A Bright Future for the Bright Yellow-2 Cell Culture

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The capacity of plant cells to revive out of a latent stage of differentiation and to start dividing in the presence of appropriate hormone concentrations has facilitated the in vitro culturing of a panoply of plant species from different tissue sources. Cell biology studies have been performed on many different cultures, but their divergent nature has complicated the integration of research information that had been collected separately. In retrospect, it has become evident that the use of Arabidopsis as a model system has created important benefits in terms of development and accessibility of novel tools and methods. With these advantages in mind, we should be encouraged also to focus on a single model system for the molecular analysis of cellular growth, including cytoskeletal organization and cell cycle control.

ADVANTAGES OF BRIGHT YELLOW-2 (BY-2) OVER ARABIDOPSIS

Arabidopsis cell cultures have been generated, but they have fallen short in cell cycle studies because attempts to synchronize these cultures with high efficiency have been unsuccessful. Moreover, because cells are very small, detailed observation of their intracellular organization and content is limited. In contrast, the tobacco (Nicotiana tabacum) BY-2 cell culture can be highly synchronized and stands out in terms of growth rate and homogeneity (Nagata et al., 1992; Samuels et al., 1998; Nagata and Kumagai, 1999).

BY-2 cells are readily transformed after protoplastation (Mathur and Koncz, 1998) or directly via particle bombardment or cocultivation with Agrobacterium tumefaciens (An, 1985; Klein et al., 1988; Rempel and Nelson, 1995). Although A. tumefaciens-mediated BY-2 transformation is performed routinely in many laboratories, we found that the efficiency in obtaining transgenic calli varies between experiments and mainly depends on the quality of the BY-2 cell culture. After synchronization, BY-2 cells in M and early G1 phase are 10-fold more susceptible for stable A. tumefaciens-mediated transformation than to cells residing in G2 (D.N.V. Geelen, unpublished data). In addition, the Agrobacterium strain LBA4404 that expresses constitutively the virG gene (van der Fits et al., 2000) is 2- to 5-fold more effective in generating transgenic calli. Typically, about 500 transgenic calli can be obtained from 4 mL of BY-2 cells cocultivated with this Agrobacterium strain (for a detailed protocol, see http://www.plantgenetics.rug.ac.be/~dagee), allowing phenotypic screening programs to be performed.

Originally, BY-2 was brought into life in an attempt to generate green factories that would produce large quantities of nicotine and other secondary metabolites in in vitro grown cell cultures. Its main raison d'être today is tied to the study of cell division-related processes, involving genes that, when ectopically expressed, can impede propagation of transformed cells. Temporal expression may circumvent the detrimental effects of the introduced gene(s) if background expression levels are tolerated. In Arabidopsis, inducible promoters that can be activated chemically have been troublesome because of leaky expression in the absence of inducer as demonstrated for the tetracycline de-repression system (De Veylder et al., 2000) or because of unspecific gene activation as observed for the glucocorticoid-dependent transcriptional activator (Kang et al., 1999; Ouwerkerk et al., 2001). For reasons that are not entirely clear, control experiments in tobacco plants and BY-2 cell cultures with the tetracycline repressor or the glucocorticoid-inducible system did not suffer the inadvertent effects reported for Arabidopsis (Criqui et al., 2000; David and Perrot-Rechenmann, 2001; Geelen et al., 2002; Nishihama et al., 2001). Unlike the tetracycline repressor, regulation by the glucocorticoid-dependent transcriptional activator is exceptionally tight and exhibits fast induction kinetics (recombinant protein can be detected within a few hours) with high inducibility, rendering this system suitable for the functional analysis of potentially toxic genes over the course of a cell division cycle (Kunkel et al., 1999; Criqui et al., 2000; Nishihama et al., 2001). The heat-inducible Arabidopsis promoter HSP18.2 may provide an alternative to the chemical-induced systems, as fast up-regulation kinetics have been also recorded in BY-2 transgenic cultures (Yoshida et al., 1995; Shinmyo et al., 1998; Joubès, personal communication).

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tion). However, prolonged exposure of BY-2 to temperatures as high as 38°C and 42°C can influence profoundly the structure of the cytoskeletal network and the cell division process (Smertenko et al., 1997). For other inducible expression systems, inducer-dependent physiological effects or inadequate control of expression have been reported (for review, see Zuo and Chua, 2000).

