Invited Review:

Cytological Studies on Proliferation, Differentiation, and Death of BY-2 Cultured Tobacco Cells

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Summary A procedure for the simultaneous isolation of mitochondrial and plastid nucleoids was first established in BY-2 cells. Biochemical analysis suggested the presence of nucleosome-like repetitive structural units in both of the plant organelle nucleoids. The isolated organelle nucleoids were also used for establishment of *in vitro* transcription/DNA synthesis systems, with which regulation of organelle genomes during proliferation and differentiation was investigated. The results revealed that transient and synchronous activation of DNA synthesis occurred in mitochondria and plastids in the initial phase of cell proliferation, which was caused by a transient activation of dually-targeted organelle DNA polymerase genes. Another series of investigations revealed a drastic difference in the transcription activity between BY-2 proplastids and leaf chloroplasts, which was brought about by differential use of bacterial- and phage-type plastid RNA polymerases. To investigate regulation of plastid gene expression further, a procedure to induce amyloplast formation in BY-2 cells was established. Various changes observed during this process were collectively similar to those observed during root cap cell differentiation. BY-2 cells were also used to develop a novel model system for programmed cell death during hypersensitive reaction (HR), in which we have succeeded in inducing programmed cell death with 100% efficiency by application of an elicitor. This system revealed that the HR cells activated anti-microbial defense mechanism by themselves and transmitted signal(s) to establish acquired resistance in neighboring cells, while executing cell-death program. Thus, BY-2 cells have taught us a lot about proliferation, differentiation, and death of plant cells.

Key words Amyloplast formation, DNA polymerase, HR cell death, Nucleoid, Nucleosome, RNA polymerase.

Tobacco BY-2, a model plant cell line

The tobacco BY-2 cell line (Fig. 1a) was established in the Japan Tobacco and Salt Public Corporation (Kato *et al.* 1972), and, thanks to Dr. Toshiyuki Nagata, became available for numerous plant scientists all over the world (Nagata 2004). Since then, BY-2 cells have contributed much to our knowledge on molecular and cell biology of plant cells (Nagata *et al.* 1992). Among the huge amount of knowledge derived from BY-2 cells, here we focus on only four topics in which we have been deeply concerned, namely (1) structure of organelle nucleoids in plant cells, (2) regulation of organelle DNA synthesis during cell proliferation, (3) regulation of plastid gene expression according to differentiation, and (4) behavior of plant cells during the process of

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Fig. 1. Images of BY-2 cells and DNA ladders. (a) A light microscopic image of BY-2 cells. Only a part of a cell cluster is shown. The scale bar represents 10 μ m. (b) DNA ladders observed for mitochondrial nucleoids (MtN) and nuclei (Nuc) isolated from BY-2. Isolated nucleoids or nuclei were partially digested with MNase and electrophoresed in an agarose gel. Numbers to the left represent the sizes of DNA markers in kbp. Adopted from Takusagawa *et al.* (2009) with modification.

programmed cell death, to illustrate what BY-2 cells have taught us.

Organelle nucleoids in plant cells may contain nucleosome-like repetitive structural units

Mitochondria and plastids contain DNAs of their own, which are folded three-dimensionally through interaction with associating proteins to form compactly organized DNA–protein complexes, called organelle nucleoids or organelle nuclei (Kuroiwa 1982, 1991, Sakai *et al.* 2004b). Because BY-2 cells are easily converted to protoplasts (Nagata *et al.* 1981), they can be homogenized mildly without severe fragmentation of cell-nuclei, which made it possible to isolate highly pure organelle-nucleoids essentially free from contaminating cell-nuclear fragments (Nemoto *et al.* 1988, Sakai *et al.* 1998c). Moreover, careful optimization of buffer composition (reviewed in Sakai 2001) made it possible to isolate organelle-nucleoids with the same compactly organized structures as observed *in vivo*. The isolated nucleoids have been used for biochemical identification of the proteins responsible for their compact organization (*e.g.*, Nemoto *et al.* 1988, 1989, 1990, 1991, Takusagawa *et al.* 2009). However, comprehensive MS-analyses did not produce convincing conclusion (our unpublished result), probably because isolated nucleoids still contain too many proteins although they are highly pure in terms of DNA species.

