DOI: 10.1007/s10535-015-0573-3 BIOLOGIA PLANTARUM 60 (1): 13-24, 2016

Practical guidelines for the characterization of tobacco BY-2 cell lines

M. SRBA[1]*, A. ČERNÍKOVÁ[2], Z. OPATRNÝ[1], and L. FISCHER[1]

Department of Experimental Plant Biology, Faculty of Science, Charles University in Prague, Viničná 5, CZ-12844 Prague, Czech Republic[1]
Institute of Applied Mathematics and Information Technologies, Faculty of Science, Charles University in Prague, Albertov 6, CZ-12843 Prague, Czech Republic[2]

Abstract

Plant cell lines represent useful models in plant cell biology. They allow simple analysis of the effects of various factors including modulated gene expression at cellular and subcellular levels. The tobacco BY-2 cell line is a favoured model due to its high proliferation rate, capability of effective synchronization, and accessibility to transformation. A relatively high uniformity of BY-2 cultures allows morphological phenotyping and assessment of growth parameters like mitotic index, viability, or cell density. Here we review already published and newly introduced optimized guidelines to carry out reliable, reproducible and efficient characterization of BY-2 cultures from suggestions of appropriate methods to acquire primary data, proper statistical treatment, and biological interpretation. The presented experimental data demonstrate the extent of natural variability and the effect of initial cell density on various cell culture features. Supportive equations allow to estimate some derived phenotypic parameters like cell cycle duration or fresh biomass of the culture and to determine the size of data sets for reliable documentation of a certain phenotypic change. The optimized protocols and accompanying discussion of weak points of different approaches should serve as practical guide for both beginners and experienced researchers working on BY-2 cells.

Additional key words: cytology, functional genomics, *Nicotiana tabacum*, plant cell phenotyping, transgenic plants.

Introduction

Effective exploitation of the extensive volume of plant genomic data depends on its proper assessment with appropriate phenotypic markers. Therefore, a huge effort has been put to uncover the relationship between the genotype and phenotype. Various large-scale procedures of plant phenotyping have been developed recently to be used for a multifactorial, rapid, and objective evaluation of complete plant organisms (Pieruschka and Poorter 2012). Unfortunately, even the best documentation of the "whole plant body" responses may not be sufficient to mechanistic interpretation that often requires understanding the behaviour of individual cells forming plant tissues and organs. For this reason, plant cell lines are used as alternative experimental models in various studies (Schaul *et al.* 1996, Richard *et al.* 2001, Orchard *et al.* 2005; reviewed in Sato 2013, Seifertova *et al.* 2014*)*. Cell lines offer the possibility of non-invasive characterization and evaluation of various cellular parameters; however, the approaches to their phenotyping have not been well established.

Phenotyping of the majority of plant cell lines is often complicated or practically impossible due to their high cell variability and formation of cell aggregates. One of the first more widely used cell lines with suitable morphological features was started in 1967 from the pith explants of *Nicotiana tabacum* cv. Virginia Bright Italia and called VBI-0 (Opatrný and Opatrná 1976, Opatrný *et al.* 2014). However, the strong need for a precise cultivation regime complicated the routine use of the VBI-0 cell line. Later on, it was replaced by the more plastic and phenotypically similar tobacco cell line BY-2 (Nagata *et al.* 1992) which was originated more than 40 years ago from seedlings of *Nicotiana tabacum* cv. Bright Yellow 2 and gradually became one of the

Submitted 21 January 2015, *last revision* 31 July 2015, *accepted* 5 August 2015.
Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; CV - coefficient of variation; DIC - differential interference contrast microscopy; FDA - fluorescein diacetate; MI - mitotic index; SBI - subculture interval; SD - standard deviation; VIA - viability; WT - wild type.
Acknowledgement: Funding was provided by the Ministry of Education, Youth and Sports of the Czech Republic (NPUI L01417). We thank Dr. Tomas Urfus for flow-cytometry analysis of BY-2 cells. We also thank Dr. Maria Barker-Mott for linguistic revision of the manuscript.
* Author for correspondence; e-mail: miroslav.srba@natur.cuni.cz

basic plant cell models. It exhibits an extreme spontaneous friability of cell files and absence of cell aggregates, consequently allowing a direct observation of individual relatively homogenous cells (Nagata *et al.* 1992, Opatrný *et al.* 2014).

BY-2 cells as well as VBI-0 cells are cytokinin-autonomous and auxin-dependent, requiring media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) as the favoured auxin (Nagata *et al.* 1992, Orchard *et al.* 2005). The average cell density of the standard BY-2 suspension culture ranges between 10^5 and 10^6 cells per cubic centimetre of the culture medium. Due to the enormous multiplication rate, reaching up to 100-fold biomass multiplication per one-week subculture interval, the cell divisions can be synchronized by transient application of various antireplicants or cytoskeletal drugs. The mitotic index in synchronized culture can reach up to 60 - 90 % (Nagata and Kumagai 1999, Kumagai-Sano *et al.* 2007, Kuthanova *et al.* 2008), which allows analysis of gene expression during the cell cycle (Schaul *et al.* 1996, Sorrel *et al.* 1999, 2001, Matsuoka *et al.* 2004).

BY-2 cells represent one of the favoured cellular models to study various aspects of plant cell physiology (Nagata 2004). In suspension, the cells are easily accessible to various chemical treatments, and individual cells or linear cell files are suitable for fluorescent immunocytochemical analyses. These features enable reproducible results to be obtained, for instance in cytoskeleton analyses (Kutsuma and Hasezawa 2002, Schwarzerova *et al.* 2003, Maisch *et al.* 2009). The cell line also allowed us to study the effects of various factors including plant hormones (Hoffmannova *et al.* 2008) and toxic metals such as cadmium (Kuthanova *et al.* 2008, Kutík *et al*. 2014) and aluminum (Krtkova *et al.* 2012). BY-2 lines also become an important tool for the functional analysis of various genes at the cellular level although assessment of the effects of gene up- or down-regulation is complicated due to high differences and variability in transgene expression in independently transformed lines (Nocarova and Fischer 2009). Therefore, the identification of the phenotypic changes and distinguishing between induced changes and natural variability of the line may not be straightforward (David and Perrot-Rechenmann 2001, Dvorakova *et al.* 2012).

The assessment of the effects of various factors including modulated gene expression on BY-2 cells can be done at various levels; determining changes in cell viability, mitotic activity, cell shape and size, polarity of both cell growth and division, and also analyzing various markers of cell differentiation and growth. To utilize this potential, a simple, routine, and reliable way for detection and measurement of these phenotypic features is needed. Such an exemplary study was performed on an *Arabidopsis thaliana* cell line by Richard *et al.* (2001) demonstrating also reliable biometric approaches. Surprisingly, the reviews devoted to the BY-2 cell line (Nagata 2004, Nagata *et al.* 2006) pay only little attention to phenotype assessment.

In order to carry out reliable, reproducible, and efficient characterization of phenotypic changes in either transgenic or treated BY-2 cell cultures, it is necessary to use appropriate methods to acquire primary data, to treat them with suitable statistical methods, and to interpret the results in a proper manner. Here we summarize basic cytological methods and mathematic procedures suitable for cell line evaluation and accompany them with potential weak points of individual approaches and special suggestions optimized for the BY-2 line. The instructions for measurement and evaluation of physiological and morphological parameters are based on our long-term experience with diverse transgenic BY-2 lines and lines treated with various compounds. We provide suitable methodological approaches for assessment of the key phenotypic parameters and guidelines to estimate a minimal necessary size of data sets for reliable comparisons. Optimization of the experimental design allows evaluation of a sufficient number of variants and repetitions for improved scientific output.

