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TECHNICAL ADVANCE

pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves

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Summary

We have constructed a matched set of binary vectors designated pGD, pGDG and pGDR for the expression and co-localization of native proteins and GFP or DsRed fusions in large numbers of plant cells. The utility of these vectors following agroinfiltration into leaves has been demonstrated with four genes from *Sonchus yellow net virus*, a plant nucleorhabdovirus, and with a nucleolar marker protein. Of the three SYNV proteins tested, sc4 gave identical localization patterns at the cell wall and nucleus when fused to GFP or DsRed. However, some differences in expression patterns were observed depending on whether DsRed or GFP was the fusion partner. In this regard, the DsRed:P fusion showed a similar pattern of localization to GFP:P, but localized foci appeared in the nucleus and near the periphery of the nucleus. Nevertheless, the viral nucleocapsid protein, expressed as a GFP:N fusion, co-localized with DsRed:P in a subnuclear locale in agreement with our previous observations (Goodin *et al.*, 2001). This locale appears to be distinct from the nucleolus as indicated by co-expression of the N protein, DsRed:P and a nucleolar marker AtFib1 fused to GFP. The SYNV M protein, which is believed to be particularly prone to oligomerization, was detectable only as a GFP fusion. Our results indicate that agroinfiltration with bacteria containing the pGD vectors is extremely useful for transient expression of several proteins in a high proportion of the cells of *Nicotiana benthamiana* leaves. The GFP and DsRed elements incorporated into the pGD system should greatly increase the ease of visualizing co-localization and interactions of proteins in a variety of experimental dicotyledonous hosts.

Keywords: protein expression plasmids, DsRed, red fluorescent protein, GFP, agroinfiltration, transient gene expression

Introduction

Although the discovery of novel genes by genome sequencing projects is advancing rapidly in plants, new tools need to be developed to characterize the functions of these genes. Protein function may be elucidated in part if cellular targeting signals and regions of subcellular accumulation can be determined. In particular, co-localization can be used to determine the effects of one protein on another (Relic *et al.*, 1998). To these ends, the expression of proteins of interest fused to autofluorescent proteins in plants has become extremely useful in protein targeting research (Hanson and Kohler, 2001; Stewart, 2001). The most commonly used of these proteins is the green fluorescent protein (GFP) from *Aequorea victoria* and the blue, yellow and red-shifted variants derived from GFP (Hanson and Kohler, 2001; Stewart, 2001). The availability of these GFP variants with distinct light absorbance and emission characteristics has allowed their successful use in a number of applications including co-localization (Kohler *et al.*, 1997; Von Arnim *et al.*, 1998), and fluorescence-radiation-energy-transfer (Mahajan *et al.*, 1998).

However, such analyses are often hampered by drawbacks such as an inability to express high levels of protein synchronously in a large number of leaf cells, the sensitivity of some GFP variants to photobleaching or the inability to clearly distinguish two types of GFP with commercially available filter-sets. Given these limitations, we have constructed three plasmid vectors, pGD, pGDG and pGDR, to permit easy cloning and to evaluate transient expression of native proteins and their fusion derivatives in a very high proportion of *Agrobacterium*-infected cells. Multiple cloning sites introduced into each of the three plasmids facilitates cloning into pGD for expression of unfused protein derivatives and to utilize pGDG to create gene fusions with the well characterized GFP protein (Hanson and Kohler, 2001; Stewart, 2001). We also have designed pGDR to utilize DsRed, a newly discovered autofluorescent protein. DsRed was originally isolated from the non-bioluminescent coral *Discosoma* sp., and it appears to have significant advantages that can be used in conjunction with several of the GFP mutant derivatives that are commercially available (Matz *et al*., 1999). DsRed has an excitation peak at 558 nm and emits light with a maximum at 583 nm. Thus, the spectral profile of DsRed is easily distinguished from the commonly used red-shifted GFPs that have absorption and emission maxima at 490 nm and 509 nm, respectively. Recently, DsRed proteins have been used successfully as vital markers in co-localization experiments in neural fibres (Verkhusha *et al*., 2001), zebrafish (Finely *et al*, 2001), *Saccharomyces cerevisae* (Rodrigues *et al*., 2001) and as unfused reporters in plants (Dietrich and Maiss, 2002; Jach *et al*., 2001; Vassilakos *et al*., 2001). Targeting of DsRed to the cytosol, endoplasmic reticulum, vacuoles and chloroplasts has been reported recently (Jach *et al*., 2001) as has expression of the *Peanut clump virus* p15 protein fused to DsRed1 (Dunoyer *et al*., 2002). Thus, these early reports suggest that both DsRed and GFP protein fusions can be used for targeting of organelles and protein co-localization experiments in plant cells.