We therefore re-analyzed the molecular organization of the organelle nucleoids isolated from BY-2 cells carefully, and found that treatment of the isolated mitochondrial nucleoids with micrococcal nuclease (MNase) resulted in the generation of a “DNA ladder” (Takusagawa *et al.* 2009, Fig. 1b) that is widely regarded as an indication of nucleosome structure in cell-nuclear chromatin (Telford and Stewart 1989). Thus, our observation suggested the presence of nucleosome-like structures in mitochondrial nucleoids for the first time. Until then, there had been few studies reporting generation of DNA ladder by partial *in organello* DNA digestion (Olszewska and Tait 1980, Salganik *et al.* 1991), and the DNA ladders obtained in those studies were rather unclear. Moreover, as far as we know, all the trials to detect DNA ladders in isolated organelle nucleoids had been unsuccessful (*e.g.*, Nemoto *et al.* 1988).

The structures responsible for the generation of the “DNA ladder” in mitochondrial nucleoids were comparable to genuine nucleosomes, in terms of its organizing principles (electrostatic

Fig. 2. Changes in the organelle nucleoids during batch-culture of BY-2 cells. Cells were harvested 0, 1, 3, and 5 d after the start of a batch-culture, converted to protoplasts, and observed by DAPI-fluorescence microscopy. Days 0 and 1 correspond to lag phase, while day 3 and day 5 correspond to mid-log phase and late-log (decelerating) phase, respectively. Large spherical fluorescent bodies represent nuclei, while small fluorescent spots represent organelle nucleoids. The scale bar represents 10 μm . Adopted from Okamura *et al.* (2002) with modification.

interaction between DNA and proteins), coverage (about 30–40% of DNA being protected from MNase), and apparent lack of sequence-specificity (manuscript in preparation). However, the DNA ladder observed for mitochondrial nucleoids was different from the ladder observed for nuclear chromatin (Fig. 1b), in terms of the number of DNA bands (usually three to five in mitochondrial nucleoids *versus* more than 10 in nuclear chromatin) and the sizes of the smallest fragments (about 75 bp *versus* 160 bp). Moreover, core histone H3, which should play a central role in the formation of genuine nucleosomes (Luger *et al.* 1997), was absent from the organelle nucleoids in BY-2 cells (Takusagawa *et al.* 2013). Thus, the structures responsible for the DNA ladders in the isolated mitochondrial nucleoids are clearly different from, though comparable to, genuine nucleosome in nuclear chromatin.

Now, our effort is directed towards estimation of the generality of the “nucleosome-like structures” and identification of protein(s) responsible for their formation. The preliminary results suggest that the nucleosome-like structures are also present in nucleoids derived from various types of plastids, and that unidentified, 17-kDa DNA binding protein(s) might be involved in the formation of the “nucleosome-like” structures in both mitochondrial and plastid nucleoids in BY-2 cells (manuscript in preparation). Thus, the nucleosome-like structures with common organizing principle might be widespread among plant organelle nucleoids.

Up-regulation of dually targeted organelle DNA polymerase is responsible for the transient and synchronous activation of organelle DNA synthesis that is necessary for starting cell proliferation

In the root apical meristem of higher plants, levels and synthetic activities of organelle DNAs are high in the lower meristematic zone (near the quiescent center) and decline towards the upper meristematic zone (Fujie *et al.* 1993, Kuroiwa *et al.* 1992, Suzuki *et al.* 1992, 1995), indicating that organelle DNAs are actively synthesized only during the initial phase of cell proliferation. This characteristic mode of organelle DNA synthesis is well preserved in batch-culture of BY-2 cells (Yasuda *et al.* 1988, Suzuki *et al.* 1992, Okamura *et al.* 2002). The numbers and the DNA contents of the organelle nucleoids increased during initial phase of a batch-culture (from lag to initial log phase, Fig. 2), due to activation of organelle DNA synthesis. The DNA synthesis in mitochondria and plastids is activated almost synchronously and only transiently; organelle DNA synthesis activities reached their maximum within 24 h after the start of the batch-culture and then declined promptly. Thus, BY-2 cells provide us with a favorable system to analyze the mechanism and biological significance of the transient and synchronous organelle DNA synthesis in the initial phase of cell proliferation.