Materials and methods

The tobacco (*Nicotiana tabacum* L.) cell line BY-2 (Nagata *et al.* 1992) was cultivated in darkness at a temperature of 27 °C in a Murashige and Skoog (1962; MS) medium supplemented with 100 mg dm^{-3} K_2HPO_4 and 1 μM 2,4-D. Pyridoxine and nicotinic acid were omitted compared to the original Murashige and Skoog's protocol. The pH value was adjusted to 5.8. The cells were cultured either in the liquid medium as suspension culture or in the form of plated suspension or inoculated pieces of the cell biomass on the agar medium. The suspension cultures were subcultured by inoculating 1 cm^3 of the previous stationary suspension culture to 30 cm^3 of a fresh medium. The cell suspension was aerated by shaking on a horizontal shaker (160 rpm).

A viability test was performed by fluorescein diacetate (FDA) staining. A staining solution (1 mm^3; 2 mg of FDA per 1 cm^3 acetone) was added to 2 cm^3 of a cell suspension. Labelling nuclei allowing automatic counting cells was done using a water solution of *Hoechst 33342*. The staining solution (0.05 mg cm^{-3} *Hoechst 33342* supplemented with 100 mg cm^{-3} *Triton X-100* to penetrate cell membranes) was added as 10 mm^3 per 1 cm^3 of sample. Density was measured by scanning of the Fuschs-Rosenthal counting chamber (see details in Results and discussion). Microscopic images were captured by *Olympus BX 51* (Tokyo, Japan) equipped with a camera *Apogee Alta U 4000* (Abingdon, UK; resolution 2048 × 2048 pixels, 16 bit colour depth).

Image analysis was performed by *NIS Elements AR v. 3.1.* (*Laboratory Imaging*, Prague, Czech Republic).

Statistical analysis was performed using *NCSS 2000* (*NCSS*, *LLC*, Kaysville, Utah USA) or *R 2.13.2* (*Bell Laboratories*, Murray Hill, NJ, USA). Specific statistical models of data processing are described in chapters of Results and discussion depicting evaluation of each parameter.

For evaluation of dilution effect on growth parameters, suspensions were prepared to a final volume of 36 cm^3 in four initial dilutions (60× = 4.9 × 10^4, 30× = 1.0 × 10^5, 15× = 1.9 × 10^5, and 7.5× = 4.1 × 10^5 cells cm^{-3}). Each dilution variant was represented by six flasks. Samples of 1.5 cm^3 were collected each day. The initial volume was set to maximally imitate a standard cultivation volume of 30 cm^3 despite repeated sampling that reduced the volume from 34.5 cm^3 (after sampling on day 0) to 24 cm^3 on day 7.

Morphological data were based on measurement of a total number of 75 000 cells, approx. 250 - 600 for each sample. Density data were based on scanning and counting six fields per replicated flask and day of sampling, covering 8.3 × 10^5 cells in total. Morphology measurement was repeated once with six replicate flasks for each dilution.

Flow-cytometry analysis was performed by a *Partec CyFlow ML* (Görlitz, Germany) cytometer. A stationary culture of tobacco BY-2 cells was compared to nuclei isolated from well-developed leaves of two month-old-plants of *Nicotiana tabacum* cv. Samsun. The samples were standardized to nuclei of *Solanum pseudocapsicum* leaf isolates.

Results and discussion

To receive reliable and reproducible data characterizing the phenotype of a particular plant cell line, one needs to understand the conditions and the character of its growth (Sato 2013). During the standard subculture interval (SBI), BY-2 cells are exposed to continually changing conditions – stationary cells are inoculated to a "raw" well-aerated medium full of nutrients. As the cell density increases, the medium is modified by the cells and the amount of nutrients and oxygen decrease. After a short initial lag phase, the cells start to divide. Cell divisions are accompanied by longitudinal growth that continues even after cessation of the mitotic activity towards the end of the SBI. The final stationary stage results from the depletion of resources and directs the cells towards senescence (during a prolonged SBI). The cell size is maximal in the stationary phase when the cell files spontaneously disintegrate (Seifertova *et al.* 2014). The behaviour of cells during the SBI is strongly affected by the initial cell density (see also bellow) which should not decrease below a critical level, where growth of the culture is partially or completely inhibited.

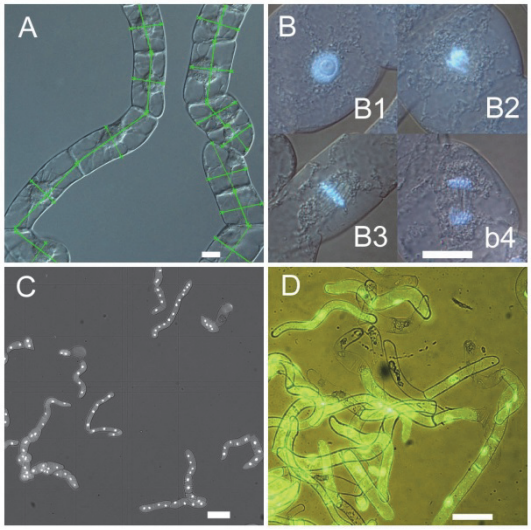

Fig. 1. Illustrative pictures showing evaluation of main phenotype features: *A* - cell length and width; *B* - mitotic index evaluation (*B1* - interphase nuclei, *B2* - prophase, *B3* - metaphase, *B4* - anaphase/telophase); *C* - cell density determination (Hoechst staining); *D* - viability (FDA staining). *Scale bar* = 10 μm in *A* and *B*, 100 μm in *C* and *D*.

The following text provides a fusion of both results and discussion. Firstly it introduces measurements of primary parameters, *e.g.*, viability, cell length, mitotic index, and cell density. Further it provides calculations of secondary parameters such as duration of cell cycle or mitosis that are based on the primary data. Differences in evaluated parameters are demonstrated within the subculture interval on illustrative experimental data obtained with a set of four wild type BY-2 suspensions differing in the initial cell density. Four different inoculum variants induced groups differing in growth dynamics. Such design imitates a hypothetical experimental design on phenotyping of treated or transgenic cell lines with altered growth parameters. Our model also demonstrates a relation among mitotic activity, cell length, and other parameters that are dependent on each other. This section also provides recommendations on a general experimental design. In addition, a special attention is paid to initial cell density, characterization of transgenic BY-2 lines, and characterization of BY-2 calli.

Viability of the untreated WT BY-2 cell population exceeds 90 % during the whole standard SBI. The determination of the proportion of dead or viable cells is the routine procedure (Seifertova *et al.* 2014). Viability of cells can be estimated by several cytological approaches with differing degrees of reliability. Dead or viable cells are commonly detected by cytological staining with trypan blue or fluorescein diacetate (FDA). Due to limited reliability of these methods, some authors discussed an alternative visual evaluation with differential interference contrast (DIC) microscopy (Widholm 1972). As this approach strongly depends on

personal experience and is time consuming, FDA staining (Fig. 1*D*) can be recommended as reproducible and simple technique for BY-2 cells (Widholm 1972, Nagata *et al*. 1992). Under standard conditions, FDA staining is sufficient approach, but under specific conditions, it is advisable first to compare the results obtained from FDA and from other techniques and then to use their combination if the results differ.

Pairwise statistical comparison of viability in two samples can be performed by proportion test (Wang and Chow 2007). A sufficient sample size can be calculated according to Equation 1a, derived after Wang and Chow (2007). For example, samples of 300 cells are sufficient to demonstrate a 5 % difference in viability compared to an untreated control with a 100 % viability at $\alpha = 0.05$.

