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Tobacco BY-2 Cells: From Cellular Dynamics to Omics

Edited by
T. Nagata, K. Matsuoka, and D. Inzé

With 102 Figures, 16 in Color, and 7 Tables

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We dedicate this volume to the late Professor Jeff Schell
for his encouragement of the development of this subject.

Preface

It is our utmost pleasure to present a new book on tobacco BY-2 cells, *Tobacco BY-2 Cells: From Cellular Dynamics to Omics*, as the 58th volume in the book series *Biotechnology in Agriculture and Forestry* (BAF). It represents an extension of the previous book *Tobacco BY-2 Cells*, vol. 53 of the BAF. Moreover, the content is rather different from the latter and includes new topics, gleaned from the First International Symposium on Tobacco BY-2 Cells held at the Plant Science Center of the RIKEN, Yokohama, organized by Nagata, Matsuoka and Inze, in September 2004. To this symposium came more than 200 people from different parts of the world to discuss issues. Although most of the contributors to the previous volume of *Tobacco BY-2 Cells* gave talks on their subjects, there were many other speakers who presented new topics and approaches. So we enjoyed the symposium very much. Thus we decided to compile a new volume on tobacco BY-2 cells which includes these new topics. In addition, towards the end of the symposium, our common understanding was that the tobacco BY-2 cell system is still important in plant biology, in particular for studying the dynamic features of plant cells. We hope this volume is useful for plant biologists.

Contents of the book are as follows: in Chapters I.1–I.6, various aspects of the cell cycle and cellular dynamics using BY-2 cells are described. In Chapters II.1–II.3, physiological and developmental aspects of BY-2 cells are discussed. In Chapters III.1–III.3, recent developments in the knowledge of intracellular traffic of BY-2 cells are described. In Chapters IV.1–IV.3, BY-2 cells as a host for infectious diseases are discussed. Chapter V.I describes the dynamic features of mitochondrial fusion and division, while Chapter V.2 discusses how BY-2 cells are useful also for elucidating the biosynthesis of isoprenoids. In Chapters VI.1–VI.3, recent developments in the omics of BY-2 cells are described. Finally, Chapters VII.1 and VII.2 include two technical advances in handling BY-2 cells.

Tokyo,
Yokohama,
Ghent, May 2006

Toshiyuki Nagata,
Ken Matsuoka,
and Dirk Inzé

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Section I Cell Cycle and Cellular Dynamics

I.1 Novel Approaches for Cell Cycle Analysis in BY-2

O.A. Koroleva, G.R. Roberts, M.L. Tomlinson, and J.H. Doonan[1]

1 Introduction

The BY-2 cell culture has proved a useful, and indeed indispensable, tool for the analysis of the plant cell cycle. This is, in large part, due to the degree to which the cells can be synchronised during the cell cycle (Nagata et al. 1982; Nagata 2004). The cell line has several other features that are advantageous for studies of basic plant cell biology. The cells are relatively large and grow as long chains. Combined with excellent optical clarity, this regular growth habit lends itself to direct microscopical observation of cellular processes and it has been used for this propose extensively. Finally, BY-2 can be easily transformed so that cellular processes can be manipulated at the molecular level, and the introduction of green fluorescent protein (GFP)-tagged constructs allows direct observation of cell division, protein localisation and organelle dynamics.

In this chapter we concentrate on the production and use of transgenic BY-2. We describe and discuss the use of a chemically inducible gene switch which, when used in combination with synchronised cells, makes BY-2 an ideal system for detailed studies of the plant cell cycle. This approach allows the up- or down-regulation of gene function at precise times in the cell cycle. Potential applications include inducible overexpression, growth analysis, dual-fluorescent labelling for flow cytometric analysis, RNAi silencing and transient expression. We provide detailed protocols and discuss how to improve throughput for transformation and analysis of the cell culture.