An *in vitro* DNA synthesis assay system using isolated organelle nucleoids (Sakai *et al.* 1999c) revealed that mitochondrial and plastid DNA polymerases are biochemically

Fig. 3. Phase-contrast with DAPI-fluorescence images of materials used for the analyses of plastid gene expression. (a), a tobacco leaf mesophyll protoplast; (b), chloroplasts isolated from tobacco leaves; (c), chloroplast nucleoids; (d), a protoplast prepared from BY-2 grown under normal culture condition; (e), proplastids isolated from BY-2 cells grown under normal culture condition; (f), proplastid nucleoids; (g), a protoplast prepared from BY-2 grown under amyloplast-inducing condition; (h) amyloplasts isolated from BY-2 cells grown under amyloplast-inducing condition; (i), amyloplast nucleoids. Photomicrographs in (a, d, g) and (b, c, e, f, h, i) are in the same respective scales. Scale bars represent 10 μ m.

indistinguishable from each other, and are distinct from DNA polymerase γ (a mitochondrial DNA polymerase in animals and fungi). Later, two homologues of bacterial DNA polymerase I, named *Ntpoli-like 1* and 2, were isolated from BY-2 cells and were identified as genes encoding organelle DNA polymerases that were dually targeted to mitochondria and plastids (Ono *et al.* 2007). During batch-culture of BY-2 cells, transcript and protein levels of *Ntpoli-like 1/2* increased transiently in the initial phase of cell proliferation, concomitantly with the elevation of DNA polymerase activities in the organelle nucleoids. Thus, the transient and synchronous activation of mitochondrial and plastid DNA synthesis in the initial phase of cell proliferation is accounted for by the transcriptional up-regulation of those dually targeted organelle DNA polymerase genes.

Pharmacological analyses using BY-2 cells revealed that organelle DNA synthesis in the initial phase of cell proliferation was essential for starting subsequent nuclear DNA synthesis and cell proliferation (Suzuki *et al.* 1996). Moreover, the organelle DNA levels that had been reached appeared to limit the capacity for subsequent cell multiplication. For the requirement of organelle DNA synthesis to start cell division cycle, involvement of tetrapyrrole retrograde signaling has been suggested (Kobayashi *et al.* 2009). However, the mechanism to up-regulate organelle DNA polymerase genes, which must occur in advance of the tetrapyrrole signaling, is presently unknown.

Differential use of RNA polymerases plays an important role in the transcriptional regulation of plastid gene expression during differentiation

Regulation of plastid gene expression, especially during greening, has been extensively examined by a number of researchers. Some of the early studies, in which newly developed run-on transcription of isolated plastids was used, suggested a limited role of transcriptional regulation and stressed an importance of post-transcriptional regulation (Deng and Gruissem 1987, Mullet and Klein 1987, Gruissem 1989). However, identification of sigma subunit genes of plastid-encoded, bacterial-type plastid RNA polymerase (Tanaka *et al.* 1996) and finding of nuclear-encoded, phage-type plastid RNA polymerases (Hedtkke *et al.* 1997) implied potential importance of the transcriptional regulation. Indeed, when chloroplast nucleoids and proplastid nucleoids were

isolated from tobacco leaves and BY-2 cells, respectively (Fig. 3a–f), and their *in vitro* transcription activities were compared, relative transcriptional activities of individual genes (chloroplast nucleoids versus proplastid nucleoids) were roughly correlated to relative abundances of corresponding transcripts (leaves *versus* cultured cells), demonstrating the importance of transcriptional regulation in plastid gene expression (Sakai *et al.* 1998b).