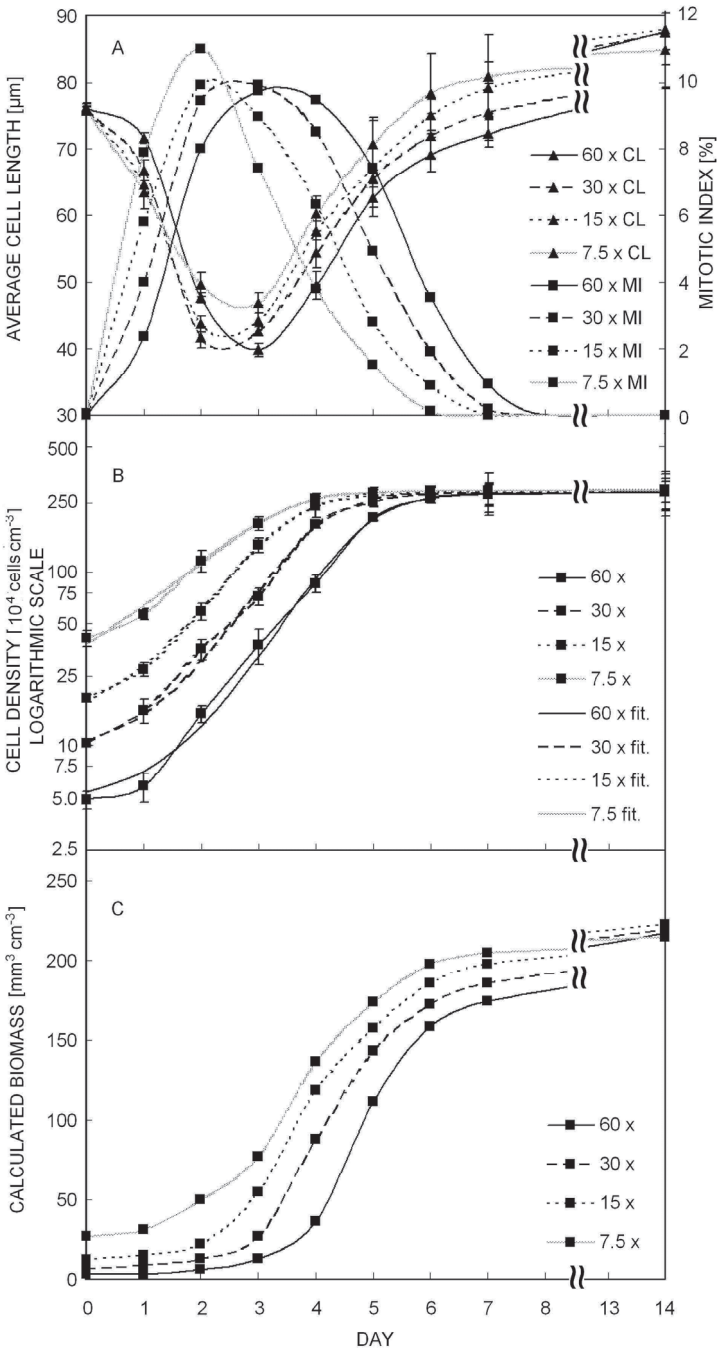

Fig. 2. Changes in selected parameters during a week subculture interval on wild-type BY-2 lines differing in initial cell density. *A* - Mitotic index and cell length; *B* - cell density; *C* - calculated biomass in suspension culture (estimated from cell size and cell density).

Mitotic index (MI) is counted as proportion of cells undergoing any phase of mitosis (Fig. 1*B*). The chromatin can be visualized by fluorescent dyes such as Hoechst 33342 or DAPI. The value of MI can be systematically shifted by a subjective evaluation of the initial period of prophase that is not very prominent, and also of late telophase that is hard to distinguish from early cytokinesis (Fig. 1*B*). Therefore, comparisons should be done with samples evaluated by the same person. In a non-synchronized culture, the MI reaches its peak of 10 - 12 % in the exponential phase usually between the second and the fourth day of cultivation and usually fades out on day 5, as also described by Nagata *et al.* (1992).

Differences in mitotic activity can be tested in the same way as viability, *i.e*., by proportion test. Orchard *et al.* (2005) used 3 × 200 cells sampling for demonstrating differences in proportions of each mitotic phase. Sample size for basic comparison of the mitotic activity of two samples can be also estimated using Equation 1a. According to this, a sample covering 300 cells is sufficient to demonstrate the difference of 5 %, 600 cells per 3 % difference, or 3000 cells per 1 % difference at the level of significance $\alpha = 0.05$.

Cell length: The length of BY-2 cells is basic morphological parameter that often changes in response to experimental treatment or as result of modulated expression of a certain gene in transgenic lines. The cell length is not constant, but gradually changes during the subculture interval due to simultaneous opposing processes: cell elongation and cell division. The difference in cell length can result from both varying cell proliferation dynamics and altered cell expansion. In the wild-type BY-2 line under standard cultivation conditions, the minimal cell length well correlates with the peak of mitotic activity (Fig. 2*A*). As both MI and cell length depend on the initial cell density (Fig. 2*A*), this parameter should be rigorously normalized. Alternatively, evaluation in the stationary phase can be recommended because of minimal cell length fluctuations in this period (see changes of measurement reliability in Fig. 3). Moreover, the overall variability of cell lengths in the stationary culture is slightly lower than in the exponential cells. The variation coefficient of cell length in the exponential phase is between 30 and 55 % (mean 40 %), whereas it reaches 28 to 40 % (mean 35 %) in the stationary culture. In stationary cells, the differences between variants reflect mostly changes in cell expansion and are not affected by cell divisions that can otherwise mask or magnify the effects of experimental treatments or a transgenic phenotype.

The average length of stationary cells reached 69 - 80 μm under our cultivation conditions. The values of cell length do not exhibit normal symmetrical distribution, but the histogram is right-skewed (Fig. 1 Suppl.). This reflects the natural limit of BY-2 cells that are rarely shorter than a certain value (30 μm for stationary cells), whereas the upper limit of cell sizes can reach surprisingly high values, especially in the case of stationary cultures (up to 350 μm). The cell length data could be transformed into a normal distribution using the *Box-Cox* power transformation. However, given that the number of data sets from metric measurements is high (> 100 observations), the overall analysis can be performed using *ANOVA* which is robust to non-normal distribution of data in such numerous data files (Milliken and Johnson 1984). Paired comparisons can be correctly performed by Tukey's test (Hoaglin *et al.* 2011).

Effect of cell affiliation to cell files: Sizes of cells measured in captured pictures are not independent but show dependence on the affiliation to individual cell files. The cell length heterogeneity is usually lower within each cell file compared to the heterogeneity of the whole cell population (Fig. 2 Suppl.). The morphological similarity of cells within a file is likely related to their common

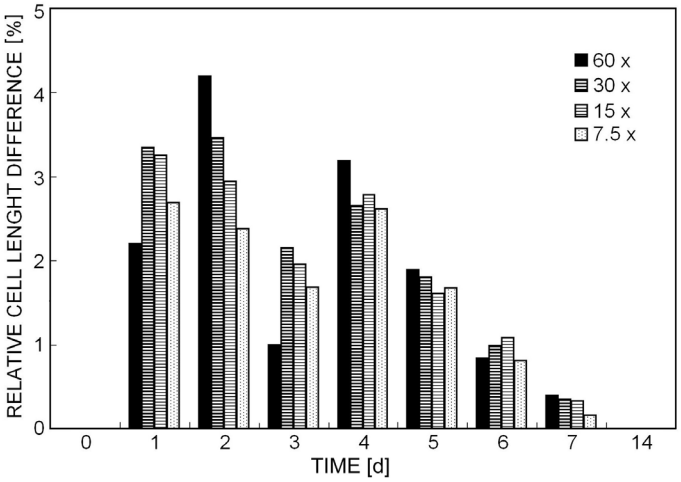

Fig. 3. Reliability of cell length measurement during a week subculture interval in wild-type BY-2 lines differing in initial cell density. The columns indicate average differences (calculated from absolute values) between the cell length in the respective time and the cell lengths 6 h before and after.