To evaluate the utility of DsRed as a vital marker in plant cells and its ability to be distinguished from GFP, we constructed GFP and DsRed fusions with three proteins from *Sonchus yellow net virus* (SYNV), a plant rhabdovirus. SYNV is the most extensively characterized species of the plant rhabdoviruses, and it appears to replicate in the nucleus and undergo morphogenesis at the inner nuclear envelope (Jackson and Christie, 1977; Jackson *et al*., 1987). Investigations of the interactions and localization patterns of these proteins can therefore provide valuable clues to the viral life cycle.

The interactions and nuclear import of the SYNV nucleocapsid (N) and phospho (P) proteins have been recently described and the proteins have been shown to engage in associations that lead to their subnuclear accumulation (Goodin *et al*., 2001). However, more detailed characterization of the interactions between these proteins, other SYNV proteins and host components requires the development of new tools to provide differentially labelled proteins in plant cells. In order to begin to develop such a system, we fused three SYNV genes, *P*, *M* and *sc4*, to the carboxy-terminus of the autofluorescent proteins GFP and DsRed. The SYNV nucleocapsid protein, N, was expressed only as a GFP fusion. The M (matrix) protein (Hillman *et al*., 1990) forms a component of the bacilliform particles of SYNV, and engages in putative associations with a glycoprotein (G) in the viral lipid envelope (Goldberg *et al*., 1991) and with the nucleocapsid core, which consists of the negative-strand genomic RNA encapsidated by the N, P and L (polymerase) proteins (Choi *et al*., 1992; Wagner and Jackson, 1997). The sc4 protein is also associated with the membrane fraction of purified virions (Scholthof *et al*., 1994), and has been predicted to function in viral cell-to-cell movement (Melcher, 2000). The P, M and sc4 proteins are similar in size, 38 kDa, 32 kDa and 37 kDa, respectively, while the N protein is 52 kDa. These proteins are likely to have different patterns of localization given their structure and their putative functions in virus replication, morphogenesis and movement. The ability to label each of the viral proteins with different fluorescent markers will facilitate additional co-expression and co-localization studies of SYNV.

To provide a distinguishable marker for use with previously existing SYNV GFP protein fusions, we have examined the expression and utility of DsRed protein fusions for subcellular studies in cells of *Nicotiana benthamiana*. The pGD plasmid expression system has great utility for SYNV analyses, and the system also provides considerable advantages for studies of the interactions of other proteins in *N. benthamiana* and other dicot hosts that can be infected with *Agrobacterium*. The fact that almost all the epidermal cells in an infiltrated leaf can be transfected with multiple proteins provides an efficient means to evaluate the localization of differentially marked proteins and to assess multiple interactions of proteins that affect their function.

Results

The pGD binary vector series enable high level transient expression of native and autofluorescent fusion proteins when agroinfiltrated into plant cells

We have devised a series of binary vectors with matched multiple cloning sites for transient expression of proteins in plants. The pGD, pGDG, and pGDR vectors (Figure 1) permit cloning of each candidate gene into individual plasmids for expression of the gene product as either a