2 A Modified alc-Inducible System for Transgene Expression in BY-2

Constitutive expression of cell cycle regulators can be difficult to interpret because compensatory changes in related processes may disguise (or confuse) changes in the actual target. Several studies indicate that constitutive overexpression of cyclins can affect the length of the S- and G2-phases, which could indirectly lead either to the observed shortening of the G1-phase or to increased length of the S- and G2-phases (Dewitte et al. 2003). Similarly, constitutive loss-of-function mutations and dominant-negative constructs suffer from similar

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problems, the most severe being observed with essential genes where early lethality can limit phenotypic analysis. Moreover, in any transgenic experiment, there is high variability between parallel transgenic lines which can lead to difficulties in interpretation of the effect of the transgene. Variable levels of silencing and effects due to the genomic context of the integration site of the transgene can contribute to overall variability. In other model systems, such as yeast, conditional mutants and gene switches are widely used to overcome these problems. Although there are a few examples where temperature-sensitive mutants have been sought and isolated from Arabidopsis, this approach has not been widely applied in plant biology. Chemically inducible gene switches, however, have been quite widely applied in both whole plants and cell cultures, and have been useful in providing insights into essential biological processes.

As compared to constitutive expression, conditional gene expression has several benefits. In the first place, it enables the primary effects of gene expression to be assayed. By application or, in some cases, withdrawal of the chemical, gene expression can be initiated at a time chosen by the experimenter. This has the advantage that the cell has less time to adapt to the presence of the gene product and one is more likely to observe the primary response. A second benefit, really an extreme example of the first, occurs if the gene product is deleterious to growth or viability. In this case, the effect of transient accumulation can be assayed without the selection against expression that would occur over successive cell generations. Finally, for complex processes such as the cell cycle or development, conditional expression allows conditional complementation of mutant phenotype at different stages. This can provide novel insights into a gene's function, which may change during the different stages of development (Laufs et al. 2003) or the cell cycle. Progression through both development and the cell cycle involves a series of interdependent sequential functions where late functions can depend on the completion of early functions. Perturbation of early functions can indirectly modify late functions and constitutive up- or down-regulation of gene function can be difficult to interpret. Work in whole plants indicated that the *alcR/alcA* ethanol-inducible regulon provided a switchable gene expression system suitable for both overexpression and complementation studies (Roslan et al. 2001; Deveaux et al. 2003; Laufs et al. 2003) and has been widely used in crop plants.

The ethanol-inducible *alcR/alcA* (alc) gene expression system is widely used for conditional gene expression studies in both plants and fungi. It is a two-component chemically inducible gene expression system, originally developed as a gene switch in *Aspergillus nidulans* (Waring et al. 1989). The *alc*R encoded transcription factor ALCR and the *alcA* target promoter constitute the two components. The ALCR transcription factor drives gene expression from the *alcA* target promoter only in the presence of ethanol or acetaldehyde (Flipphi et al. 2001). It provides high speed of induction and the volatile nature of the inducer allows its removal from the system, resulting in a highly dynamic switch. However, for longer periods of induction ethanol needs to be supplied constantly. The ethanol switch has numerous advantages for a large-scale agri-

cultural application, using a non-toxic, cheap, stable chemical. Although it would be very useful to be able to use the same system in both plants and cell cultures, the utility of the ethanol switch in suspension cells is severely limited as gene expression seems to be constitutive even in the absence of exogenous inducer. Under certain physiological conditions, notably anoxia, it is thought that substances are produced by the cells that mimic the effect of ethanol. This "leaky" expression is apparent when the alc system is used in plant suspension or callus cell cultures (Roberts et al. 2005) and, while it is possible to reduce the level of expression by additional aeration, this background expression is difficult to eliminate.

A modified alc system, the alc-GR system, was generated by fusing the rat glucocorticoid receptor (GR) domain to the ALCR transcription factor (Roberts et al. 2005). Fusion of the GR domain to a protein restricts the protein to the cytoplasm until dexamethasone (dex) is applied. Then the fusion enters the nucleus. This is widely used in plants to produce dex-inducible transcription factors (Wagner et al. 1999; Gallois et al. 2002; Gomez-Mena et al. 2005). Tests on BY-2 have shown that the alc-GR system is tightly dex-inducible. Exogenously applied ethanol is not required for dex-inducible transgene expression and ethanol does not induce expression in the absence of dex.