Because of their amenability for microscopic analysis, BY-2 cells have become popular for the subcellular localization of proteins through green fluorescent protein (GFP) tagging. Using organelle-targeted GFP markers, cytoskeleton and membrane compartments have been visualized in life BY-2 cells, revealing novel insights into Golgi dynamics unique to plants (Kost et al., 1998; Nebenführ et al., 1999; Granger and Cyr, 2000). Along with its advantages, BY-2 bears a number of limitations relating to the unavailability of mutant lines. Given the lack of mutants, the functionality of the analyzed GFP-tagged proteins is to be tested in appropriate mutants of other species like yeast (Saccharomyces spp.), as shown recently for MedusaCDK-A;2-GFP (Weingartner et al., 2001). The activity of GFP-tagged proteins may follow also from dominant phenotypic effects due to misexpression or inadvertent protein-protein interactions (Nishihama et al., 2001).

BY-2 AS A MODEL FOR CYTOSKELETON STUDIES

Early after BY-2 cultures had been introduced, they were used especially by Japanese groups as a suitable alternative to Lloyd’s carrot (Daucus carota) cell suspension cultures (Lloyd, 1999) to study and visualize the plant cytoskeleton (Traas et al., 1987; Nagata et al., 1992; Fairbairn et al., 1994; Hasezawa et al., 1998; Kumagai and Hasezawa, 2001). Distinct microtubule arrays occur during the cell cycle: a cortical network in interphase cells, a preprophase band during the G2 to early M phase, and a spindle and phragmoplast in cells undergoing mitosis. Fluorescence imaging of BY-2 expressing GFP-tagged tubulin and actin-binding proteins confirmed the structural organization of the cytoskeletal network and the transitions between consecutive configurations in dividing cells (Kost et al., 1998; Granger and Cyr, 2000). A more in-depth statistical analysis of a large number of BY-2 cells expressing the GFP-microtubulin-binding domain (MBD) assessed the structure/function relationship of unusual preprophase bands occurring in a subpopulation of cells, correlating the location of the preprophase band to the positioning of the nucleus and of the cell plate (Granger and Cyr, 2001).

Unraveling the molecular mechanisms that underlie the organization and functioning of the cytoskeleton requires the identification and isolation of cytoskeleton elements. New, plant-specific constituents are anticipated because of unique structural properties of the plant cytoskeleton (Lloyd and Hussey, 2001). Interphase cells contain a cortical array unique to plants that is implicated in the transfer of external stimuli across the plasma membrane, and in organizing the deposition of newly synthesized cellulose fibers (Wasteneys, 2000). The cortical microtubule and actin arrays are physically linked to the plasma membrane (Sonobe and Takahashi, 1994; Collings et al., 1998; Sonobe et al., 2001), and attempts to identify cross-bridging proteins have been undertaken (Marc et al., 1996). The cortical microtubules are usually aligned in parallel in single cells from suspension cultures as in cells that are integrated into whole plant tissue and form a dynamic network that responds to various agents or physiological stimuli by rearranging from a transverse orientation to a more oblique or fully longitudinal alignment (Yuan et al., 1994). These structural reorganizations depend on stabilizing and destabilizing microtubule-associated proteins (MAPs) and on motor proteins that move microtubules relative to each other (Lloyd and Hussey, 2001). Recently, a breakthrough in our understanding of the molecular aspects of the plant cytoskeleton has been established by cloning tobacco BY-2 cDNAs encoding three proteins of the MAP65 family (Smertenko et al., 2000). MAP65 proteins share no sequence similarity with MAPs identified in yeast or animal species; they represent a novel class of cytoskeleton-associated proteins that may contribute to the unique properties of the plant microtubular network. Significant similarity has been found with nine open reading frames from Arabidopsis without reported function (D.N.V. Geelen, unpublished data).