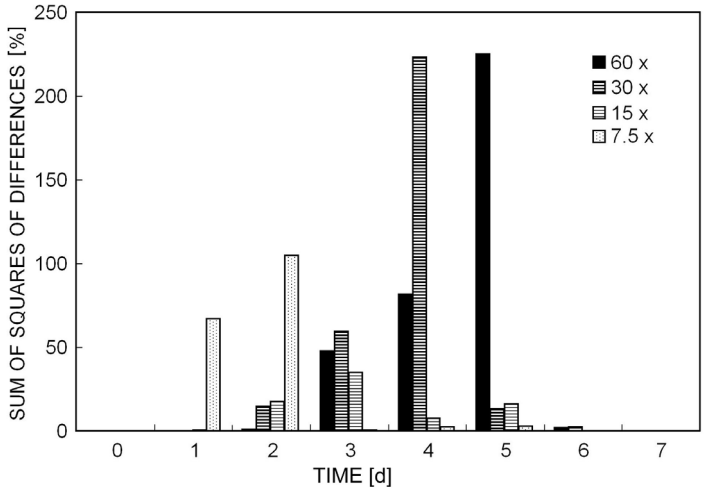

Fig. 4. Relative importance of density measurement in each day of subculture interval in wild-type BY-2 cultures differing in initial cell density. The columns indicate increases in the sum of squares of differences (on every day) between the curve obtained from all data (see Fig. 2*B*) and the curve constructed with the omission of the particular day.

ancestor and symplastic interconnections (Laporte *et al.* 2003) which were demonstrated to mediate a mutual physiological synchronization of cells within a file (Campanoni *et al* 2003, Maisch and Nick 2007).

Data sets of cell length can be analyzed by a basic linear modeling of the mentioned tests. One of the assumptions of such a model is independence of the measurements. However, the values of cell length are not fully independent. Hence, the most correct model for the data is a random effect model, with the cell-file affiliation as the random effect. Comparison of the two approaches (simple linear and random effect models) can be done *via* the Akaike information criterion (AIC). It was found that the random effect models are much better for the data (*e.g.*, AIC = 10116 for the simple linear model and AIC = 9606 for the random effect model).

However, from a practical point of view, the registration of cell file affiliations is problematic when cell files overlap, and it increases the time required for sample preparation and image analysis (Table 1). The upcoming question is, how large should the sample of cells be so that we can obtain reasonable results, when neglecting the cell file affiliation. Since we will compare two independent samples, we will use the Equation 1b rising from the two-sample *t*-test (Chow *et al.* 2003). This equation expects that we work with independent data. When the data are structured into cell-files, we can expect that the observed variability is lower than that of independent data. Hence, if we evaluate this equation with a greater variability [the term standard deviation (SD) in Equation 1b], we will obtain the necessary sample size for our situation. The other parameters of the equation remain the same regardless the data are dependent or not. The remaining question is, how much we should increase the variability. When comparing the two linear models, with and without the random effect of cell affiliation, we can check how much the models change the explained variability. The simple linear model explains 12 % of the variability, whereas the model with the random effect for cell affiliation explains 77 %. Hence, the observed variability can be reduced up to by 65 % (77 - 12 %) comparing to the situation where the data are independent. The right sample size can be obtained when using the observed SD multiplied by 100/35 = 100/(100-65).

Cell density measurement: The measurement of BY-2 cell density using conventional counting chambers (*e.g.*, Burker's or Thoma's) is complicated by a relatively large size of BY-2 cell files. An average thickness of a BY-2 cell is around 30 μm, a length of a stationary cell is about 80 μm (Sorrel *et al.* 1999, Kouri *et al.* 2003), which makes the cell file dimension reaching hundreds of micrometers or can exceed even one millimeter. The suspension of such "bodies" is prone to form dense clumps of cell files inside the counting grid or aside of it, both heavily affecting the accuracy of a single measurement. Irregular spread of measured particles can be expected especially when counting chambers with a 100 μm depth are used. To assess the effect of chamber depth, we compared the results obtained by using four counting chamber types; Fuchs-Rosenthal's (200 μm depth), Burker's (100 μm), Neubauer's improved (100 μm), and Thoma's (100 μm). The differences between cell densities estimated in these chambers were not significant ($\alpha = 0.05$) when counting 30 images containing approx. 12 000 - 22 000 cells in total, but the data sets greatly differed in variation (Fig. 3 Suppl.) that was lowest in the case of Fuchs-Rosenthal's chamber. Thus, in this chamber, cell density can be estimated from a smaller number of images. According to Equation 1b, we can estimate that approximately 6-times more images are required when using 100 μm-deep chambers to obtain cell density estimation in accuracy comparable to Fuchs-Rosenthal's chamber.

Fuchs-Rosenthal's chamber is deep enough to enable

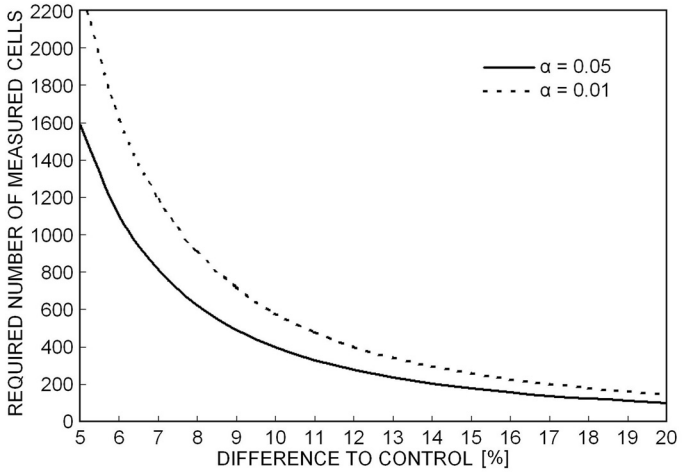

Fig. 5. Estimation of the minimal sample size based on Equation 1b. X-axis indicates the length difference between a control and evaluated variant (in %) to be demonstrated. Y-axis indicates the minimal number of cells needed for evaluation. The *solid line* is applicable for $\alpha = 0.05$, the *dotted line* is applicable for $\alpha = 0.01$.