The alc-GR system has proven to be a good switch for cell synchrony studies. First, dex activation of the ALCR-GR transcription does not appear to perturb cell cycle progression. The inert nature of the ALCR-GR transcription factor in cell synchrony experiments provides a good basis for experiments where transgenes are expressed in cell cycle synchronised cultures. Therefore, using the alc-GR system, any changes to the duration of cell cycle phases can be directly apportioned to the induced transgene. Second, induction is very rapid – the expressed protein appears as soon as 1 h after induction (Koroleva et al. 2004; Roberts et al. 2005).

3 Use of the alc System to Define the Time of Action of Induced Protein

We used the alc-GR expression system to monitor the consequences of D1 cyclin gene expression. D cyclins are known to be rate-limiting components for progression through G1, as association of these unstable proteins with the cyclin-dependent kinases (CDKs) leads to phosphorylation of the Rb protein, an event thought necessary for release of the E2F transcription factors and activation of the S-phase (Oakenfull et al. 2002). We addressed whether *CycD1* could promote cell cycle progression in BY-2. We used timed *CycD1* expression to define which stages of the cell cycle were affected. Cooper (1998) argued that it is not possible to identify G1 cyclins by the overexpression induced changes in the lengths of individual cell cycle phases without considering changes in the overall cellular growth rate. Any shortening of the G1-phase could be due

to an increase in the rate of mass synthesis in all phases of the cell cycle. Our experimental approach allowed us to solve this logical problem by inducing extra D1 cyclin at specific times of the cell cycle and measuring the immediate effect. We were able to temporally dissect the function of D1 cyclin during the G1/S- and S/G2-phases of the cell cycle.

4 An Example of Functional Analysis Using the AlcR-GR Gene Switch

Briefly, transgenic lines of BY-2 already carrying the gene switch cassette 35S-*alc*R-GR (Roberts et al. 2005) were retransformed with a second cassette containing the *CycD1* gene or tagged HA:*CycD1* under control of *alc* A promoter, using a hypervirulent strain of *Agrobacterium tumefaciens* (LBA4404.pBBR1MCSvirGN54D) (van der Fits et al. 2000) as described in Koroleva et al. (2004) and Protocol 1 (see Sect. 7.1).

4.1 D1 Cyclin Inducible Over expression in Synchronised Cells

Suspension cultures can be synchronised at several different stages during the cell cycle using different methods. Stationary cells are arrested in the G0 phase by withdrawing nutrients from the media, such as sucrose (Menges and Murray 2002). Entry into S phase can be reversibly blocked by aphidicolin, a potent inhibitor of DNA polymerases α and δ (Sala et al. 1983; Nagata et al. 1992). Furthermore, the cells can be synchronised at the onset of mitosis by the microtubule-disrupting drug propyzamide (Nagata and Kumagai 1999). Using two types of synchrony, either G0 or S-phase arrest, we determined the effect of D1 cyclin expression at the different time points during the cell cycle (Koroleva et al. 2004).

To test whether *CycD1* had a specific effect on cell cycle progression, we first expressed *CycD1* in cells released from stationary phase. Sucrose-starved or stationary cultures can be induced to re-enter the cell cycle by addition of fresh media and they proceed in a semi-synchronous manner into S-phase and mitosis. If *CycD1* was induced at the same time as cells are sub-cultured into fresh media, entry into mitosis was accelerated, as judged by mitotic index (MI) measurements, by at least 2 h. *CycD1* induction led to not only faster progression into mitosis but also a significantly increased MI. The induced culture entered mitosis and reached its peak value 2 h earlier and also achieved an approximately 4% higher peak value of MI compared to the control. Thus, overexpression of *CycD1* from G0 significantly accelerated cell cycle progression through S-phase to mitosis.

