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Secretory Pathway Research: The More Experimental Systems the Better

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TRANSIENT GENE EXPRESSION, in plant protoplasts or specific plant tissues, is a key technique in plant molecular cell biology, aimed at exploring gene products and their modifications to examine functional subdomains, their interactions with other biomolecules, and their subcellular localization. Here, we highlight some of the major advantages and potential pitfalls of the most commonly used transient gene expression models and illustrate how ectopic expression and the use of dominant mutants can provide insights into protein function.

INTRODUCTION

Transient gene expression, either in plant protoplasts or using specific plant tissues, has been a key technique in plant molecular cell biology ever since the introduction of reporter gene fusions in the late 1980s. In contrast with gene identification work, which is routinely based on differential gene expression or loss-of-function mutants in whole-plant models, transient gene expression is aimed at experiments with the gene products and modifications thereof to explore functional subdomains, their interactions with other biomolecules, and their subcellular localization. With this technique, progress in our understanding of protein sorting in the plant secretory pathway accelerated since sorting signals could be routinely studied by deletion and fusion experiments within a matter of weeks. Moreover, quantitative coexpression studies and dose–response assays permitted the characterization of sorting machinery components in conjunction with cargo molecules for various destinations in the pathway.

In this article, we highlight the major advantages of the most commonly used transient expression models and illustrate how ectopic expression and the use of dominant mutants can provide further insights into protein function after the involvement of a gene in a specific process has already been established. We also seek to correct some popular misconceptions about transient expression in general and protoplasts in particular and provide some recommendations with respect to good experimental practice to help others avoid some of the pitfalls inherent in the use of these techniques.

PROTOPLASTS: A VIABLE AND VALUABLE SYSTEM FOR TRANSIENT EXPRESSION STUDIES

The cell wall and the vacuole are integral components of the plant cell (Robinson, 1991), and freshly prepared protoplasts fall short of this definition as they no longer possess a cell wall. However, unlike mitochondria and plastids, plant cell walls can be synthesized de novo. After 24 h of protoplast culture, at least a partial cell wall is already present around protoplasts, and the resulting cells can be regenerated to entire plants with normal fertility (Nagy and Maliga, 1976; Shillito et al., 1983; Shahin, 1985); therefore, protoplasting as such does not cause irreversible damage to the plant cell. This observation together with the ease with which protoplasts can be generated has led to their ubiquitous use in generating transgenic mono- and dicotyledonous plants (Shillito et al., 1985; Cocking and Davey, 1987; Shimamoto et al., 1989).
culture medium permits highly quantitative and reproducible secretion assays (Denecke et al., 1990), which were rapidly exploited to study sorting signals for retention in intracellular compartments (Bednarek et al., 1990; Matsuoka and Nakamura, 1981; Neuhaus et al., 1991; Holwerda et al., 1992; Denecke et al., 1992).

Although electroporation and other chemical transfection methods for naked DNA transfer exhibit relatively low overall efficiencies, typically just 10% of the total protoplast population (Denecke et al., 1989), the efficiency of independent plasmid cotransfection is known to be extremely high. This was deduced from early studies aimed at using protoplast transfection for the generation of stable transformants via nonhomologous (illegitimate) integration of the plasmid-borne gene and subsequent plant regeneration. Although the stable transformation efficiency is several orders of magnitude lower than the transfection efficiency, it is remarkable that up to 80% of the regenerated plants receive just one and not the other plasmid are very low. Due to this feature, protoplast transfections have become a key system to carry out biochemically controlled experiments for testing potential roles of candidate factors controlling the secretory pathway (Denecke and Vitale, 1995). Thus, protein trafficking can now be studied in vivo in a matter of days by coexpressing cargo molecules with regulatory genes that control specific transport steps and the reproducibility of the transport assays results from the average behavior of a minimum of 10^5 transfected cells in a sample.