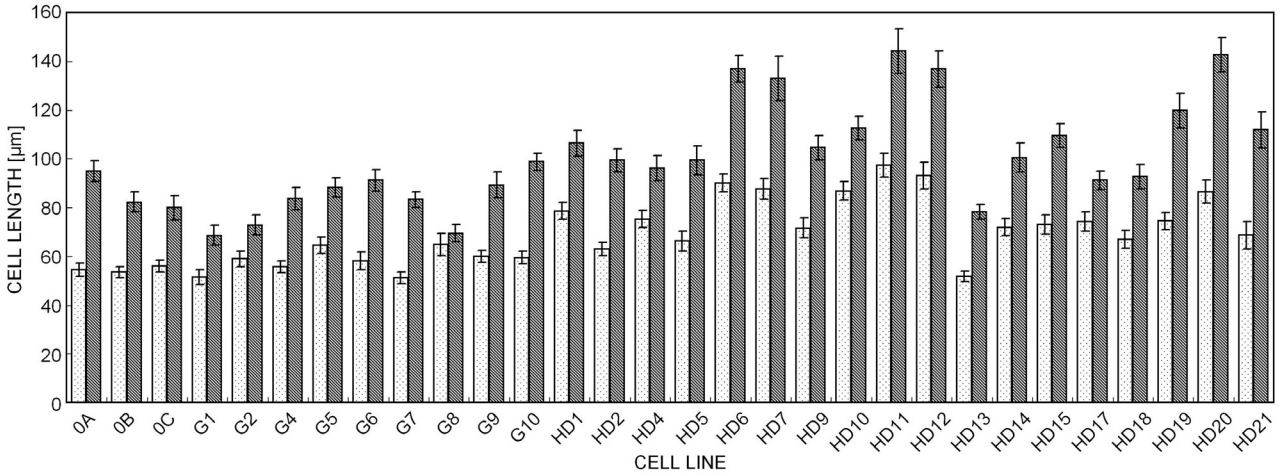

Fig. 6. Variability of cell length in wild-type and transgenic BY-2 lines. The graph presents three independent suspensions of untransformed BY-2 obtained by dispersion of independent *calli* (0A - 0C), nine lines overexpressing GFP (G1 - G10), and 18 lines overexpressing a modified cell wall protein NtHyPRP1 (H1 - H21; adapted from Dvorakova *et al.* 2012). Columns indicate cell length in the 3rd and 7th day (*light gray* and *dark gray*, respectively).

an almost accurate movement of an applied sample of BY-2 cell suspension, and it is also shallow enough to be acceptably focused with an objective with a 4- or 10-fold magnification. Such a low magnification enables scanning of a large field of the counting chamber with a microscope camera. We recommend collecting images of cells with fluorescent labelled nuclei (Hoechst 33342 or DAPI) which can be subsequently quantified by a (semi)automatic image analysis software (Fig. 1*C*). Resolution of the images must be high enough to cover any nucleus with at least five pixels to enable the software to reliably detect nuclei in contrast to the background and also to separate close nuclei from each other. It is not necessary to quantify the cells according to the counting grid of the chamber. The counting chamber can be used just as instrument providing a proper Z dimension (depth), and XY dimensions of the analyzed volume are defined by the screen area of the camera. Cell density can be calculated according to the total volume of screened area. The most appropriate data are obtained when the whole grid of Fuchs-Rosenthal's chamber is covered with 150 - 1 500 cells. Application of a more dense suspension into the counting chamber leads to formation of clumps of cell files that increase variation/inaccuracy of a single measurement. It can be seen from the comparison of the variation of a properly dense (diluted) sample and a 5-times denser (undiluted) sample (Fig. 3 Suppl.). Therefore, dense suspensions (usually since the 3rd day of SBI) should be diluted to the recommended value between 150 and 1 500 cells within the counting grid.

Suspensions of isodiametric individual cells are usually applied in excess and the redundant liquid flows out to marginal drains of the chamber. This procedure cannot be adopted in the case of BY-2 cells because their movement within the counting chamber is irregular,

highly affecting the numeric data obtained. The sample of BY-2 cells should optimally be applied as single drop of appropriate size (approx. 3 mm in diameter, *i.e.*, 15 to 20 mm^3) using a wide-cut tip. The sample should not overflow the counting area of the chamber.

An alternative approach to overcome the obstacles discussed above is adoption of some maceration technique. These techniques are frequently used for separation of cells from a tissue (Johansen 1940, Brown and Rickless 1949). Maceration is generally based on modification of cell wall solidity and especially loosening the middle lamella (Johansen 1940, Němec 1962). Acidic maceration (*e.g.*, 15 % (m/v) HCl + 15 % (v/v) ethanol; Johansen 1940) or H_2CrO_4 treatment (5 % (m/v) aqueous solution) described by Brown and Rickless (1949) can be applied on a BY-2 cell suspension leading to softening and partial splitting long cell files (Sorrel *et al.* 1999). We confirmed that maceration with H_2CrO_4 made even a very dense BY-2 suspension suitable for reproducible pipetting. It resulted in a decreased variation of data obtained from the counting chamber. However, in the case of diluted samples, the maceration did not decrease variation compared to the results obtained with our optimized procedure described above (Fig. 3 Suppl.). Moreover, the maceration needed an extra treatment of samples and in the case of H_2CrO_4, handling with an extremely toxic and carcinogenic compound (Dayan and Paine 2001). Other maceration techniques using less harmful agents (*e.g.*, HCl + ethanol) require additional staining cells (*e.g.*, with Orcein) for subsequent image analysis (H_2CrO_4 causes shrinkage of protoplasts into dark brown particles that can be easily counted).

Taking together these results, we recommend an optimized procedure based just on a counting chamber of 200 μm depth (*e.g.*, Fuchs-Rosenthal's, Sorrel *et al.* 1999), a proper dilution, and pipetting investigated samples stained with Hoechst or DAPI. Such a procedure provided reliable data and was less time consuming compared to the maceration. Gentle processing cells also enabled evaluation of morphological parameters and the MI from the same images.

Qualitative parameters of cell density data: The variation of obtained density data is very high. The coefficient of variation calculated for 194 available data files was within the range of 7 - 50 %, the average value of this data parameter was 23 %. Based on this value, we can estimate the required size of a data set (the number of scanned fields) for statistical comparison of two samples (*e.g.*, the *t*-test). A modified Equation 1c (see the section Equations) can be used for such estimation. The size of a data set for the significant detection of a 10 % difference in density between two samples was calculated to be 177. It is practically impossible to investigate cell density by such extensive sampling. However, when such a number of scanned fields is used to cover the whole subculture interval, we can obtain a highly representative growth curve describing the growth features of investigated lines. Such an approach is described and discussed bellow. The time required for evaluation of basic growth and physiological parameters of one sample is summarized in Table 1.

Growth curve calculation – data fitting: Due to the high internal variability of the primary density data, the representative growth curve can be obtained either from high numbers of scanned fields (see above) or by fitting the primary data (*e.g.*, by the method of least squares) to a modified logistic curve according to Equation 2. Keeping an acceptable degree of inaccuracy, this approach enables to reduce the number of required primary data and to smooth the curve. The main features such as the slope and duration of the exponential phase are maintained by this approach (Fig. 2*B*).

Fitting data to the logistic curve has several critical

Table 1. The list of growth and physiological parameters of BY-2 lines and a time required for evaluation of a sample.

Parameter	Sample processing	Subsequent analysis	Postponed processing	Special comment
Viability	5 - 10 min	5 - 15 min	yes, from saved image data	Simple, exact and well reproducible results.
Mitotic index	5 - 10 min	5 - 15 min	yes, from image	Simple, exact and well reproducible results.
Cell length	5 - 10 min	20 - 40 min	yes, from image	Exact and highly reproducible results, moderately laborious.
Cell width	5 - 10 min	20 - 40 min	yes, from image	Exact and highly reproducible results, moderately laborious.
Density	10 - 15 min	30 - 60 min	yes, from image	Cell files are too large to behave normally in a counting chamber; application of a small culture volume reduces variability, a good scanning equipment and software for image analysis helps to accelerate density evaluation.
Fresh mass	< 5 min	< 5 min	no	Non-reproducible separation of intact cells from the medium; we recommend estimating fresh mass as density multiplied with an average cell volume.
Dry mass	< 5 min (+ 1-h waiting)	< 5 min	yes, from frozen samples	Simple, exact, and well reproducible results.

points. The first of them is "rooting", *i.e.*, fitting the curve to the initial point. Since the lag-phase is usually very short in BY-2 cells, the lower asymptote of the curve has too low amount of numeric data to be fitted properly (the density values are missing before the starting point). Therefore, we suggest to calculate the confidence interval ($\alpha = 0.05$) for the density value at time 0 and to set this interval as restriction for the initial value of the calculated curve. The confidence interval can be easily calculated from primary density data using Equation 3.