We used flow cytometric measurement of DNA content to follow the dynamics of S-phase entry, with or without expression of *CycD1*. Stationary BY-2 cells had a mainly G1 nuclearcontent. Even at 6 h after release from G0, the

induced culture had a much higher proportion of cells in S-phase compared to non-induced culture. By 10 h the proportion of nuclei in the G2 peak exceeded the number in the G1 peak in induced culture while G1 nuclei still predominated in non-induced culture and much fewer cells contributed to the G2 peak (Koroleva et al. 2004). Screened at 8 h after induction, 8 out of 8 *CycD1* lines and 4 out of 8 *HA-CycD1* lines had a remarkable increase of cells in S- and G2-phases compared to non-induced controls. This marked acceleration of S-phase entry caused by overexpression of *CycD1* represents a 2 h advance and, therefore, could fully account for the accelerated progression to mitosis. However, this does not rule out the possibility that S- and G2-phases were also affected by *CycD1*.

To test whether the cell cycle acceleration was specific to G1 phase, we assessed the effect of *CycD1* expression on S-phase cells by release from aphidicolin arrest. We induced expression of *CycD1* during either early S-phase (dex added together with aphidicolin and then again after the removal of aphidicolin) or late S-phase/G2-phase (dex was added immediately after removal of aphidicolin). The cultures that were induced on release from the S-phase block progressed faster into mitosis and achieved a higher maximal value of MI than the non-induced control. The number of mitotic cells by 7–10 h was significantly increased and we estimated that induction of *CycD1* expression at this stage accelerated entry into mitosis by about 1 h, suggesting an effect either in late S-phase or on the G2/M transition. We analysed the appearance of newly divided cells by flow cytometry to follow progression into and completion of mitosis. By 8–9 h after release, the number of cells that had passed through mitosis (as judged by the G1/G2 index) became significantly higher in cultures induced by dex, and the difference was maintained during several following hours. This effect could be due to the shortening of either the S- or G2-phase, as HA-CYCD1 protein could be detected as early as 1 h after induction, which should correspond to the peak in DNA synthesis in the S-phase after the release from aphidicolin (Sorrell et al. 1999). The observed effect of accelerated entry into mitosis could result in *CycD1* effect in either late S- or G2-phase.

Therefore, we tested the effect of earlier induction by inducing both during and after the aphidicolin block. In this case, the acceleration into mitosis was even greater, suggesting either that a long time was required for protein levels to accumulate or that *CycD1* promotes progress through S-phase even in the presence of an aphidicolin block. The MI increased much earlier in cultures under continuous induction, with a significant increase in the number of cells in mitosis at 4 h after the aphidicolin block was released, indicating that progression into mitosis was faster (Koroleva et al. 2004). The initially higher speed of cell cycle progression in the cultures continuously induced by dex was most likely achieved due to accelerated progression through the S-phase. However, this acceleration does not speed up completion of the cell cycle as the number of cells that had passed through mitosis (as judged by the G1/G2 index) became equal in cultures induced by dex once or twice. By 12 h after release, the G1/G2 index became equal in control cultures and cultures induced

by dex twice, but lower than the cultures induced once, indicating that cultures induced in early S-phase entered mitosis earlier but spent a prolonged time in mitosis. This was not the case when expression was induced at the time of aphidicolin release. The faster progression through mitosis in these cultures therefore could be attributed to late S- or G2-M-specific action of *CycD1*, and earlier expression during an S-phase block may lead to premature mitotic entry that then leads to a delay in mitotic progression.

