there might be an enrichment of mitogenic factors that would be reaching optimal levels to facilitate cell division of leaf protoplasts. We intended to identify these mitogenic factors, but our facility was not sufficient to resolve anything at the biochemical level. Many years later, when I read a paper on phytosulfokine (PSK) by Matsubayashi and Sakagami (1996), I thought this could have been at least part of our perceived mitogenic stimulus. Our paper (Nagata and Takebe 1971) was also selected in Citation Classics by ISI (1985), in which we demonstrated that totipotency of plant somatic cells in tissues is nearly 100%. In a seminal paper by Steward et al. (1963), totipotency of plant cells was demonstrated, but the induction of cell division in somatic cells was shown to be only at ca. 5–8% (Israel and Steward 1966). This poses a question about the remaining 92–95% of cells in the somatic tissues. In contrast, we have shown that cell division can be induced in almost all cells in leaf tissue at the optimal condition. So in the essay added to the Citation Classics (1985), I wrote explicitly that this is the real start of somatic cell genetics and totipotency of plant cells.

3 Interim Period or Wandering Years

3.1 Germany

After receiving a Ph.D. from the University of Tokyo in 1973, I was appointed as an assistant professor in the Department of Pure and Applied Sciences of the University of Tokyo. Immediately after that, Professor Georg Melchers at the Max Planck Institute for Biology invited me to work in his laboratory in Tübingen, Germany and I went there as an Alexander von Humboldt Fellow. At the time, I proposed a project on transformation of protoplasts with *Agrobacterium tumefaciens*, as the transformation of plant cells should be a next target for our adventure. Actually, shortly before my departure to Germany, I read with great interest a paper by Professor Jeff Schell and others (Van Larebeke et al. 1974), in which Ti plasmid was identified as the causative agent for crown gall disease. However, because of the general interests in the laboratory and available facilities, Professor Melchers asked me to do work on cell fusion. So I did a kind of theoretical analysis on protoplast fusion in relation to cell–cell interactions (Nagata and Melchers 1978; Nagata and Nagata 1984a). In fact, I measured surface charges of protoplasts by cell electrophoresis using an apparatus made by a technician in the laboratory using an old cathetometer that Professor Carl Correns had used. Around that time, I also found that polyvinyl alcohol of low molecular mass is an agent for inducing cell fusion (Nagata 1978), just like polyethylene glycol whose effect was discovered by Kao and Michayluk (1974).

I returned to Japan in 1975, but I went back and forth to Tübingen and some other places in Germany until the passing away of Professor Melchers on November 22, 1998. He was an extraordinary man and trusted me to the extent that he called me his "fourth son." Somewhat later, Professor Fritz Melchers, his eldest son and former Director of the Basel Institute of Immunology, visited me and told me that although his father had many guests and coworkers in his laboratory, I was the only one that his father called his son. So from that moment, Fritz and his two other brothers and I became a kind of quasibrotherhood. Around a year before he died, Prof G. Melchers sent me a whole set of his publications with a letter saying that if some day he would die, I would be asked to write an obituary. Even after his death, I have been in Tübingen several times and in 2006, on the anniversary of what would have been his 100th birthday, I attended a symposium in his honor where Professor Detlef Weigel gave a plenary lecture on "florigen." This was timely because the florigen concept was first proposed by three scientists; Melchers, Chailakhyan, Kuiper in 1937. For almost 70 years, the biochemical identity of florigen remained elusive but at last was proven to be a product of the *FT* gene (Turck et al. 2008). So, I wrote a kind of obituary to Professor Melchers together with reporting this new finding in a Japanese journal on Heredity (Iden in Japanese).