Data presented in Fig. 2 show the growth characteristics of WT BY-2 cells in one-day sampling. The pattern of sampling can be adjusted. Periods of special interest (*e.g.*, a lag-phase) can be sampled more frequently (*e.g.*, every 8 or 12 h). On the other hand, some days can be omitted from the sampling schema. An analysis of importance of each sampling day within the 7-d SBI was performed on our dataset. It shows that the values of days 1 and 2 are of a low importance if the curve is "rooted" into the confidence interval of the day 0. However, we do not recommend omitting the measurement on day 1 because this value can indicate altered dynamics of the initial growth, if affected (not documented in our illustrative dataset). Day 2 can be skipped when the initial period of growth is not a subject of interest. Also day 6 is of a low importance for data fitting. In contrast to this, it is very important to correctly measure the day when the culture abandons exponential growth and slows its metabolic activity, usually the 4th or 5th day (Fig. 2*B*). Omitting the value from this "turning point" causes the most significant distortion of the fitted curve shape (Fig. 4).

Cell cycle and mitosis duration: The period of constant exponential growth is apparent as linear part of growth curves when cell density is displayed in a logarithmic scale. Provided that practically all cells undergo cell divisions within this period, the doubling time of the cell density should equate to the duration of the cell cycle (see Equation 4; Richard *et al*. 2001). The calculated values between 18.6 and 20.2 h well correlate with the values documented in literature (Nagata *et al.* 1992). Cell cycle duration is obviously basic parameter that can be changed under various experimental treatments or by genetic modifications.

The duration of the mitotic phase of the cell cycle can be estimated from the proportion of the MI during this period of exponential growth (*i.e.*, cell cycle duration × MI; Equation 5). The calculated value of about 2 h is again in agreement with literature (Granger and Cyr 2000).

Biomass and the rate of cell file elongation: Measurement of fresh mass of a BY-2 culture is complicated due to the inefficient and non-reproducible separation of the cultivation medium from intact cells using either sedimentation or filtration (vacuum or capillarity-based filtration). The fresh mass can be estimated indirectly by using the biometric and cell density data, as cumulative cell volume, *i.e.*, the average cell size multiplied with the cell number (Equation 6). The curve of calculated biomass (Fig. 2C) clearly shows a rapid increase which is also visually apparent between the 4th and 5th day and is caused by a synergic effect of a high mitotic activity and the highest rate of cell elongation in this period.

Image data storage and subsequent analysis: Direct evaluation of growth parameters during the experiment limits the number of samples that can be processed and brings high demands on work performance, since the quality of the samples changes over time. Therefore, it is highly advisable to separate sample processing, scanning, and storing images from subsequent evaluation and numerical data processing. This approach allows reliable capturing the rapidly changing parameters, such as viability or the MI, and inclusion of a larger number of parallel samples or replicates.

Capturing universally applicable images can also reduce the time required for sample analysis. In this respect density, MI, and cell sizes can be evaluated from the same fluorescent-labelled image captured in Fuchs-Rosenthal's counting chamber (for density assessment). For MI determination, images must have a sufficient resolution to allow evaluation of the mitotic activity, at least 1 pixel = 1 μm. Images of FDA labelled cells for viability assessment must be captured separately, but their number need not be high because approx. 300 cells is sufficient to demonstrate a 5 % decrease in viability at $\alpha = 0.05$.

Estimation of a minimal sample size: Appropriate calculation of numeric sample size is essential step in the building of experimental design. It is advisable to state/estimate in advance the minimal difference between the variants that we would like to demonstrate. This statement or estimation is researcher's choice: "How large difference is relevant for testing my scientific hypothesis? Am I going to demonstrate the difference of 15 or 0.01 % in cell length?" Processing too large sample size may lead to a highly significant approval of negligible differences that are as tiny as they are practically/biologically irrelevant (Fay and Gerow 2013). On the other hand, the data sets have to be large enough to identify and demonstrate a certain difference between the compared samples. Underestimation of sample size may lead to a weak or completely unsuccessful statistic approval of experimental results. However, an appropriate reduction of the number of analyzed cells saves time and thus allows analysis of a larger number of experimental variants that is highly advisable.

The required sample size can be thereafter predicted by Equations 1a,b,c. For transgenic/treated variants, whose morphological variability is not dramatically different from a control, the estimation can be done on the basis of the chart which is based on Equation 1b (Fig. 5, Chow *et al.* 2003).

Initial cell density – dilution of the culture: Nagata

(2004) described the ability of the BY-2 line to increase its biomass 100-times during one-week SBI and therefore recommended the week subculture with a 100-fold dilution. The growth of the BY-2 line below this multiplication ratio is suboptimal and the culture conditions need to be optimized (Nagata and Kumagai 1999, Nagata 2004). In most laboratories, however, the BY-2 lines grow in lower volumes and using a different mode of aeration - shaking instead of rolling used in Nagata's work. Under such conditions, the cultures are diluted only 20 to 30× per week (Koukalova *et al.* 1997, Schwarzerova *et al.* 2003, Seifertova *et al.* 2014). As clearly visible from Fig. 2*B*, lower dilutions resulted in a shorter lag phase, but longer time of "stagnation" in the stationary, physiologically less active state. Maximally diluted suspensions showed a practically constant multiplication rate for 4 d, whereas this period decreases in less diluted suspensions (see the duration of the phase of exponential multiplication, *i.e.*, the linear part of the curve in Fig. 2*B*). A decreased growth activity of the lines causes amongst others a significant decrease of efficiency in cell cycle synchronization (Samuels *et al.* 1998, Nagata and Kumagai 1999). Our results also show that an increased dilution (up to 60×) had a positive effect on morphological uniformity of cells (Fig. 2*A*). Consequently, the overall reproducibility of the results of morphological analysis can be significantly improved.

Moreover, Kovarik *et al.* (2012) demonstrated dramatic alterations of karyotype (variation between 39 to 47 chromosomes) in a BY-2 cell line grown for a long time under a low dilution rate (the culture was diluted 10× by week subculture). As the nuclear DNA content changes can affect the morphological uniformity of cells, we performed cursory flow-cytometry analysis in our BY-2 cells compared to leaf cells of *Nicotiana tabacum* cv. Samsun. Although this approach did not allow a precise karyotype assessment, the results did not indicate dramatic karyotype alterations and any ploidy shift in our cultures standardly diluted 50× by week subculture (Fig. 4 Suppl.).

Analysis of phenotypic variability of transgenic lines: The BY-2 line is valuable tool for functional genomics, especially in cases when the phenotypic change resulting from modulated expression of a certain gene is masked by compensatory mechanisms *in planta* (Dvorakova *et al.* 2012). Differences between the phenotype of transgenic and wild type lines have to be demonstrated carefully due to natural variability of both WT cell lines and transgenic BY-2 cell lines that can either reduce or escalate the real phenotypic change in a suboptimal experimental design. This variability has several sources.

The first source of variation arises from the cultivation of transgenic BY-2 in the form of calli resulting from the transformation procedure. We observed that the BY-2 cell lines transferred from suspension to solid media and reverted again several weeks later to the suspension could be slightly altered in morphologic features (Fig. 6). Although these shifts are minor, they can be statistically significant when the data matrix is large.

The second cause of variation is related to the transformation event itself. The T-DNA can be inserted into various chromosomal locations where it can interfere with a flanking DNA resulting in varying expression patterns (Nocarova and Fischer 2009). The stress conditions connected with the transformation procedure and subsequent antibiotic selection can also affect the phenotype of the cells.