Although transient expression levels can be controlled by the plasmid concentration and the type of promoter fusion (Bottanelli et al., 2012), there is a common misconception that transient expression always involves overexpression, particularly when the strong cauliflower mosaic virus 35S promoter is used. However, careful timing of the experiment permits analysis of cells that produce recombinant protein levels from near to the detection limit right up to the full steady state level when an equilibrium between de novo synthesis and turnover is reached (Phillipson et al., 2001). Time-course experiments are particularly easy to perform with protoplasts as the moment of DNA transfer is fixed in time and gene products can be detected as early as 4 h after gene transfer, followed by a linear increase in the first 24 h (Phillipson et al., 2001). It is therefore possible to work with extremely low expression levels merely by choosing short incubation times. The experimental limit is dictated by the detection method, and when endogenous protein levels are beyond the detection limit in Arabidopsis thaliana, heterologous expression in tobacco (Nicotiana tabacum) can be explored (Gu and Innes, 2011).

LOCATION OF FLUORESCENT CONSTRUCTS IN PROTOPLASTS

The onset of live-cell imaging using fluorescent protein fusions has also led to the use of protoplasts for rapidly testing the subcellular localization of a fluorescent gene chimera in comparison with known fluorescent markers. However, it is important for researchers to understand the limitations of this system. The functionality of fluorescent protein fusions can only be assessed on an individual basis, and overexpression artifacts must be avoided by carefully designing the parameters of the experiment. Steady state expression levels are usually reached between 30 and 50 h of incubation after gene transfer, even when low copy numbers of plasmids are present in the transfected cells. Depending on plasmid concentrations used during DNA transfer, 16 to 24 h of gene expression usually offers a practical range of expression levels from which low-expressing protoplasts can be routinely chosen for high-quality imaging.

It is also essential to check cell viability by monitoring organelle motility and cytoplasmic streaming. Cell viability is likely to be compromised when organelles such as mitochondria and Golgi bodies lose directional mobility and merely wobble on the spot due to Brownian motion. Lack of such directional organelle motion is often accompanied by organelle aggregation, another unmistakable sign of cell mortality. These two features are often correlated by a loss of the spherical shape. Cells showing any of these features or an oval or corrugated cell surface in bright-field microscopy must be excluded from further study. When these simple guidelines are followed, protoplasts represent a reliable model system to obtain fast subcellular localization data on new chimeric constructs. Regardless of the expression system, the functionality of the fusion protein remains a major point to be considered and can only be assessed case by case.

Although leaf protoplasts contain only a thin layer of cytosol sandwiched between the plasma membrane and the tonoplast, the spherical nature of protoplasts permits easy distinction between these two major membranes of the cells. Figure 1A shows a typical plasma membrane signal recognized by the smooth circular shape or even fluorescence intensity. For the best images, the focal plane should be in the very center of the protoplast, which can be achieved by focusing along the z axis until the maximum diameter is reached. Under these conditions, the plasma membrane can be distinguished at a glance from the tonoplast because the latter shows a corrugated shape at the cell periphery as it bends inwards around the various cytosolic organelles (Figure 1B). This is particularly noteworthy with respect to the large nucleus, the chloroplasts, and transvacuolar strands, which may contain cytosol and endoplasmic reticulum (ER) but never the plasma membrane.

If a peripheral signal from the center of the protoplast appears to be interrupted and highly variable in fluorescence intensity as well as thickness, then the signal will originate from cytosolic localizations or other cellular organelles, such as the ER. Cytoplasmic green fluorescent protein (GFP) is usually accompanied by
Figure 1. Confocal Imaging of Organelles of the Secretory Pathway.

(A) and (B) Comparison of fluorescent signals for the plasma membrane (YFP:SYP121) and tonoplast (YFP: SYP22) in optical median sections. (C) to (E) The parallelism of actin filaments (labeled with GFP:Talin-5 and cortical ER tubules (labeled with BIP:Cherry:HDEL) is unaffected in Arabidopsis leaf protoplasts. (F) to (H) Organelles of the secretory pathway easily can be recognized in mesophyll protoplasts from tobacco leaves. The tubular ER network and nuclear envelope (labeled with GFP:HDEL) and punctate Golgi stacks (labeled with Man1:RFP) are immediately distinguishable (F). Golgi stacks are randomly distributed and can easily be recognized by cis-cisternae markers (Man1:RFP) or TGN markers (YFP:SYP61) (G). The same applies for the TGN and prevacuolar compartments (labeled by VSR2:RFP) (H). It should be noted that the YFP:SYP61 signal is not exclusively present at the TGN, and it also is seen at the plasma membrane.