3.2 Enzymes

As noted in the beginning, my scientific carrier started with culture of protoplasts from tissues, and knowledge about enzymes became obligatory. As may be well known, enzymes that are used for the preparation of protoplasts have been mostly and are still produced by Japanese manufacturers. The enzymes that have been used for this purpose were found first by Dr. Takebe (Takebe et al. 1968); Macerozyme R10 and Cellulose Onozuka R10 (Yakult Pharmaceutical Co. Tokyo). When I was working at the Max Planck Institute for Biology in 1976, I knew a Japanese researcher Dr. S. Ishii of Kikkoman Co, who had reported a novel type of pectin-degrading enzyme, pectin lyase, based on the information from Chemical Abstract. I visited him after I returned to Japan. The enzyme named Pectolyase from *Aspergillus japonicus*, which is used for a fermentation process of soya source, was found to be very much powerful for degrading plant tissues. This is most likely to be due to the presence of both *endo*-pectin lyase and *endo*-polygalacturonase (Ishii 1976). However, the initial sample that I obtained from Dr. Ishii was very toxic to plant cells. After a partial purification by Dr. Ishii, it became useful for preparing protoplasts from a wide variety of plant tissues. Particularly with this enzyme when combined with cellulose, rapid preparation of protoplasts became possible. I tried to persuade Kikkoman Co. to produce and sell this enzyme but the initial reply was negative. However, a paper was published by Nagata and Ishii (1979) using an enzyme mixture of Pectolyase and cellulose; DuPont Co. ordered it from Kikkoman Co. without even asking the price and the company the decided to

produce and sell it under the trade name of Pectolyase Y23 through Seishin Pharmaceutical Co., a subsidiary of Kikkoman Co.

Another example is Cellulose Onozuka RS. From 1978 to 1981, I used protoplasts prepared from *Catharanthus roseus* cultured cells, which are easily produced by using a combination of Pectolyase Y23 and Cellulose Onozuka R10. When we started to use tobacco BY-2 cells, something rather odd occurred. A graduate student was working with tobacco BY-2 cells and was studying the infection of protoplasts with TMV and was almost able to perfect the technique of protoplasts infection with TMV, which was later published by Kikkawa et al. (1982). However, on one occasion, he told me that the infection technique was no longer reproducible. When I looked through his whole experimental procedure and my experimental notes, it turned out that successful infection of the protoplasts with TMV was only achieved with a kind of probe enzyme material that the manufacturer, Yakult Pharmaceutical Co., offered us. When I asked them what was the difference between the probe material and the commercially available one, they replied that it was produced from a mutated strain of *Trichoderma viride* by UV irradiation. It was also revealed that the enzyme prepared from the mutated *T. viride* actually contained a wider spectrum of enzymes including hemicellulose. In fact, with this enzyme cocktail, protoplasts could be prepared from various plant species. Again we asked the manufacturer to produce it and it became available under the brand name of Cellulose Onozuka RS by the Yakult Pharmaceutical Co. (Nagata et al. 1981). Since then, I have had a close relationship with these manufacturers and from time to time am asked to examine the quality of their enzymes.

3.3 Move to Nagoya

In 1979, I moved to Nagoya University. In fact, Dr. Takebe was offered with a full professor position at Nagoya University. A prerequisite for him to accept it was that I could go with him to Nagoya as he did not have much experience at the university. So I said yes and moved to Nagoya. Our intention was to establish a delivery system of genetic materials into protoplasts by any means. To this end, first we did liposome-mediated gene delivery into protoplasts prepared from *C. roseus* (Fukunaga et al. 1981). This was demonstrated more clearly by using tobacco BY-2 cells (Nagata et al. 1981). I worked for more than 27 years with tobacco BY-2 cells and have accumulated a lot of information on this line. However, as mentioned in the Introduction, stories of experiments we have done on the tobacco BY-2 cell will not be described here (see Nagata et al. 2004, 2006).

Our main aim was to establish systems to transform protoplasts with *A. tumefaciens* or Ti plasmid. Because of our poor facility at that time, Ti plasmid was offered by Professors Milt Gordon and Nene Nester, University of Washington, Seattle, USA. However, before our success with this trial, a Dutch group reported a series of their successful trials (Márton et al. 1979).

3.4 Miles International Symposium

In May 1982, I was invited to give a talk at the 14th Miles International Symposium at Johns Hopkins University, Baltimore, USA (Nagata 1984b). This meeting was unusual and extremely interesting; animal, plant and microbial scientists assembled to discuss cell fusion and transformation techniques and their potentials. Here, I learned about the frontiers of animal cell transformation, which was a very competitive field at the time. One unforgettable meeting was with Professor Jeff Schell, Max Planck Institute for Plant Breeding, Cologne, Germany, although I knew about him through a series of seminal works. Since then, we developed a close scientific relationship and we met at many occasions in many locations until his untimely death in 2002; later, he recommended me as an associate member of the EMBO in 1998.