The differences in the transcription activity between chloroplast nucleoids and proplastid nucleoids involved division of labor between two types of plastid RNA polymerase, *i.e.*, bacterial-type and phage-type (Sakai *et al.* 1998a). Overall transcription activity of the isolated chloroplast nucleoids was high and highly sensitive to tagetitoxin, a transcription inhibitor specific to bacterial RNA polymerase (95–99% reduction at 10 μ M tagetitoxin). In contrast, transcription activity of the isolated proplastid nucleoids was low (less than 5% of the activity in the chloroplast nucleoids) and relatively resistant to tagetitoxin (only 40–50% reduction at 10 μ M). Those observations indicated that tagetitoxin-sensitive (bacterial-type) RNA polymerase was overwhelmingly major in chloroplasts, while tagetitoxin-insensitive (phage-type) RNA polymerase occupied about 50% or more of the activity in proplastids, and that increase in the level of bacterial-type RNA polymerase was responsible for the elevation of overall transcription activity in chloroplasts. Nonetheless, detailed analyses also showed that the gene preferences of the two RNA polymerases changed according to plastid differentiation (Sakai *et al.* 1998a). The mechanism for such change is presently unclear, but involvement of differential use of transcription factors (including sigma subunits) and/or changes in the template accessibility due to structural changes in plastid nucleoids is supposed.

Amyloplast differentiation in BY-2 cells: similarity to root cap cell differentiation

In the studies described above, a comparison was made between proplastids in BY-2 cells and chloroplasts in leaf cells because induction of chloroplast development in BY-2 cells was impossible. If we could induce differentiation of “proplastids” in BY-2 cells into any other type of plastids by any means, it should provide us with a good opportunity to investigate the changes in the plastid gene expression continuously during the course of plastid differentiation. We could indeed establish such a system, thanks to an advice from Dr. Masaya Satoh, by modifying the hormonal contents of the culture medium of BY-2 (Sakai *et al.* 1992, Fig. 3g–i). BY-2 cells are usually maintained in a medium containing auxin (0.2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid). In this medium, BY-2 cells proliferate rapidly and include undifferentiated “proplastids”. When BY-2 cells were grown in the auxin-free culture medium that contained cytokinin (1 mg L⁻¹ benzyl adenine), the plastids in the cells accumulated a large amount of starch and differentiated into amyloplasts within two days. During this process, overall plastid transcription activity declined, but the declines in transcription activities of *psbA* and *psaA/B* genes encoding reaction center proteins of photosystems II and I, respectively, were relatively small (Sakai *et al.* 1999b). The mechanisms, as well as the physiological significance, of this differential inactivation of plastid genes are presently unclear and should be examined in a future work.

Amyloplast formation in BY-2 cells is primarily triggered by the depletion of auxin and facilitated by addition of cytokinin (Sakai *et al.* 1996), and requires continuous expression of nuclear genes such as those encoding starch synthase (Sakai *et al.* 1997, 1999a, Miyazawa *et al.* 1999). Moreover, it was found that the amyloplast formation in BY-2 cells was always accompanied by a reduction in cell multiplication rate, increase in cell volume (Sakai *et al.* 1996), shorter lifetime, weaker cell–cell adhesion (Sakai *et al.* 2004a), and alterations in the secretion properties (unpublished result). The changes observed under amyloplast-inducing conditions resembles to what observed during differentiation of root cap cells, as has been suggested from the study of Golgi differentiation (Staehelin *et al.* 1990, Winicur *et al.* 1998). Thus, the behavior of BY-2 cells under amyloplast-inducing conditions (in the absence of auxin) might mimic the process of differentiation of the cells in the root cap, whereas the behavior under normal culture condition

Fig. 4. Fragmentation of nuclear DNA during cryptogeiin-induced cell death. (a), TUNEL assay. DAPI-stained images of nuclei (top row) and TUNEL-positive signals (bottom row) of the cells at 0, 24, 48, and 72 h after cryptogeiin application are shown. The scale bar represents 100 μ m. (b) DNA ladders. Total DNA was extracted 0, 24, 48, and 72 h after cryptogeiin application, and electrophoresed in an agarose gel. M, marker. Numbers to the left represent the sizes of DNA markers in kbp. Adopted from Sawai *et al.* (2010) with modification.