MAP65 proteins are also expressed in dividing cells and localize to the preprophase band, the central area of the spindle, and the phragmoplast, where the positive ends of the antiparallelly arranged microtubules overlap (Smertenko et al., 2000). The localization of MAP65 to the positive ends of microtubules suggests that in addition to its capacity to bundle microtubules (Chan et al., 1999), in vivo MAP65 may function as a cross-linking molecule of antiparallel microtubules (Smertenko et al., 2000). In semipermeabilized BY-2 cells, phragmoplast microtubuli incorporate (fluorescent) tubulin molecules at the positive ends, indicating that MAP65 must interact dynamically and be displaced continuously toward newly polymerized tubulin termini, perhaps through phragmoplast-associated kinesin motor proteins (Asada et al., 1991). Kinesin TKRP125 is a likely candidate because it has been implicated in minus-end translocation of phragmoplast microtubuli in permeabilized BY-2 cells (Asada and Shibata, 1994; Asada et al., 1997). It would be interesting to see whether MAP65 colocalizes with TKRP125 and whether it can influence TKRP125 activity in vivo.

Because of its potential for high synchronization, BY-2 has been used also to determine the ultrastruc-
tural features of separate steps during cytokinesis (Samuels et al., 1995) and have been promoted as the system of choice to analyze cytokinesis proteins that have been identified through genetic screens of Arabidopsis mutants. Localization of the Arabidopsis KORRIGAN protein, a membrane-bound β-1,4-d-glucanase involved in cell elongation and cytokinesis, as GFP-tagged recombinant proteins in BY-2 cultures, indicated a requirement for the LL and YXXO motifs (O, bulky hydrophobic amino acid) for proper subcellular localization to the vesicular compartment of tobacco phragmoplasts, suggesting that the polar targeting mechanism is conserved (Zuo et al., 2000). The new, separating cell wall initially consists primarily of callose synthesized by a callose synthase that specifically accumulates to the cell plate and forms a complex with phragmoplastin, UDP-Glc transferase, and the small GTP-binding protein Rop1 (Hong et al., 2001). Once again, the subcellular localization of the phragmoplastin (originating from soybean [Glycine max]) and the UDP-Glc transferase (originating from Arabidopsis) was determined in a BY-2 background, leading to the conclusion that at least in these instances, proteins are correctly targeted, and that targeting signals must be preserved (Gu and Verma, 1997; Hong et al., 2001). An alternative route to the center of the cell may involve the direct binding of phragmoplast microtubules (for instance, MAP65) or the interaction with a microtubulin-binding protein. Kinesins may exhibit such activity because they possess a cargo-binding domain in addition to the microtubulin-binding domain. In line with this suggestion, a cytokinesis-related mitogen-activated protein kinase kinase NPK1 has been proposed to be targeted to the spindle and the phragmoplast microtubules via the tobacco kinesin-like protein NACK1 (Nishihama et al., 2001).

Do kinesins or other MAPs embody the specificity determinants for regional localization to different microtubular configurations? To address this question, we have localized kinesin subdomains by coupling them to enhanced GFP (EGFP). Figure 1A shows a fluorescence image of a BY-2 cell transformed with a motor-EGFP construct under the control of the 35S promoter. The EGFP-tagged motor domain, cloned from a kinesin (AtKLP2) that has previously been found to interact with the Arabidopsis CDKA;1 kinase in a yeast two-hybrid assay (De Veylder et al., 1997), decorates the cortical array similar to GFP-MBD (Fig. 1B). However, cells expressing the GFP-tagged AtKLP2 motor did not survive and featured abnormal divisions, implicating intrinsic differences between these two microtubule-binding proteins.