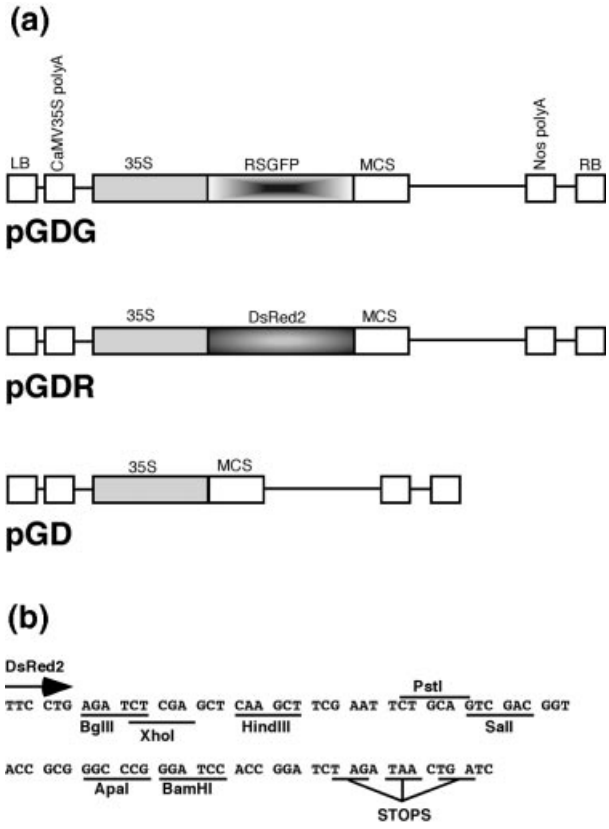


Figure 1. Maps of the pGD vector series. All three vectors are derivatives of the binary vector pCAMBIA-1301.
Sources of the 35S promoter and autofluorescent proteins are described in the Experimental procedures section.
(a) Schematic representations of pGDG, pGDR and pGD. LB and RB denote left and right borders of the T-DNA, respectively. The pGD vectors retain both the CaMV35S polyA and nopaline synthase (Nos polyA) polyadenlylation signals present in pCAMBIA-1301. However, only the Nos polyA signal is utilized in these vectors.
(b) Sequence of the multiple cloning site of pGDR. The reading frame is identical in pGDG and pGD and contains the same set of restriction sites. Restriction sites common to all three vectors are shown beneath the multiple cloning site sequence. The PstI site can be used only in pGDG and pGD because the DsRed2 coding sequence contains a PstI site.