(I) to (N) Monitoring for overexpression artifacts.

(I) and (J) At low plasmid DNA concentrations, the trans-Golgi marker ST:YFP is typically punctate. At 10-fold higher concentrations, a clear signal in the ER can be discerned.

(K) to (N) RFP:p24 is a membrane marker for the ER, although it cycles between the ER and the Golgi apparatus (COPI-mediated retrograde transport is highly efficient; Langhans et al., 2008). Under a huge range of plasmid DNA concentrations, it always gives rise to an ER labeling pattern.

All images were taken from protoplasts that had been incubated for 20 to 30 h after polyethylene glycol—mediated transformation ([C] to [E]) or electroporation (all others).
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labeling of the nucleoplasm. For all other cases, imaging the cell surface at much higher magnification is needed to distinguish between the ER and other organelles. For punctate structures, reliable identification requires dual fluorescence imaging of the test object with known organelle markers. Figures 1C to 1E show convincingly the close alignment of the majority of tubular ER with parallel actin filament networks and closely resemble the situation in epidermal cells (Boevink et al., 1998). Under these conditions, organelle identity is well preserved, and no unspecific aggregation is observed. The constant remodeling of the ER network and high lateral motility of tubules that are not in contact with the actin network is also easy to distinguish in viable protoplasts.

Figures 1F to 1H document that punctate signals representing individual Golgi stacks are by no means clumped or aggregated as long as viable cells are imaged. Golgi stacks, the trans-Golgi network (TGN), and endosomes, as well as mitochondria and peroxisomes, usually show directional motion, sometimes in opposite directions to one another, indicating active cytoskeleton-mediated transport in addition to random cytoplasmic streaming. These are clear indications for cell viability and should be used as criteria for inclusion in the analysis. In thin sections of protoplasts fixed for electron microscopy, there is also no indication of an unusual distribution of organelles (Figure 2). These organelles have as sufficient space to move around as they have in the cells from which they were derived. In conclusion, with the appropriate attention to the quality of imaging, protoplasts allow for excellent visualization of the organelles of the secretory and endocytic pathways and, therefore, for the unequivocal localization of fluorescent constructs. This has been demonstrated repeatedly in the past and recently, for example, by Scheuring et al. (2011) and Wolfenstetter et al. (2012).

ARE PROTOPLASTS STRESSED?

The idea that protoplasts are undergoing secretory pathway stress and that data obtained on them therefore might be flawed is a frequent concern of many researchers. Certainly, protoplasts are actively engaged in building new cell walls and as such are expected to have an active secretory pathway. Although protoplasts have been shown to retain many signal transduction pathways found in the cells from which they are derived (Sheen, 2001), it stands to reason that the process of protoplasting will be accompanied by changes in the mRNA profiles (Birnbaum et al., 2005). For instance, compared with tobacco leaf cells, leaf mesophyll protoplasts exhibit higher mRNA levels of chaperone immunoglobulin binding protein (BiP) (Denecke et al., 1995), which is induced in response to stress and often considered as a marker for stress. However, in the same study, it was shown that this increase is actually very small compared with other tissues, such as petals, anthers, and stigmas, which show much higher mRNA levels of BiP and other ER residents. In fact, the highest BiP levels were found in young seedlings 4 d after onset of imbibition. At this stage, many of the cells are in the process of cell expansion and are synthesizing new cell wall material, but there are no grounds for considering them to be stressed. More importantly, BiP mRNA induction by the process of protoplast isolation is dwarfed by the additional induction that can be observed by the unfolded protein response (UPR) in protoplasts (Denecke et al., 1995). Furthermore, genes induced in Arabidopsis root protoplasts do not include typical UPR targets (Birnbaum et al., 2003).

The UPR is a specific response to the accumulation of folding intermediates or permanently misfolded proteins in the ER that occurs during unfavorable growth conditions, disease, or certain drug treatments. BiP is then trapped in protein complexes that trigger a complex network of pathways leading to rapidly increased de novo BiP synthesis. UPR-inducing agents, such as tunicamycin, cause massive induction of BiP mRNA levels in protoplasts (Denecke et al., 1995; Frigerio et al., 2001b). The limiting factor is the chaperone itself because overexpressed BiP alleviates the cells from the UPR (Leborgne-Castel et al., 1999). This clearly indicates that while protoplasts are fully equipped to respond to ER stress, they only exhibit modest increases in stress-related mRNA levels compared with a variety of other plant tissues.