4.2 The Activity and Potential Role of D1-Associated Kinase

The BY-2 cell system is also amenable to biochemical studies, allowing protein complexes to be isolated from cells at defined stages of the cell cycle. To define the in vivo partners of D1 cyclin, we transformed BY-2 with epitope tagged versions of CycD1. Using an HA-tagged version, we immunoprecipitated proteins associated with CYCD1, from total soluble protein extracts. Western blot analysis with antibodies against the "PSTAIR" motif (specific for A-type CDKs) clearly demonstrated that CDKA had co-precipitated with CYCD1 (Koroleva et al. 2004). To assess whether CYCD1 bound to CDKB, the mitosis-specific CDKs, we probed immunoprecipitates with antibodies against CDKB1;1. Although, the antibody recognised the CDKB1 protein in whole cell extracts, we failed to detect it in the HA immunoprecipitates. However, data from yeast two hybrid analysis indicated that CYCD1 and CDKB2;1 can interact (O.A. Koroleva, M.L. Tomlinson, J.H. Doonan, unpublished). Therefore, it remains possible that CYCD1 does interact with both classes of CDK in vivo. CDKA remained at a constant level during the cell cycle, as reported previously by Sorrell et al. (2001), and this level was not changed significantly by *CycD1* expression. CDKB1 protein was undetectable in stationary cells. After re-entering the cell cycle from a G0 release, CDKB1 could be first detected in late S-phase and then its levels increased gradually, reaching a peak during mitosis. Therefore, it is unlikely that CDKB would have mediated the *CycD1*-induced acceleration in the G0 synchrony experiment.

To determine the substrate specificity of the CYCD1-associated kinase at different phases of the cell cycle during G1 synchrony experiment, we assayed HA-associated kinase activity towards two known substrates, histone H1 and Rb, and compared this with the total fraction of endogenous tobacco CDKs purified on $p13^{Suc1}$ beads. We found that the expression of HA-CYCD1 led to an initial increase in HA-associated histone H1 kinase activity, followed by decline and second peak of activity at the time of mitosis. The non-induced control culture had a very low background level of HA-associated histone H1 kinase activity that did not change significantly over the time-course. HA-dependent phosphorylation of Rb protein increased steadily from G1 to mitosis in the induced culture. The Rb kinase activity in non-induced culture remained low.

We measured total histone and Rb kinase activity to estimate whether ectopic expression of *CycD1* led to global changes in CDK activity towards either of

these substrates. The kinase activity associated with p13^{Suc1} beads was about an order of magnitude higher towards both substrates, compared to the HA-CYCD1-interacting fraction precipitated on HA beads. Total histone H1 kinase activity in the induced culture was dramatically increased compared to the non-induced control. The non-induced culture showed a steady increase in kinase activity from G1- through S-phase into mitosis. Kinase activity towards Rb protein followed a similar trend of steady increase through G1- and S-phase, and then a slight decrease at the time of mitosis in both induced and non-induced cultures, but the induced culture had higher levels of Rb kinase activity at each time point (Koroleva et al. 2004). These data indicate that the HA-associated CYCD1-dependent kinase makes a significant contribution to the increase in total CDK activity, and its contribution appears to be amplified, probably by activation of downstream kinases.

4.3 CYCD1 Protein Localisation Studies

BY-2 cells also provide an excellent system by which to study protein localisation. Protein localisation can often provide clues as to its function or regulation. The living cell is a complex of morphologically distinct compartments with discrete and well-defined functions. At the intracellular level, compartmentation is achieved by the existence of multiple membrane-bound organelles, each containing a spectrum of biomolecules and set of proteins forming the organelle-specific "proteome".

An N-terminal fusion of GFP to *CycD1* was made using the GATEWAY system (Invitrogen) and pGWB6 expression vector (a gift of Dr. T. Nakagawa, Shimane University, Japan) and expressed in lines of BY-2, using a hypervirulent strain of A*grobacterium* (Koroleva et al. 2005). GFP-CYCD1 is primarily present in the nuclei of interphase cells but was much reduced or absent from the spindle domain during mitosis (Koroleva et al. 2004), where it may get degraded by a ubiquitin-dependent pathway: MG132 proteasome inhibitor treatment of cells led to increased levels of CycD1 on Western blots, which is consistent with CycD1 being an unstable regulator of CDK activity in the nucleus.