To avoid misinterpretations, we suggest including two types of control in the experiment. The first, a few (*e.g.*, three) suspensions obtained by independent resuspension of wild type BY-2 calli which were at least two months (≈ three subcultures) grown in a callus form. The second control should cover several (> 3) lines transformed with an empty vector (or a gene construct with a known absence of phenotypic effect, *e.g.*, a gene encoding free cytosolic GFP; Seth and Viersta 1998, Stewart 2001). All suspensions should be established from calli of comparable size, rigidity, and age (one to two weeks after subculture).

The set of investigated transgenic lines should be relatively numerous (at least 5 to 10 lines) due to a high variability within the primary transgenic population. As mentioned above, the T-DNA can be inserted in different chromosomal contexts, in various numbers of insertions, and transgene expression can be epigenetically modified in the lines (Nocarova and Fischer 2009) or even expression of internal genes can differ between the lines as result of feed-back regulation (Dvorakova *et al.* 2012).

Subsequent statistical analysis of the transgenic phenotype can be tested using the General Linear Model of *ANOVA*. Each cell line tested within a certain genotype class should be implemented into the statistical model as „random factor".

Calli as alternative model for phenotype characterization: Alteration in cell size or proliferation rate of the investigated (transgenic) lines can be documented in the alteration of the biomass curve. Quantification of these parameters provides exact data, but is relatively laborious. Bigger alterations in growth of biomass can be also observed under cultivation of BY-2 cells in the form of calli. In the case of transgenic lines, such a phenotypic change can be seen already on the selection medium a few weeks after transformation. We repeatedly observed larger calli after transformation of BY-2 cells with constructs which caused an enhanced cell elongation (Dvorakova *et al.* 2012). However, differences in growth of calli can be masked or extended by the effect of callus density on a plate; growth of calli is inhibited when their density is too low or too high. This can be overcome by cloning transgenic suspension cells and plating them in various densities (Nocarova and Fischer 2009). An altered growth/cell size could be demonstrated as difference in callus diameter as shown in Dvorakova *et al.* (2012). A pairwise comparison of cell size and callus diameter (after cloning) of five selected BY-2 cell

lines provided a highly comparable statistical output (Fig. 5 Suppl.). Also correlation between cell length and callus size was proven by testing a linear regression model at $\alpha = 0.05$ ($P = 0.013$).

Equations:

1a (MI/VIA req. sample size): $n_i = (Z_{1-\alpha} + Z_{1-\beta})^2/{}_{\Delta}p^2 \times [2p_0 + {}_{\Delta}p - p_0^2 - (p_0 + {}_{\Delta}p)^2]$
1b (required sample size): $n_i = 2 \times (Z_{1-\alpha} + Z_{1-\beta})^2/[(\mu_1 - \mu_2)/SD]^2 + Z^2_{1-\alpha}/4$
1c (cell density req. sample size.): $n_i = 2 \times (Z_{1-\alpha} + Z_{1-\beta})^2/[(\mu_1 - \mu_2) * 0.23]^2 + Z^2_{1-\alpha}/4$
2 (logistic curve): $Y(t) = A + (K - A)/(1 + Q \times 2.718^{-2\times(t - M)})^{1/\upsilon}$
3 (confidence interval): $CI = Z_{1-\alpha \times} SD/(\sqrt{n})^2$
4 (cell cycle duration [h]): $t = (t_2 - t_1)/\log_2(density_{t2}/density_{t1})$
5 (mitosis duration[h]): $t = t_{(cell\ cycle\ duration)} \times MI$
6 (biomass volume [cm^3/cm^3]): $V = D \times \pi \times L \times W^2/(4 \times 10^{12})$

where: A = lower asymptote (initial density level), D = measured cell density [cells/cm^3], K = upper asymptote (terminal density), L = measured cell length [μm], W = measured cell width [μm], Q, M, υ = logistic curve parameters to be computed to fit the curve to the experimental data, t_1, t_2 = time of measurements [d], p_0 = proportion of events in control sample (*e.g.*, viability/mitosis of control sample), ${}_{\Delta}p$ = proportion difference to be tested, $Z_{1-\alpha}$: 1 - α = quantile of normal distribution (α = significance level), a theoretical value that can be obtained by *MS Excel*: $Z_{1-\alpha}$ = NORMINV (1-α;0;1), $Z_{1-\beta}$: 1- β = quantile of normal distribution (1- β = power of the test), a theoretical value that can be obtained by *MS Excel*: $Z_{1-\beta}$ = NORMINV(1-β;0;1), SD - expected standard deviation (the value for the stationary cell length of unaffected wild type BY-2 is approx. 35 μm, the variability can be estimated by a preliminary measurement), $\mu_1 - \mu_2$ = difference to be demonstrated between means of the measured values of the compared variants

References

Brown, R., Rickless, P.: A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and of nutrients. - Proc. roy. Soc. London B Biol. Sci. **136**: 110-125, 1949.

Campanoni, P., Blasius, B., Nick, P.: Auxin transport synchronizes the pattern of cell division in tobacco cell line. - Plant Physiol. **133**: 1251-1260, 2003.

Chow, S.C., Shao, J., Wang, H. (ed.): Sample Size Calculations in Clinical Research. - Marcel Dekker, New York 2003.

David, K.M., Perrot-Rechenmann, C.: Characterization of a tobacco Bright Yellow 2 cell line expressing the tetracycline repressor at a high level for strict regulation of transgene expression. - Plant Physiol. **125**: 1548-1553, 2001.

Dayan, A.D., Paine, A.J.: Mechanisms of chromium toxicity, carcinogenicity and allergenicity: review of the literature from 1985 to 2000. - Human exp. Toxicol. **20**: 439-451, 2001.

Dvorakova, L., Srba, M., Opatrny, Z., Fischer, L.: Hybrid proline-rich proteins: novel players in plant cell elongation? - Ann. Bot. **109**: 453-462, 2012.

Fay, D.S., Gerow, K.: A biologist's guide to statistical thinking and analysis. - In: WormBook: the Online Review of *C. elegans* Biology. Pp. 1-54. The *C. elegans* Research Community, published on-line 2013.

Granger, C.L., Cyr, R.J.: Microtubule reorganization in tobacco BY-2 cells stably expressing GFP-MBD. - Planta **210**: 502-509, 2000.

Hoaglin, D.C., Mosteller, F., Tukey, F.W. (ed.): Exploring Data Tables, Trends, and Shapes. - John Willey & Sons. New York 2011.

Hofmannova, J., Schwarzerova, K., Havelkova, L., Borikova, P., Petrasek, J., Opatrny, Z.: A novel, cellulose synthesis inhibitory action of ancymidol impairs plant cell expansion. - J. exp. Bot. **59**: 3963-3974, 2008.

Johansen, D.A. (ed.): Plant Microtechnique. - McGraw-Hill Book Co., New York - London 1940.

Koukalova, B., Kovarik, A., Fajkus, J., Siroky, J.: Chromatin fragmentation associated with apoptotic changes in tobacco cells exposed to cold stress. - FEBS Lett. **414**: 289-292, 1997.

Kouri, T., Gyory, A., Rowan, R.M.: ISLH recommended reference procedure for the enumeration of particles in urine. - Lab. Hematol. **9**: 58-63, 2003.

Kovarik, A., Lim, K. Y., Souckova-Skalicka, K., Matyasek, R., Leitch, A. R.: A plant culture (BY-2) widely used in molecular and cell studies is genetically unstable and highly heterogeneous. - Bot. J. Linn. Soc. **170**: 459-471, 2012.