The UPR is not the only mechanism by which BiP and other ER chaperone expression levels can be upregulated. In barley (Hordeum vulgare) aleurone layers, the hormone gibberellic acid elevates ER chaperone levels together with those of starch hydrolases and exhibits a slightly more sensitive dose response compared with the genes encoding the secreted enzymes (Denecke et al., 1995). Also, during plant pathogen interactions, BiP mRNA levels are rapidly upregulated in a UPR-independent manner that is systemic throughout the plant and occurs well before the onset of pathogenesis-related protein synthesis (Jelitto-Van Dooren et al., 1999). Both examples illustrate UPR-independent mechanisms of BiP induction because the UPR is a feedback response that occurs after misfolded proteins accumulate. If increased secretory protein synthesis were the causal agent for increased BiP expression, then BiP induction would be delayed compared with that of the hydrolases and the pathogenesis-related proteins, but the opposite was observed. These examples show that plants actively avoid ER stress by upregulating the key machinery required for increased secretory protein synthesis. Therefore, increased BiP levels alone cannot be regarded as an indicator for ER stress in plants.

Apart from the difficulty in agreeing on criteria for stressed cells, it could also be argued that increased secretory protein synthesis and cell wall synthesis can be an advantage in the study of the secretory pathway. Similar to the process of autophagy (Yoshimoto et al., 2010), cell wall synthesis is a constitutive property of plant cells, and both are best studied under conditions where the process is upregulated. Protoplasts have been shown to be perfectly capable of coping with correct protein sorting in the cell, as documented by efficient ER retention and vacuolar sorting of cargo molecules. When the natural...
secretory reporter molecule α-amylase (amy) is modified by the addition of sequence-specific vacuolar sorting signals (amy-spo and aleu-amy) or ER retention signals (e.g., amy-HDEL), sorting fidelity remains at a high level when cargo dosage is maintained below specific thresholds. This was demonstrated by time-course studies designed to illustrate that saturation of ER retention or vacuolar sorting are not inherent properties of the protoplasts but strictly cargo dosage dependent (Neuhaus et al., 1994; Phillipson et al., 2001; Pimpl et al., 2003).

Targeting of membrane proteins also appears to occur normally in protoplasts, and any mistargeting observed may be caused by overexpression. Since this has been mainly studied by fluorescence microscopy, the natural variance in transient expression levels in individual transfected protoplasts makes it easy to monitor for overexpression artifacts directly. Overexpression per se does not automatically lead to localization artifacts. Figures 1I to 1N present such an analysis with an ER protein (RFP-p24Δ5) whose location is not affected by overexpression, together with a Golgi protein (sialyl transferase), which can be seen in transit via the ER at high expression levels. The p24 protein is of instructional value in this regard because it is a membrane protein whose steady state localization in the ER is a result of its highly efficient COPI-mediated retrieval from the cis-Golgi (Langhans et al., 2008). Thus, even at high expression levels, the recycling machinery is not saturated and the protein does not overflow into the Golgi. This is not the case with ST-(X)FP constructs, the localization of which varies significantly with expression levels.

**PROTOPLASTS VERSUS AGROBACTERIUM-INFILTRATED LEAVES**

The technical demands for efficient and routine transient expression in protoplasts may be one of the factors that led to a certain reluctance by many in the field to accept the method. As an alternative to protoplasts, it is also possible to carry out transient expression within intact tissues of entire plants, either via naked DNA transfer using ballistic methods or using Agrobacterium
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tumefaciens as a vector for stable gene integration. This is generally easier to learn and can be achieved with greenhouse-grown plants, which are often more easily accessible compared with the in vitro–grown plants needed for efficient protoplasting. Best characterized is the tobacco leaf infiltration system with Agrobacterium strains, leading to long-term proliferation of the plant transformation–competent bacteria in the spongy parenchyma cavities, culminating in highly efficient transformation of the leaf cells (Sparkes et al., 2006). Due to its high popularity in the field, we wish to compare this model system with the protoplast model and highlight specific advantages.