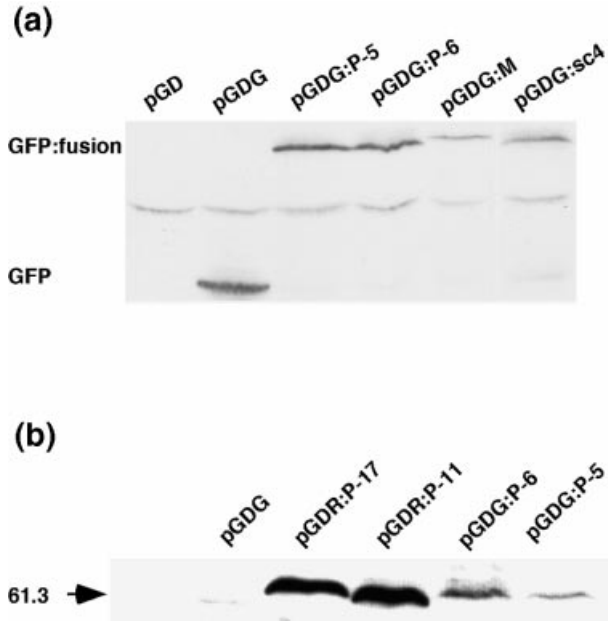
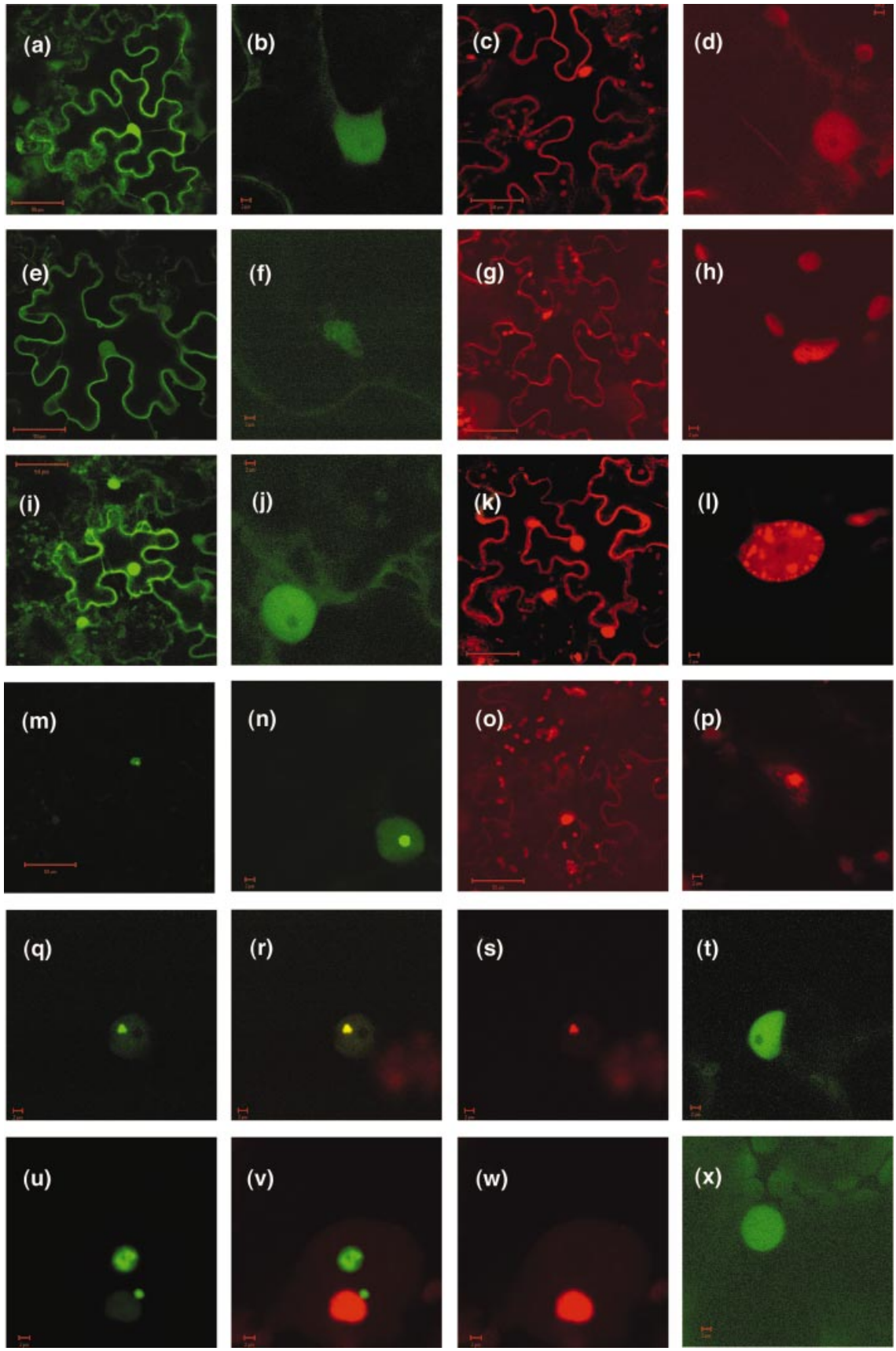


Figure 2. Immunoblot detection of independent clones of GFP and DsRed fusions to SYNV proteins expressed from pGDG and pGDR vectors in agroinfiltrated leaves.
(a) Detection of GFP and GFP fusions with a GFP monoclonal antibody; Lane 1, pGD control; Lane 2, pGDG control; Lane 3, pGdG:P-5; Lane 4, pGDG:P-6; Lane 5, pGDG:M; Lane 6, pGDG:sc4;
(b) DsRed and GFP fusions of SYNV P protein detected using P protein antisera; Lane 1, pGDG control; Lane 2, pGDR:P-17; Lane 3, pGDR:P-11; Lane 4, pGDG:P-6; Lane 5, pGDG:P-5; nearest size marker indicated on left. Note that all of the pGDR and pGDG constructs that gave the expected sized products on Western blots also gave identical results in localization studies. Only pGDR-5 and pGDG-5 were used to produce Figures 3 and 4.