5 Transient Protein Expression

5.1 Comparative Analysis of Applications for Tobacco BY-2 vs Arabidopsis Col-0 Cell Cultures

Stable transformation of BY-2 cells using inducible promoters to drive gene expression in a highly controlled manner, therefore, allows detailed functional analysis of genes involved in cell cycle regulation. Synchronised cell populations provide insight into the biochemistry, function and interactions of the transgenic protein. However, using current methodology, the number of genes

that can be analysed in this way is limited. In part, this limitation is due to the high variability between independent transgenic cell lines carrying the same construct and makes necessary the rigorous statistical verification of the observed characteristics across several cell lines so that the phenotype can be correlated with the expression of the transgene. Thus, if we wish to use cell suspensions for systematic analysis of gene function on a whole genome scale, then new methods must be developed.

In an effort to solve these problems, we have investigated the possibility of using transient transformation for understanding gene function. This approach is widely used in mammalian cells where viral vectors are used to deliver genes to whole populations of cultured cells. Unfortunately, most plant DNA viruses have limited tolerance in terms of the size of gene that they will accept and this has limited their development as vectors. Transient expression in plant cells, however, is achievable and has been widely used to dissect gene function. Perhaps the most commonly employed technique uses transformation of protoplasts with naked DNA and this is both effective and efficient in BY-2. This method can produce 25–30% of cells expressing protein within 18–24 h of transfection with GFP, and similar levels 36–40 h after transfection with the slow-maturating fluorescent protein dsRed (Bhat and Thompson 2004). The other method exploits *Agrobacterium*-mediated transformation and this has the advantages that whole cells can be transformed and the same plasmids and strains can be used subsequently to produce transgenic plants. However, we found that the efficiency of transient transformation of BY-2 cells by Agrobacteria is quite low: the percentage of cells that express the transgene is usually less than 0.01% of the cell input, which is more than adequate for the selection of stable transgenic lines but sub-optimal for use in transient transformation. However, the rate of transient transformation may vary depending on the culture conditions, length of co-cultivation with Agrobacteria and the nature of the introduced transgene. *Agrobacterium* strains are known to vary with respect to their ability to transfect particular hosts, so it is hoped that more efficient strains can be found.

However, since transient expression using *Agrobacterium* offers so many advantages, in terms of both increased throughput and better integration into whole plant studies, we investigated other cell cultures as possible systems. Arabidopsis Col-0, originally derived in C. Koncz's laboratory, had shown promise for transient expression of proteins for biochemical studies (Mathur et al. 1998). In contrast to BY-2, the Col-0 Arabidopsis cell culture has a much higher susceptibility to transformation by Agrobacteria and the proportion of cells transiently expressing the transgene can be up to 70% (Koroleva et al. 2005). We have developed a protocol (Protocol 3) that allows systematic protein localisation (Koroleva et al. 2005), which also has the potential for functional and biochemical analysis (Koroleva et al. 2004; Chan et al. 2005; Korolev et al. 2005; Mao et al. 2005). This method complements the traditional range of applications of the BY-2 cell culture: one strategy is to use Arabidopsis cell culture for fast screening of transient expression, and continue with detailed analysis

Table 1. Comparative analysis of a range of applications for BY-2 vs Col-0 cell cultures, using transformation by *Agrobacterium* for both. The total number of *asterisks* indicates the relative merit of the different cell types

	Transformation by *Agrobacterium*		Advantage	
Option	**Stable expression in BY-2**	**Transient in Col-0**	**BY-2**	**Col-0**
Maintenance of the culture	Easy	Not easy	*	–
Transformations per week	36	> 96	–	*
Time for expression	2–4 weeks	3–4 days	–	*
Time for analysis	Unlimited	3–5 days	*	–
GFP localisation	Good resolution	Brighter signal	*	*
Cell cycle analysis	Yes	Yes for some	*	*
Growth analysis	Yes	No	*	–
Inducible expression	Yes	No	*	–
RNAi	Yes	Not yet	*	–
Problems	– High variability in cell size/growth	– Larger cell clusters	–	–
	– GFP fusions are not bright	– Poor resolution of cell structures	–	–
Score	–	–	7	4

of “interesting” genes in BY-2 later. Table 1 summarises the comparative characteristics and advantages of both cell cultures for analysis of transgene effect after transformation by Agrobacterium. Some particular types of analysis will be discussed below.