3.5 Move to Okazaki

In December 1983, I moved to Okazaki, as an associate professor at the interuniversity facility of the National Institute for Basic Biology. I worked in the Cell Biology Division headed by Professor Tsuneyoshi Kuroiwa, who was enthusiastic to see molecules including DNA under the microscope. He developed a very sensitive DNA detection system using fluorescence microscopy on samples stained with 4′,6-diamidino-2-phenylindole (DAPI). Thus, I learnt how to detect traces of DNA upon fertilization in plants by DAPI staining. Although maternal inheritance was discovered by Carl Correns and was confirmed in many organisms, molecular

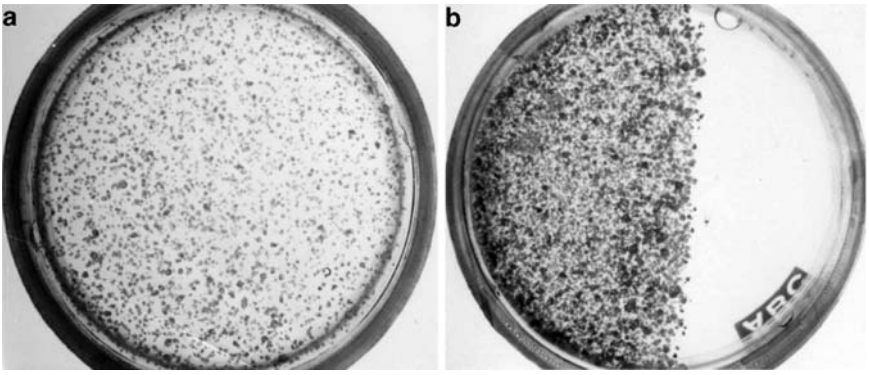

Fig. 3 Fluorescence microscopy of tobacco mesophyll protoplasts after staining with DAPI. (**a**) A freshly isolated protoplast. Whereas location of DNA in nucleus and chloroplasts was clearly demonstrated, DNA was also observed in mitochondria. Note that red fluorescence in chloroplasts is originated from chlorophyll. (**b**) Protoplasts culture for 36 h. When nuclear division was observed, red fluorescence in chloroplasts became darker

proof for this was insufficient. Kuroiwa et al. (1984) provided molecular proof for maternal inheritance in *Chlamydomonas reinhardtii*. By using these techniques, we examined the fate of organelle genomes upon fertilization in various plant species (Miyamura et al. 1986, 1987). Unexpectedly, Professor Lloyd Morgensen (Northern Arizona University) wrote to me that our work was the first clear proof of this phenomenon. I also followed the fate of early stages of cultured tobacco mesophyll protoplasts after staining with DAPI (Fig. 3), which clearly demonstrated morphological changes to the protoplasts at the subcellular level.

4 Settlement at Tokyo

I was appointed as a full professor at the University of Tokyo in 1990 and worked there until my retirement in 2007. During this period, my first focus was on the establishment of tobacco BY-2 cells as the model cell line of higher plants, on which I refer here only to the points that have not been included previously (Nagata et al. 2004, 2006). Secondly, I tried to understand the molecular basis of the early events that occur when tobacco mesophyll protoplasts undergo G_0 to S phase transition. In particular, I studied the effects of auxin and cytokinin on this process. Thirdly, I devoted time to understand habituation, whose molecular basis has not been clarified yet and which remains the most enigmatic phenomenon in plant cell culture even since its discovery by Gautheret (1942). Further, I spent time studying heterophylly, which is an ecophysiological phenomenon but is a good system to understand the relationship between an environmental cue and leaf morphogenesis. At the University of Tokyo, I was also involved in administration of the Botanical Gardens as a director for 8 years and did some work on biodiversity, but space does not permit any description of this work here.

4.1 Tobacco BY-2 Cells

When I arrived to Tokyo, the first task was to describe details on the handling of tobacco BY-2 cells. We discovered that BY-2 cells could be incubated with aphidicolin (a reversible inhibitor of replicative DNA polymerase α) and following removal of this drug, we could observe between 60 and 70% of cells reaching mitosis synchronously. Up to that time, published reports of cell synchrony in plants was no more than 10% (max). In fact we first established this system that could yield high levels of synchrony earlier in 1982. However, it was not necessarily reproduced by others, even in Japan! Some people seemed to doubt what we had done because they could not reproduce it. Professor Hiroh Shibaoka of Osaka University, who is now in retirement, knew me well and sent one of his younger staff

Dr. Tatsuo Kakimoto, to learn the technique and how to handle the BY-2 cells. However, after returning to Osaka, he called me 3–4 weeks later to say that he could not reproduce what he did in our laboratory. It took the Osaka group another 6 months to reproduce our results. The key reason why the technique could not be reproduced elsewhere was because new users of the cell line did not culture the cells under the precise conditions with which these cells must be grown: exactly 130 rpm at 27°C in darkness in specially modified Murashige and Skoog medium. I personally maintained the cell line for more than 27 years, but after retirement from the University of Tokyo, I stopped for one and a half years but recently reestablished it in my new laboratory. When I see the cells under microscopy, I can tell the condition of the cells.