native protein or as in-frame C-terminal fusions with the autofluorescent proteins GFP or DsRed. Since the native form of DsRed has the capacity to form oligomers that might affect the correct localization of fusion proteins (Baird *et al.*, 2000), a mutant form of DsRed developed at Clontech (Palo Alto, CA, USA), called DsRed2, that has a substantially reduced propensity to oligomerize relative to the wild-type protein, was used for the construction of pGDR. Following agroinfiltration of whole leaves, nearly all cells expressed up to three proteins from 2 to at least 7 days post infiltration (data not shown). In every experiment, large numbers of cells showed similar levels of high intensity fluorescence, which was specific for the fusion partner. The accumulation of protein fusions was determined using immunoblot analyses of total protein extracts from agroinfiltrated leaves. (Figure 2a). GFP:P, GFP:M and GFP:sc4 fusions appeared to accumulate to similar levels at 45 h post agroinfiltration as visualized by a GFP monoclonal antibody (Figure 2a). Because an antibody able to detect DsRed C-terminal fusions was not available at the time this research was conducted, a P protein-specific antiserum was used to detect DsRed:P fusions, as well as to confirm the accumulation of GFP:P fusion protein seen with the GFP monoclonal antibody (Figure 2b). Both the DsRed:P and GFP:P fusion proteins accumulated to high levels in agroinfiltrated leaves when assayed after 60 h and 45 h, respectively,(Figure 2b), and expression of native N and/or P proteins, expressed from pGD, was determined with antisera against intact virions or P protein (data not shown). The molecular weight of pGDG:M appeared to be greater than those of the P and sc4 fusions despite the fact that M is at least 4 kDa smaller than P and sc4 (Figure 1a). However, P, M and sc4 are phosphoproteins (unpublished data), so it is possible that the degree to which these proteins are phosphorylated *in vivo* could affect their migration on SDS-PAGE gels.



DsRed is a useful vital marker in protein localization studies utilizing laser-scanning confocal microscopy

A protein expression study using the pGD vector series was conducted to determine whether DsRed and GFP had similar utilities in plant cells (Figure 3). The expression patterns of unfused GFP and DsRed from pGDG and pGDR were qualitatively the same, both at the whole cell level (Figure 3a,c), and in the nuclei (Figure 3b,d). The sc4 putative movement protein also showed similar patterns of expression when fused to either GFP or DsRed (Figure 3e-h). Sc4 fusions were targeted primarily to the periphery of epidermal cells and exhibited lower fluorescence in the nucleus than unfused GFP or DsRed. SYNV P had similar cellular localization patterns when fused to GFP or DsRed (Figure 3i,k). However, when nuclei were viewed at higher magnifications, it became apparent that DsRed:P fusions often formed aggregates that accumulated at or near the nuclear membrane, whereas the GFP:P fusions had a more uniform nuclear fluorescence pattern (Figure 3j,l). Despite this difference, DsRed:P and GFP:P exhibited similar subnuclear localization patterns when co-expressed with the SYNV N protein (compare Figure 3m,n with Figure 3o,p). These patterns appear to be quite similar to those shown with GFP:P and N co-expressed in yeast and plant cells (Goodin *et al.*, 2001) and they indicate that GFP and DsRed fusions can provide useful partners to evaluate co-localization of proteins.

DsRed and GFP fusion proteins can be distinguished in co-localization studies

In order to evaluate the utility of the pGD vectors in co-localization experiments, leaves were infiltrated with mixtures containing equal amounts of *Agrobacterium* cells harbouring pGDG:N and pGDR:P plasmids (Figure 3q-s). Observations with the green filter to reveal the fluorescence pattern of GFP:N (Figure 3q) and with the red filter to visualize interactions of DsRed:P (Figure 3s) indicated that both signals, i.e. both proteins, accumulated in the same subnuclear location (Figure 3r). Taken together, these patterns of fluorescence suggest a potential interaction between N and P proteins that result in a subnuclear accumulation of these proteins. The GFP:N fusion protein when expressed alone localized to completely to the nucleus (Figure 3t). M protein expressed as a GFP fusion accumulated solely in the nucleus in the absence of N and P (Figure 3x). In contrast, we were unable to detect fluorescence from DsRed:M fusions (data not shown). It is possible that the matrix properties of M result in a tendency to aggregate and, since DsRed exists as a tetramer, we suspect that the combined effects of M aggregation and DsRed oligomerization may result in a non-fluorescent DsRed:M fusion.