5.2 Use of Arabidopsis Cell Culture for Systematic Protein Localisation

The function of a particular protein is best understood in the context of its micro-environment. Where the protein is located will often affect its function, by restricting access either to substrates or to interacting partners. Interacting partners will determine in part how multi-protein complexes are constructed and how they respond to extraneous signals from the environment or neighbouring cells. Information about a protein’s location or “compartmentation” is therefore essential to fully understand its function. Despite the existence of many software programs for prediction of putative protein localisation, it may not always be possible to predict localisation from the primary amino acid sequence because of the major role played by protein–protein interactions. A protein may be delivered to a given location as part of a multiprotein complex, and be anchored there by such interactions. Some motifs are relatively easy to recognise, but even they do not necessarily determine the ultimate location. For example, the presence of nuclear localisation signal (NLS) or nuclear export signal (NES) indicates probable nuclear or cytoplasmic location, but some NES sequences can have differential activity and some NES-like sequences are

inactive (Henderson and Eleftheriou 2000). Studies aimed at defining specific nuclear localisation or nuclear export signals rely on experimental comparison of localisation of wild type with that of mutant proteins with introduced amino acid replacements in the tested motif (Henderson and Eleftheriou 2000). Moreover, many proteins change their location in response to signals or altered physiological states, so experimental verification will always be necessary.

Previous approaches aimed at defining protein localisation have tended to be very laborious and time-consuming, allowing analysis of only a small number of proteins. Ideally, initial information on protein localisation could be obtained from a systematic screen that would utilise existing genomic resources and be cheap and convenient to implement. Excellent genomic resources are now available for Arabidopsis: the SSP ORF collection contains about 10,500 publicly available and verified Arabidopsis full-length ORFs, trimmed at the 5′ translational start site (Yamada et al. 2003).

We have taken advantage of this collection of clones and used GATEWAY technology for the fast and efficient production of GFP translational fusions with Arabidopsis proteins for localisation studies in model suspension culture cells via Agrobacterium-mediated transient transformation. For more detailed cell cycle studies some of these have been transferred in BY-2 cells.

Related proteins often have different preferred subcellular localisation which reflect function: for example, mammalian B cyclins accumulate in cytoplasm in interphase and first appear on centrosomes in prophase (Jackman et al. 2003). We have shown that different CDKs (CDK C;2, CDK D;1, CDK D;2, CDK D;3 and CDKF;1) and cyclins (CycD3;1, CycA2;3, Cyc A3;2 and CycT1) have distinct patterns (Koroleva et al. 2005). Even closely related CDKs that are localised within the nucleus have subtly different GFP-fusion localisation patterns, although the functional significance of this is presently unclear. The use of EDE1-GFP fusions in transient assays has revealed novel insights into microtubule behaviour in the plant cell cortex (Chan et al. 2003) and in organisation of the mitotic spindle (Chan et al. 2005).

Ideally, the GFP tag should be introduced into the native genomic copy of the gene. Comprehensive screens in yeast have already exploited site-specific integration to precisely place GFP in the correct genomic context, but this option is not yet possible in higher plants since site-specific integration is a very rare event (Lutz et al. 2004). Site-specific integration of GFP to give translational fusions in the correct genomic context has been reported in the moss *Physcomitrella* (Kiessling et al. 2000), but genome-wide surveys are not yet possible, due to the fragmentary nature of the cDNA collections available for this species (Reski and Cove 2004).

Recently, Tian et al. (2004) demonstrated the use of multiple 35S enhancers in combination with native promoters to generate brighter fluorescent signals than provided by the native promoters alone. Apparently, the addition of the 35S enhancers and transformation into varied genomic contexts did not change either the tissue or cell type distribution or subcellular localisation and, in most cases, moderately increased levels of expression should provide