After Shibaoka's group published their seminal work on the preparation of phragmoplasts (Asada et al. 1991) using highly synchronized tobacco BY-2 cells, our method was noted by workers particularly those outside of Japan. First, Professors Anne-Marie Lambert and Claude Gigot at the Institut de Biologique Moléculaire des Plantes du CNRS at Strasbourg were very enthusiastic about using our method and obtaining the cells from me. Given the lack of success of some laboratories with our line, I decided that it was absolutely necessary to describe full details of the correct way to handle and synchronize the cells. So, I wrote a review paper including technical details (Nagata et al. 1992). The paper has been cited more than 549 since the publication and the use of the cell line has spread to at least 35 countries world-wide; later, I decided to supplement this paper with another review (Nagata and Kumagai 1999). My hope was that the BY-2 cell line could be the plant HeLa cell line, a hope that has been fully realized.

4.2 Dedifferentiation

Dedifferentiation is a process, in which differentiated nondividing cells become meristematic. The molecular basis of dedifferentiation is poorly understood. I decided to study this process using tobacco mesophyll protoplasts. With this system, I could study cell division in protoplasts that could be induced to divide synchronously in a simple medium only supplemented with auxin and cytokinin (Nagata and Takebe 1970; see above). Thus, we followed auxin-induced genes during the first day of culture. These genes encode glutathione *S*-transferase (Takahashi et al. 1989; Takahashi and Nagata 1992), and are first expressed shortly after the addition of auxin, and their expression lasts up to 12 h. They are components in a mitogenic signaling process (Takahashi et al. 1995), although the precise function of GSTs in this process remains to be clarified. Among various types of GSTs (Marrs 1996), we discovered parA that is localized in the nucleus and is classified as a Type III GST, while parB that is localized in the cytoplasm belongs to a Type I GST. With a similar strategy, a cytokinin-induced gene was also identified (Iwahara et al. 1998), whose function also remains to be clarified.

Regarding this issue, we were always searching for plant hormone-regulated genes under the assumption that auxin and cytokinin are necessary for the induction of cell division. When I looked at what happens if auxin or cytokinin alone was given to the protoplasts, intriguing effects were observed. Regarding cell cycle progression, almost nothing happened with auxin alone, while the sole addition of cytokinin induced a small but significant level of DNA synthesis; however, this DNA synthesis did not go further and was not followed by cell division. The addition of auxin around 24 hr after the addition of cytokinin resulted in full cell cycle progression (our unpublished results). However, although the sole addition of cytokinin induced cells to undergo the transition from G_0 to S phase, cells did not progress into mitosis. It seems that during the early stage of dedifferentiation, there is a cytokinin-responsive stage shortly after the addition of cytokinin (G_0/S) and then, an auxin-sensitive stage (G_2/M) that follows. Currently, we are looking for what kind of molecular components of the cell cycle machinery are involved in this process.

4.3 Habituation

Habituation is an epigenetic change observed in cells in culture where after a prolonged continuous culture with a plant hormone like auxin, they develop the competence to grow on an IAA-depleted medium. It was discovered by Gautheret (1942), but its molecular basis has not been clarified although there have been many publications on this issue. Thus, habituation is a most enigmatic issue in plant cell and tissue culture. Regarding this, I have been culturing the tobacco 2B-13 cell line since 1988, which is derived from tobacco BY-2 cell line. The presence of auxin is obligatory for propagation of tobacco BY-2 cells, whilst depletion of auxin from the culture medium causes cell division to stop (Ishida et al. 1993). However, the 2B-13 cells can grow without auxin and thus, it shows a classic feature of habituation. After several trials to find differences between the two cell lines, it was found that addition of the culture filtrates from 2B-13 cells to auxin-starved nondividing BY-2 cells caused the induction of cell division. The search for factors that induced such cell division in this fraction revealed that they were glycoproteins, which when further purified by column chromatography and eluted with SDS-PAGE, were shown to be a single band of 30 kDa. It was finally identified by MALDI/TOF-MS analysis to be a kind of ATP-binding cassette (ABC) transporters named P-Glycoprotein (PGP) (Shimizu et al. 2006). However, it was also found that there are similar glycoproteins in the culture filtrates of tobacco BY-2 cells, but they had different molecular sizes of 25 and 40 kDa than those isolated from 2B-13 cells. Further characterization of these factors should give clues to understand the molecular basis of habituation. Regarding this issue, the molecular mechanism of auxin action has been well characterized and its signaling pathway has also