Given the results shown in Figure 3q-s, we were concerned that the immature form of DsRed, which fluoresces green (Gross *et al.*, 2000), might interfere with co-localization studies if the authentic GFP signal could not be distinguished from the green emission of immature DsRed. This was important because the immature DsRed exhibits a GFP-like excitation at 475-nm and emission maximum at 500-nm (Gross *et al.*, 2000), and hence could interfere with co-expression of GFP. Therefore, experiments were conducted to co-express DsRed:P and N with the *Arabidopsis thaliana* nucleolar marker, Fib1:GFP (Barneche *et al.*, 2000). Co-expression of DsRed:P and N clearly revealed that the subnuclear accumulation of DsRed:P is distinct from that of Fib1:GFP (Figure 3v). The two differentially marked subnuclear locales were then used to enable direct comparisons of the potential interference of the green immature DsRed signal with GFP detection and *vice versa*. Figure 3u shows the green channel of the proteins shown in Figure 3v. Note that the faint green fluorescence from the area illuminated by DsRed in the red channel was extremely weak compared to the GFP signal from Fib1:GFP. Similarly, red fluorescence was not detectable from Fib1:GFP in the red channel (Figure 3w). Hence, GFP failed to interfere with the DsRed:P signal shown in Figure 3w. Co-expression of DsRed:P/N also failed to interfere with the fluorescence pattern of Fib1:GFP. Fib1:GFP, which localizes to the nucleolus in *A. thaliana* and onion epidermal cells (Barneche *et al.*, 2000), shows a similar nucleolar localization in *N. benthamiana* epidermal leaf cells, where two discrete accumulation sites can be identified (Figure 3u). These data therefore provide confidence that the results observed in Figure 3q-s are due to authentic co-localization of GFP:N with the DsRed:P rather than an artefact resulting from the immature form of DsRed.

Figure 3. Laser-scanning confocal micrographs showing fluorescence of leaf cells following infiltration with Agrobacteria carrying pGD, pGDG or pGDR plasmids expressing the autofluorescent proteins GFP, DsRed, SYNV proteins and their autofluorescent protein fusions, and Fib1-GFP.
Scale bars are 50 μm and 2 μm for whole cell and nuclear image capture, respectively. Panels (a) to (p) depict pairs of micrographs showing expression patterns in selected whole cells (25× magnification) and nuclear close-ups (100× magnification), respectively. (a) and (b) GFP expressed from pGDG; C and (d) DsRed expressed from pGDR; (e) and (f) GFP:sc4 expressed from pGDG; G and (h) DsRed:sc4 expressed from pGDR; (i) and (j) GFP:P expressed from pGDG; (k) and (l) DsRed:P expressed from pGDR; (m) and (n) Co-expression of GFP:P and N; (o) and (p) Co-expression of DsRed:P and N; (q), (r) and (s) nucleus of a cell in which GFP:N, and DsRed:P were co-expressed; (q) Green channel showing location of GFP fluorescence; (r) Overlay of images Q and S; (s) Red channel showing localization of DsRed; (t) Nucleus of a cell in which GFP:N was expressed; (u), (v) and (w) Co-expression of Fib1:GFP, DsRed:P and N; (u) Green channel showing GFP localization; (v) Overlay of micrographs u and w.; (w) Red channel showing cell DsRed localization. (x) Nucleus of a cell in which GFP:M was expressed.