In contrast with naked DNA transfer in protoplasts and ballistic DNA transfer in tissues or suspension-cultured cells, agroinfiltration of leaves does not lead to a specific moment in time in which gene transfer occurs. Agrobacteria can infect plant cells over a long period as the cells can proliferate within the apoplastic water layer rich in minerals and Suc. For this reason, expression is not as easily controllable through bacterial OD as it can be with DNA concentrations in naked DNA transfer. The timing of cell entry of single-stranded transcriptionally inactive T-DNA coated with VirD2 and VirE2 followed by nuclear targeting and genome insertion is not well defined and may occur shortly after infiltration or up to days after infiltration. Proliferation of Agrobacterium in the apoplast, as well as its infectivity is highly variable due to differences in leaf physiology, even within subdomains of the same leaf. For this reason, variation between independent leaf infiltrations is higher than in transfected protoplasts. Compared with protoplast systems, it is thus more difficult to carry out time-course experiments; furthermore, a transfected cell population cannot be split into equal portions, for example, to test chemical dose responses against a control.

However, the nature of Agrobacterium-mediated plant transformation offers specific advantages that can be exploited to enrich the experimental toolbox of the field. Unlike naked DNA transfer where the introduced genes are transcribed from extrachromosomal DNA fragments in the nucleoplasm, Agrobacterium mediates highly efficient stable integration of the desired genes. The mechanism of T-DNA insertion into the genome is unknown (Gelvin, 2010; Pitzschke and Hirt, 2010), but it is likely that the VirD2 and VirE2 gene products provide more than just protection of the single-stranded T-DNA and nuclear targeting because these proteins can mediate integration of synthetic T-DNA complexes, even into the genome of mammalian cells (Pelczar et al., 2004). There is no experimental evidence demonstrating the formation of double-stranded extrachromosomal T-DNA copies that mediate transient expression, and it cannot be ruled out that instability of transgene expression could be caused by rapid gene silencing after T-DNA insertion. For example, in Nicotiana benthamiana, parallel infiltration with the p19 viral suppressor of RNAi of Tomato bushy stunt virus can increase expression levels of a heterologous protein severalfold (Voornet et al., 2003). Further research is necessary to establish if transgene expression after leaf infiltration with Agrobacterium originates mainly from extrachromosomal or genomic T-DNA.

Expression levels in leaf cells 24 to 48 h after Agrobacterium infiltration are generally lower and less variable than in protoplasts after naked DNA transfer. The main cause for variation in transgene expression in individual cells is likely to be the timing of the transformation events after infiltration, although position effects and copy number after gene insertion cannot be ruled out. Cells can be cotransformed by different Agrobacterium strains, and the timing of each individual transformation event is spread over the whole incubation period until analysis. This is why mixing two Agrobacterium strains leads to a much lower degree of cotransformation compared with naked DNA transfer, and relative gene expression from genes introduced by separate agrobacteria is not correlated in individual cells as it is in protoplasts. To overcome current problems of variable expression in agroinfiltrated leaves (Yang et al., 2000; Wroblewski et al., 2005; Conley et al., 2011), dual expression vectors have been developed to guarantee cotransformation of untagged dominant mutants with fluorescent markers (Bottanelli et al., 2011).

The transformation efficiency of the Agrobacterium-infiltration method is much higher compared with the transfected protoplasts and can amount to nearly 100% of the cell population depending on the incubation time. One spends much less time looking for fluorescent cells and the rate of data acquisition is high compared with protoplasts because most cells are transformed and quite a good deal of these express at low or moderate levels if the timing is adequate. However, the success of Agrobacterium-mediated gene transfer also depends on the plant’s repertoire of cell surface receptors to perceive pathogen-associated antigens, which in the case for Arabidopsis drastically reducing the rate of Agrobacterium-mediated transformation of leaf cells (Zipfel et al., 2006).