**BY-2 AS A MODEL FOR CELL CYCLE REGULATION AND CELL GROWTH**

A balanced cocktail of the phytohormones auxin and cytokinin sustains permanent growth of in vitro cultured, undifferentiated plant material by stimulating the checkpoint control mechanisms of the cell cycle. Unlike wild-type tobacco cell cultures, BY-2 cells grow without added cytokinins by synthesizing their own hormones during the G2/M transition (Nagata et al., 1992; Redig et al., 1996). The cytokinin synthesis pathway and accumulation of zeatin at G2/M are inhibited in the presence of the cell division blocker lovastatin, revealing a dependence on cytokinins to proceed through the M phase (Crowell and Salaz, 1992; Laureys et al., 1998). Cell cycle checkpoints at G1/S and G2/M are controlled through a phosphorylation program executed by cyclin-dependent kinases (CDKs) and their principal cyclin regulators (Joubès et al., 2000; Stals and Inzé, 2001). Cyclin kinases have been suggested to modulate the phosphorylation status and kinase activity of CDKs (Kakimoto, 1996; Zhang et al., 1996). In line with a promotive effect on DNA replication and cell division, a G1/S checkpoint D-type cyclin cycD3 is up-regulated by cytokinin (Riou-Khamlichi et al., 1999). Accumulation of zeatin in BY-2 G1/S and G2/M cells may therefore trigger cyclin synthesis when it is needed. In fact, cyclins show oscillatory transcriptional regulation peaking at different intervals, hence their name (Pines, 1999). Cyclic expression of tobacco, Arabidopsis, and periwinkle (Catharanthus roseus) cyclins and other cell cycle-controlled plant genes has been demonstrated in BY-2 cell cultures, indicating the existence of a common M phase-specific regulatory machinery that is conserved in these species (Reichheld et al., 1996; Shaul et al., 1996; Ito et al., 1997; Combettes et al., 1999; Tréhin et al., 1999). Cyclin abundance has been shown also to be controlled in a cell cycle-dependent manner in BY-2 cultures via the ubiquitin degradation pathway, involving the recognition of a destruction box present in cyclins (Genschik et al., 1998; Criqui et al., 2000).

The effect on growth by auxin is complex and less well understood at the molecular level. When auxin is omitted from the BY-2-culturing medium, cell division ceases and differentiation sets in, associated

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**Figure 1.** Confocal image of BY-2 cells transformed with a EGFP-tagged motor domain from AtKLP2 (A) and with GFP-MBD (B). An asterisk indicates accumulation of fluorescence in the nucleus; the arrow points out a prophase band. Bar = 20 μm.
with morphological changes of the Golgi, an accumulation of starch, and an increase in cell length (Winicur et al., 1998; Miyazawa et al., 1999). In fact, low concentrations of auxin stimulate BY-2 cell elongation, whereas high concentrations reduce the average cell length by promoting cell division (Hasezawa and Syo-no, 1983). Transgenic BY-2 cultures in which the auxin-binding protein ABP1 is suppressed no longer respond to auxin and fail to show auxin-induced cell elongation (Chen et al., 2001). This observation demonstrates that control of the elongation process by auxin is cell autonomous. In addition to playing a role in determining cell length, ABP1 is needed for normal positioning of the division plane in developing embryos (Chen et al., 2001). The precise actions of auxin to modulate cell division are not understood but may involve proteolytic steps, because several Arabidopsis auxin mutants are affected in genes that encode proteins with similarity to the ubiquitin degradation pathway (Callis and Vierstra, 2000).

**GENE DISCOVERY IN BY-2**

The identification of plant genes involved in cell cycle regulation has hitherto depended heavily on homology-based gene isolation strategies (Mironov et al., 1999). However, the various types of protein activities needed for cell division appear redundant and occur in a variety of processes unrelated to division, leaving us with little clues from sequence information alone to predict which member of a protein family performs what task. Therefore, it is far more beneficial to identify genes through their expression patterns or subcellular localizations. In our department, we have taken on a project using cDNA-amplified fragment-length polymorphism technology to characterize and sequence BY-2-derived DNA fragments that correspond to mRNA that is differentially expressed from the S phase through the G1 phase of the cell cycle (Breyne and Zabeau, 2001). Samples are prepared from 12 time points every hour after release from the aphidicolin block and used to generate over 18,000 amplified fragment-length polymorphism tags, of which 10% exhibit a modulated cell cycle profile. The corresponding full-length cDNAs of the latter are now being isolated and further characterized by gene suppression and protein localization experiments. The majority of these cell cycle-modulated genes cluster in three large functional classes that relate to the S, G2, and M phases of the cell cycle. Although more than half of the genes isolated have no similarity to sequences in the available databases or are homologous to genes with unknown functions, the presence of typical genes, such as cyclins and tubulins, validate the clustered data sets. The “unknowns” are to be characterized functionally either through genetic analysis of mutations in the corresponding orthologs in Arabidopsis or, preferentially, through the suppression of the endogenous genes of the BY-2 cells by a gene-silencing strategy. Such efforts will certainly boost the cell cycle and cytoskeleton studies in BY-2 cells and bring this model system right into the spotlight of plant cell biology research.

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**LITERATURE CITED**