(in the presence of auxin) might mimic the process of cell proliferation in the meristem (Sakai *et al.* 2004a).

Induction of programmed cell death in BY-2 cells

Plants recognize pathogen invasion by sensing signal molecules called elicitors. The elicitor sensing leads to activation of several defense mechanisms, including programmed cell-death at the site of infection, named HR (hypersensitive reaction). In addition, accumulation of antimicrobial substances (phytoalexins) and PR (pathogenesis-related) proteins with various anti-microbial functions is induced both locally and systemically (Mittler *et al.* 1997). As a result, more prompt and efficient response to subsequent infection becomes possible both locally and systemically, which are called local acquired resistance (LAR) and systemic acquired resistance (SAR), respectively. Thus, the cells that first received the elicitor signal and activated the internal cell-death program (referred to as “HR cells” hereafter) might play multiple roles; they might not only produce phytoalexins and PR proteins (direct defense) but also generate signal(s) to induce LAR and/or SAR (signal transmission), while executing the cell-death program.

What kinds of activities are turned on in the HR cells before they finally die? To analyze this problem biochemically, plant organs have some problems as experimental system; because they are composed of heterogeneous cell populations behaving differently during HR, the observed biochemical response should be a mixture of those different behaviors. Such a problem may be avoided by using homogeneous, suspension-cultured cells as experimental material. Several researchers indeed developed “model systems” to induce HR-like cell death in suspension-cultured cells in response to application of elicitors (Dorey *et al.* 1999, Suzuki *et al.* 1999, Kadota *et al.* 2004). However, the efficiency of cell-death induction in those “model systems” had never reached 100%. This problem was finally solved by the establishment of our novel HR model system in which BY-2 and an oomycete-derived proteinaceous elicitor, cryptogeiin were used. In this system, programmed cell-death was induced with 100% efficiency in BY-2 cells in response to cryptogeiin application (Sawai *et al.* 2010), which, for the first time, allowed us to investigate the “pure” behavior of HR cells, by completely excluding surviving cells that are not committed to cell death.

The 100% efficient cell-death induction was achieved only when stationary-phase cells were diluted to 1×10^5 cells mL^{-1} with conditioned medium before cryptogeiin application, indicating that both growth phase and cell density were important factors. The cryptogeiin-induced cell-death was

accompanied by nuclear DNA fragmentation (Fig. 4) and induction of genes related to HR cell death (*Hin1* and *HSR203J*). Genes related to phytoalexin synthesis (*PAL*, *HMGR*, and *OMT*) and a subset of PR protein genes was also activated during the process (Sawai *et al.* 2010). In addition, the HR cells were found to secrete unidentified chemical signal(s) that led to induction of defense-related genes in neighboring cells (as reported by Chappell *et al.* 1997). The signal(s) also appeared to cause high sensitivity to the second cryptogin application in the neighboring cells (our unpublished results). The changes induced by the unidentified signal(s), *i.e.*, induction of defense-related genes and high responsibility to the second infection signal, were comparable to so-called acquired resistance. Thus, the HR cells appeared to activate direct antimicrobial mechanism of their own and transmit infection signal(s) to induce acquired resistance in other cells while executing the cell-death program.

Concluding remarks

As summarized in this review, the tobacco BY-2 cell line has taught us a lot about cytological aspects of plant cells, even though limited to the research fields in which we have been deeply involved. The high growth rate and high homogeneity have made the BY-2 cell line an ideal experimental material for biochemical and physiological analyses, which led to the finding of nucleosome-like structures in organelle nucleoids. Moreover, the BY-2 cell line has been shown to be useful not only as a model of undifferentiated cells, but also as a model of proliferating cells in meristematic tissues, differentiating cells in root caps, or cells undergoing programmed cell-death in hypersensitive reaction, which has further extended the potential usefulness of this experimental material. From now on, this cell line will continue to guide us towards further understanding of plant cell life.

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