Differences in expression levels within individual cells can be actively exploited to identify low expressing cells that show unbiased subcellular localizations, and just as with protoplasts, it is possible to test if overexpression leads to mistargeting. The responsibility lies with the scientist to carry out the appropriate controls and to study if systematic changes in results can be attributed to levels of expression. In general, the sooner after infiltration one analyses the cells, the more likely that overexpression will not be a problem, but even 48 h after infiltration, low-expressing cells can be readily identified.

One of the major differences between protoplasts and intact tissues is the presence of a cell wall, regardless of the form of gene transfer. Protoplasts have lost all polarity that may have been present in the tissue from which they were derived. Obviously, when polarized localization patterns are to be studied, intact tissues are essential. However, it can be of interest to ask what happens to polar domain–localized proteins when they are expressed in a nonpolar cell. For example, if polarized transport to plasma membrane subdomains is achieved by selective exocytosis, then one might observe retention in an earlier compartment because the required machinery for selective export is missing. If selective endocytosis is the main contributory mechanism to polar localizations, the protein may still accumulate in
the plasma membrane despite loss of polarity. Similarly, proteins with a specialized function during cytokinesis (e.g., KNOLLE) (see Lauber et al., 1997; Reichardt et al., 2007) may provide interesting insights into protein targeting mechanisms when ectopically expressed in nondividing cells like protoplasts. Thus, the more experimental models we use, the more we can learn about a specific biological process.

HOW OVEREXPRESSION CAN HELP US TO UNDERSTAND THE SECRETORY PATHWAY

An important general argument in favor of transient expression is that it is easy to overexpress protein constructs through high plasmid copy numbers, strong promoters, and long expression times. This is an advantage when higher protein levels are required to test transport models. Thus, mis-sorting by overexpression can be a useful experimental tool to verify the next step in the pathway. Does an overexpressed membrane protein mistarget to an earlier compartment, or does it proceed to a more distal compartment in the trafficking system? Answers to these questions can shed light on specific targeting mechanisms as well as the functioning of the entire pathway and are as such just as valuable as the initial characterization of a knockout mutant.

Classic loss-of-function approaches to identify new genes in new pathways have served the community well and will doubtlessly continue to do so. However, dominant genetic experimental strategies via overexpression or construction of mutant molecules with altered or novel functions can be equally valuable. This is particularly true when it is established that a gene is necessary for a specific process, and it remains to be uncovered how the gene product works and with which cellular components it may interact. For instance, it has been shown that the inhibition of vacuolar sorting by the drug Wortmannin is caused by increased receptor degradation due to lack of recycling, which is supported by the observation that overexpression of wild-type receptors can suppress the effect of the drug (daSilva et al., 2005). If Wortmannin instead inhibited forward trafficking of the receptor, overexpression of receptor molecules would not be expected to restore vacuolar sorting.

Overexpression strategies should be grouped together with dominant mutants as both overshadow the endogenous gene, enabling one to carry out hypothesis-driven experiments to investigate further details of protein function and binding partners. The latter has been illustrated using the guanosine exchange factor Sec12, which titrates its partner GTPase Sar1 when overexpressed (Phillipson et al., 2001). This type of study (e.g., dose-dependent titration and reconstitution by additional Sar1 gene expression), would be nearly impossible to carry out in whole plants, as it would require the use of two different inducible promoter systems and plant transformations followed by supertransformations or crosses, which would take years instead of a few weeks to complete. In addition, quantitative secretion assays in plant tissues have yet to be established.

OTHER ADVANTAGES OF DOMINANT APPROACHES

It is another common misconception that loss-of-function mutations have specific effects with direct causal links, while dominant mutants are pleiotropic and unspecific/indirect. In fact, both type of mutations have precisely the same chance of yielding direct as well as indirect effects and should be considered on a case-by-case basis without prejudice. The main difference is that a common working routine is available for loss-of-function mutants, mapping of which is simple as it is not hampered by dosage effects. Gene identification is strictly proven by a combination of complementation analysis and establishment of the causal mutation by sequencing. However, this only confirms that the gene corresponding to the mutant has been cloned, and this is where the direct relationship ends. All further interpretations of the mutant phenotypes are just as risky as with dominant mutants, and there is no guarantee that the observed morphological phenotype reflects a direct gene function. It could just as well reflect a long cascade of events brought about by interfering with a housekeeping function of the cell.