Krtkova, J., Havelkova, L., Krepelova, A., Fiser, R., Vosolsobe, S., Novotna, Z., Martinec, J., Schwarzerová, K.: Loss of membrane fluidity and endocytosis inhibition are involved in rapid aluminum-induced root growth cessation in *Arabidopsis thaliana*. - Plant Physiol.Biochem. **60**: 88-97, 2012.

Kumagai-Sano, F., Hayashi, T., Sano, T., Hasezawa, S.: Cell cycle synchronization of tobacco BY-2 cells. - Nature Protocols **1**: 2621-2627, 2007.

Kuthanova, A., Fischer, L., Nick, P., Opatrny, Z.: Cell cycle phase specific death response of tobacco BY-2 cell line to cadmium treatment. - Plant Cell Environ. **31**: 1634-1643, 2008.

Kutík, J., Kuthanová, A., Smertenko, A., Fischer L., Opatrný, Z.: Cadmium-induced cell death in BY-2 cell culture starts with vacuolization of cytoplasm and terminates with necrosis. - Physiol. Plant. **151**: 423-433, 2014.

Kutsuma, N., Hasezawa, S.: Dynamic organization of vacuolar and microtubule structures during cell cycle progression in synchronized tobacco BY-2 cells. - Plant Cell Physiol. **43**: 965-973, 2002.

Laporte, C., Vetter, G., Loudes, A.M., Robinson, D.G., Hillmer, S., Stussi-Garaud, C., Ritzenthaler, C.: Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of grapevine fanleaf virus

movement protein in tobacco BY-2 cells. - Plant Cell **15**: 2058-2078, 2003.

Maisch, J., Fiserova, J., Fischer, L., Nick, P.: Tobacco Arp 3 is localized to actin-nucleating sites *in vivo*. - J. exp. Bot. **60**: 603-614, 2009.

Maisch, J., Nick, P.: Actin is involved in auxin-dependent patterning. - Plant Physiol. **143**: 1695-1704, 2007.

Matsuoka, K., Demura, T., Galis, I., Horiguchi, T., Sasaki, M., Tashiro, G., Fukuda, H.: A comprehensive gene expression analysis toward the understanding of growth and differentiation of tobacco BY-2 cells. - Plant Cell Physiol. **45**: 1280-1289, 2004.

Milliken, G.A., Johnson, D.E. (ed.): Analysis of Messy Data: Volume 1 Designed Experiments. - Van Nostrand Reinhold, New York 1984.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio assays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nagata, T.: When I encountered tobacco BY-2 cells! - In: Nagata T., Hasezawa S., Inzé D. (ed.) Tobacco BY-2 Cells. (Biotechnology in Agriculture and Forestry Volume 53). Pp. 1-5, Springer, Berlin - Heidelberg 2004.

Nagata, T., Kumagai, T.: Plant Cell biology through the window of the higly synchronized tobacco BY-2 cell line. - Methods Plant Sci. **21**: 123-127, 1999.

Nagata, T., Matsuoka, K., Inze, D. (ed.): Tobacco BY-2 Cells: From Cellular Dynamics to Omics. (Biotechnology, Agriculture and Forestry. Vol. 58.) - Springer, Berlin - Heidelberg 2006.

Nagata, T., Nemoto, Y., Hasezawa, S.: Tobacco BY-2 cell line as the 'HeLa' cells in the cell biology of higher plants. - Int. Rev. Cytol. **132**: 1-30, 1992.

Němec, B. (ed.): [Botanical Microtechnics]. - Nakladatelství Československé Akademie Věd, Praha 1962. [In Czech]

Nocarova, E., Fischer, L.: Cloning of transgenic tobacco BY-2 cells; an efficient method to analyse and reduce high natural heterogeneity of transgene expression. - BMC Plant Biol. **9**: 44-55, 2009.

Opatrny, Z., Opatrna, J.: The specificity of the effect of 2,4-D and NAA on the growth, micromorphology, and occurence of starch in long-term *Nicotiana tabacum* L. cell strains. - Biol. Plant. **18**: 359-365, 1976.

Opatrny, Z., Nick, P., Petrasek, J.: Plant cell strains in fundamental research and applications. - In: Nick P., Opatrný Z. (ed.): Applied Plant Cell Biology. Pp. 455-481. Springer, Heidelberg - New York - Dordrecht - London 2014.

Orchard, C.B., Siciliano, I., Sorrell, D.A., Marchbank, A., Rogers, H.J., Francis, D., Herbert, R.J., Suchomelova, P., Lipavska, H., Azmi, A., Van Onckelen, H.: Tobacco BY-2 cells expressing fission yeast cdc25 bypass a G2/M block on the cell cycle. - Plant J. **44**: 290-299, 2005.

Pieruschka, R., Poorter, H.: Phenotyping plants: genes, phenes and machines. - Funct. Plant Biol. **39**: 813-820, 2012.

Richard, C., Granier, C., Inzé, D., De Veylder, L.: Analysis of cell division parameters and cell cycle gene expression during the cultivation of *Arabidopsis thaliana* cell suspensions. - J. exp. Bot. **52**: 1625-1633, 2001.

Samuels, A., Meehl, J., Lipe, M., Staehelin, L.A.: Optimizing conditions for tobacco BY-2 cell cycle synchronization. - Protoplasma **202**: 232-236, 1998.

Sato, F.: Characterization of plant functions using cultured plant cells, and biotechnological applications. - Biosci. Biotechnol. Biochem. **77**: 1-9, 2013.

Schaul, O., Mironov, V., Burssens, S., Van Montagu, M., Inze, D.: Two *Arabidopsis* cyclin promoters mediate distinctive transcriptional oscillation in synchronized tobacco BY-2 cells. - Proc. nat. Acad. Sci. USA **93**: 4868-4872, 1996.

Schwarzerova, K., Pokorna, J., Petrasek, J., Zelenkova, S., Capkova, V., Janotova, I., Opatrny, Z.: The structure of cortical cytoplasm in cold-treated tobacco cells: the role of the cytoskeleton and the endomembrane system. - Cell Biol. Int. **27**: 263-265, 2003.

Seifertova, D., Klima, P., Parezova, M., Petrasek, J., Zazimalova, E., Opatrny, Z.: Plant cell lines in cell morphogenesis research. - In: Zarsky, V., Cvrckova, F. (ed.): Plant Cell Morphogenesis. Pp. 215-229, Springer, New York - Heidelberg - Dordrecht - London 2014.

Seth, J.D., Vierstra, R.D.: Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. - Plant mol. Biol. **36**: 521-528, 1998.

Sorrell, D.A., Combettes, B., Chaubet-Gigot, N., Gigot, C., Murray, J.A.H.: Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco Bright Yellow-2 cells. - Plant Physiol. **119**: 343-351. 1999.

Sorrell, D.A., Menges, M., Healy, J.S., Deveaux, Y., Amano, C., Su, Y., Nakagami, H., Shinmyo, A., Doonan, A.H., Sekine, M., Murray, J.A.: Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar Bright Yellow-2 cells. - Plant Physiol. **126**: 1214-1223, 2001.

Stewart, C.N.: The utility of green fluorescent protein in transgenic plants. - Plant Cell Rep. **20**: 376-382, 2001.

Wang, H., Chow, S.C.: Sample size calculation for comparing proportions. - In: D'Agostino, R., Massaro, J., Sullivan, L. (ed.): Wiley Encyclopedia of Clinical Trials. Pp. 1-11. John Wiley & Sons, New York 2007.

Widholm, J.M.: The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. - Biotech. Histochem. **47**: 189-194, 1972.