been described (Dharmasiri et al. 2005); however, its relevance relating to the above-mentioned factors remains to be clarified. This is one of my current interests at Hosei.

4.4 Heterophylly

I was interested in studying heterophylly that is observed in plants growing along the riverside that can tolerate submergence. This is because submergence causes morphological changes to the leaves. As certain environmental changes cause such distinct morphological changes, it is quite intriguing to follow the signaling pathway downstream from an environmental cue to morphological changes. This study began when Asuka Kuwabara joined us as a graduate student. We first established a system, in which induction of heterophylly in *Ludwigia arcuata* (Onagaraceae) can be conducted in sterile conditions and consequently, experiments can take place throughout the year, in contrast to the previous experiments in natural conditions, in which material sometimes deteriorates upon infection by microbes. Then, we found that ethylene induced elongated submerged-type leaves, while abscisic acid (ABA) induced round terrestrial-type leaves (Kuwabara et al. 2001, 2003). Most previous works on heterophylly had been explained by the elongation of leaf epidermal cells; these two plant hormones induced cell division but form two types of leaves of elongated and round shape leaves.

So again I encountered "cell division" during leaf development. Although cell divisions causes the increase in cell numbers in tissues and the increase of tissue mass, the plane of cell division axis to the long axis of leaves was different between ethylene and ABA treatments. This observation enabled us to analyze the processes involved in forming leaf shape mediated by cell division and as regulated by ethylene or ABA. This process can be divided into a few steps; first, how the direction of cell division plane would be determined by either a condition of submergence or terrestrial condition and then, how the respective cell division events contribute to affect leaf shape. These analyses revealed that there is a gradient in developmental stage in *L. arcuata* leaves; namely, leaf shape determination, starts from the tip to the base. Thus, at a certain stage of leaf development, differentiation in the tip is fixed, while at the base, it is still responsive to cues. When an intermediate stage of leaf is transferred from a terrestrial to a submergence habitat, or from submergence to terrestrial, leaf shape becomes spoon or spear head shaped, respectively (Kuwabara and Nagata 2006), which is a kind of developmental plasticity. These analyses have allowed us to first construct a mathematical model of how respective cell division events contribute to leaf shape and to ask what might be the molecular basis of this process. Since almost nothing is known about the *L. arcuata* genome, we are trying to understand these processes in *Arabidopsis thaliana* in collaboration with Dr. A. Kuwabara and Professor Andrew

Fleming, Sheffield University. As a kind of complementary evidence, I add that changes described above were caused at temperatures above 28°C, while below 21°C, cell elongation contributes to determine leaf shape (Sato et al. 2008). This study should give clues to understand the role of cell division in leaf shape determination.

5 Concluding Remarks

I am now trying to bring this chapter to an end. Normally, such a review would be from a retired perspective, looking back at one's career. However, as mentioned above, I now have further opportunities to continue my work. I would be happy if I could provide answers to questions on dedifferentiation, habituation, and heterophylly. All of these phenomena are found to be related to cell division. Throughout my career, I have first tried to study certain phenomena at the physiological level and then at the cellular level. Repeatedly I arrive at a road-sign that says "cell division." If one speaks of cell division at the present time, it is mostly related to publications on genes that regulate cell cycle progression but, as I explain here, there are many other phenomena in which cell division is involved particularly in relation to plant morphogenesis. So I would be extremely happy, if I could give some reasonable answers to exactly how cell division is linked to developmental processes. Finally, I thank people, who have worked with me in the past and those who are currently working with me in my new surroundings. Without their help I would not have been able to make progress in my scientific journey at any location or any situation. Thus far, I have enjoyed my journey in science but as such the journey never-ends; I hope I am only half-way.

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