In the case of dominant mutants, mapping can be more difficult because most dominant mutants are not truly dominant after critical analysis and their influence on cellular processes may vary with expression levels. This often makes them more difficult to interpret, but their value, similar to that of suppressors, lies in their ability to expand the network of interactions in which a certain gene may operate. The main reason for their lower popularity is likely that no common working practice can be defined for the creation of dominant mutants simply because every protein is different. For instance, replacement of the lumenal ligand binding domain by fluorescent reporters in vacuolar sorting receptors gave rise to a semidominant mutant that interferes with vacuolar sorting (daSilva et al., 2005). This feature was successfully exploited to identify loss-of-function mutations affecting anterograde transport toward and retrograde transport from the prevacuolar compartment (daSilva et al., 2006; Foresti et al., 2010). It is doubtful whether such detailed insights into receptor transport could have been obtained by studying vacuolar sorting receptor knockouts. There are countless other examples in the literature that illustrate how dominant mutants provide a cornerstone to test hypotheses inspired by specific knowledge about a gene product (e.g., Batoko et al., 2000; Takeuchi et al., 2000; Jin et al., 2001; Bolte et al., 2004; Lee et al., 2004; Watanabe et al., 2004; Haas et al., 2007; Snowden et al., 2007).

What most of these have in common is that long-term overexpression of certain wild-type genes or stable low-level expression of dominant mutants leads to cell death when housekeeping functions are targeted. However, this is also true for knockouts of essential genes, such as the ER chaperone BiP and the HDEL receptor ERD2, or an essential SNARE in the transport between the ER and the Golgi. Vacular sorting and ER retention are typical examples of essential housekeeping functions. Transient time-course experiments in protoplasts can be an excellent way of overcoming long-term toxicity problems and may lead to specific insights that would not be possible or practical otherwise. For these reasons, it does not come as a surprise that mutants of

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essential secretory pathway machinery components lead to erroneous developmental or embryo-lethal phenotypes (Rojo et al., 2001), and one should therefore be careful before assigning a specific function in cell morphology and development. When cell biologists choose to avoid gene knockouts, it is often because there are good reasons to assume that the mutant or knockout will be lethal. Knocking out the essential ER chaperone BiP is likely to be just a futile as knocking out the Golgi-ER recycling receptor ERD2 or for that matter an essential SNARE in transport between the ER and the Golgi. On the other hand, many Arabidopsis proteins are members of multigene families, and multiple knockouts may be necessary before any phenotypic effect is observed. Examples of this are the myosins, reticulons, tonoplast integral proteins, and vacuolar sorting receptors, where individual family members show functional redundancy (Peremyslov et al., 2010; Zouhar et al., 2010; Gattolin et al., 2011). Nevertheless, knockout studies remain an excellent way to begin analyzing the function of unknown genes and provide the best platform for functional analysis of fluorescent gene fusions by complementation analysis.

HETEROLOGOUS EXPRESSION AND THE USE OF ARTIFICIAL CARGO

Another argument against transient expression studies stems from a concern over the use of heterologous promoters, heterologous expression, and non-native cargo molecules. A corollary to this is the belief that as long as one uses an endogenous promoter, the transgenic plant will exhibit native expression levels. This is far from true, as position effects (e.g., outside the endogenous promoter region) have been exploited effectively in enhancer trap approaches to isolate new promoter isoforms and transcriptional enhancers (Campisi et al., 1999). Plants seem to exhibit an upper limit of stable transgene expression beyond which silencing becomes a limiting factor (Schubert et al., 2004). However, this appears to be more pronounced for multiple insertions of virus promoters that are naturally designed to work in multiple copies during infection. Weaker promoter constructs may be influenced quite dramatically by flanking sequences in the genome and may titrate out limiting transcription factors when present in multiple copies. Therefore, using an endogenous promoter is no guarantee for adequate expression when it is randomly inserted by T-DNA insertion.

Weak expression can be achieved with weaker promoters (Bottanelli et al., 2012), and this is independent of the heterologous nature. Even strong promoters can yield low expression levels when the experiment is timed before steady state levels are reached. The take-home message is that neither the promoter nor the plant model can be blamed for inappropriate expression; only the scientists themselves can be responsible for carrying out a careful case-by-case analysis and choosing the most appropriate and convenient experimental conditions for the particular biological problem under study.

It is also important to realize that artificial cargo molecules can have experimental advantages over endogenous proteins. Some of the most significant advances in our knowledge of the secretory pathway in mammalian cells have been achieved using the vesicular stomatitis virus G-protein, a viral membrane protein heterologously and transiently expressed in a variety of mammalian cell types (Lippincott-Schwartz et al., 1998). In plant cells, the concept of secretory bulk flow originated from the heterologous introduction of cytosolic proteins from prokaryotic origin into the ER via fusion to a signal peptide (Denecke et al., 1990). The fact that these were secreted was unlikely due to specific ER export signals that several prokaryotic cytosolic proteins would have acquired by accident. Using endogenous proteins for this experiment would have required extra steps to distinguish the modified proteins from endogenous proteins.

Similar arguments can be raised for all fluorescent protein fusions for live fluorescent bioimaging, for example, fusions to the N terminus and transmembrane domain of sialyl transferase (a mammalian enzyme that targets to the trans-Golgi cisterna but is without function in plants; Daskalova et al., 2009) and other reporters, such as the barley α-amylase protein used for quantitative transport assays in vivo (Phillipson et al., 2001). They may be regarded as neutral proteins that are transported by the secretory pathway and act as gratuitous markers. Just like fusions between a synthetic auxin response element DR5 and β-glucuronidase, which have become a major tool in auxin response studies (Ulmasov et al., 1997), artificial cargo molecules report on the functioning or malfunctioning of the secretory pathway. This is exemplified by the analysis of partial knockout plants of vacuolar sorting receptors using artificial fluorescent vacuolar cargo (Craddock et al., 2008). This approach permitted experiments in which the type of sorting signal was the sole variable, which would have been impossible with different types of endogenous cargo.

There are also examples in which heterologous expression is simply the only viable way forward. Transient expression in protoplasts has proven to be an irreplaceable system for the study of toxins, such as ricin (reviewed in Frigerio and Roberts, 2010). Ricin is a potent cytotoxin from Ricinus communis, with the ability to depurinate plant ribosomes, thus inhibiting protein synthesis. This makes stable expression of ricin (in particular of its cytotoxic A subunit) problematic even in heterologous systems. However, transient expression of prorcin and its A and B subunits was achieved successfully in tobacco protoplasts (Frigerio et al., 1998). This expression system therefore proved very useful to elucidate the intracellular route of prorcin, its vacuolar sorting signal (Frigerio et al., 2001a), and the trafficking of its individual subunits (Frigerio et al., 1998; Chamberlain et al., 2008). In particular, the study of the fate of ricin A chain in protoplasts provided one of the earliest indications that a bona fide ER-associated degradation pathway exists in plant cells (Di Cola et al., 2001, 2005).

CONCLUSIONS

As we have discussed above, the use of protoplasts and other transient expression systems for the in vivo analysis of organelles
in the secretory and endocytic pathways are valuable tools to complement the more classical whole-organism studies using loss-of-function mutants and gene knockouts. Live fluorescent imaging drastically increases the limit of detection and permits dynamic studies with native organelle structures in the most biological relevant configuration: a living cell. Overexpression and dominant mutants may be used constructively in the same way as drugs can help us to understand certain processes. Employing them can further our understanding of protein function and facilitate the identification of interacting partners. Heterologous transient expression not only simplifies experimental procedures, it can sometimes be the only way to understand the location and function of toxins or the assembly of viral coat proteins in the host cell.

The use of multiple plant models and cell systems like protoplasts brings an important degree of diversity and versatility to plant research. While functional complementation using endogenous promoters may be the most elegant way to validate the location of a fluorescent protein, it has the potential caveat that expression levels may be too low for protein detection so that one has to resort to heterologous transient expression, as recently demonstrated by Gu and Innes (2011). It should also not be forgotten that many plants, especially agriculturally valuable ones, cannot be as easily transformed as Arabidopsis. We encourage the plant cell biology community to exploit the full range of experimental methods that have been made available over the last few decades.

AUTHOR CONTRIBUTIONS
All authors contributed to writing this article.

REFERENCES


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