DsRed is a useful vital epifluorescence microscopy marker

In addition to laser scanning confocal microscopy, we investigated the utility of DsRed as a reporter in tissues to be examined by standard epifluorescence (Figure 4). As noted by Dietrich and Mais (2002), autofluorescence emission from chlorophyll possibly could interfere with the detection of DsRed in plants. To partly overcome this problem, the localization of proteins expressed from pGDR was examined in leaf epidermal cells because these cells lack chloroplasts and hence the majority of the autofluorescence arises indirectly from mesophyll cells beneath the epidermal layer. In addition, the Rhodamine-X (Chroma, Brattleboro, VT, USA) filter sets described below in the Methods section provided a substantial reduction in the chloroplast interference over other available filters (data not shown). A comparison of Figure 4a-f with those of the controls shown in panels Figure 4g,h shows that fluorescence from the DsRed protein and the DsRed fusion proteins could be easily detected in epidermal cells without chlorophyll interference. In particular, the fluorescence patterns verified that the localization of the DsRed protein is not affected by co-expression with SYNV P or by co-expression of native P and N expressed from pGD (Figure 4a,b). However, expression of the DsRed:P protein resulted in nuclear accumulation of DsRed fluorescence and a corresponding marked reduction in the amount of cytoplasmic signal (Figure 3c). In contrast, and as predicted from Goodin *et al.* (2001), co-expression of DsRed:P with native N protein virtually eliminated the cytoplasmic signal and shifted the fluorescence into an intense subnuclear locale (Figure 4d). The localization patterns of DsRed:P or DsRed:P/N in nuclei of cells expressing DsRed:P or DsRed:P/N could be clearly distinguished in the absence (Figure 4e) and in the presence (Figure 4f) of the native N protein expressed from pGD (Figure 4e,f). The negative controls (Figure 4g,h) indicate that the background fluorescence is negligible. These results thus show that with appropriate filters, GFP and DsRed can be clearly distinguished by epifluorescence microscopy of epidermal cells and that pGD, pGDG and pGDR provide valuable tools that can be used in conjunction with epifluorescence microscopy to evaluate localization patterns of the DsRed fusion proteins.

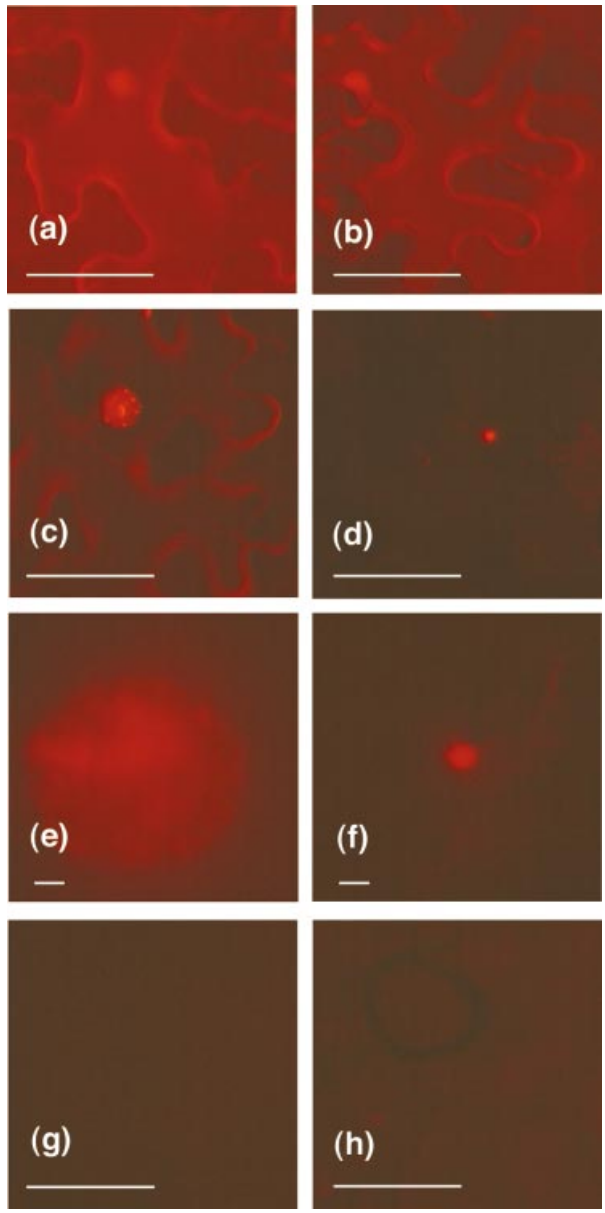


Figure 4. Epifluorescence micrographs of agroinoculated leaf cells expressing DsRed fusions and SYNV proteins.
Long and short scale bars represent 50 and 2 μm, respectively. Panels (a)-(h) show (a) Co-expression of DsRed and P; (b) Co-expression of DsRed, N and P; (c) Expression of the DsRed:P fusion; (d) Co-expression of DsRed:P and N; (e) DsRed:P expression from nuclei. (f) Nucleus of a cell in which DsRed:P and N was co-expressed; (g) pGD control examined with Rhodamine-X filter. (h) GFP expressing cells examined with Rhodamine-X filter.

Discussion

We have constructed and tested the utility of a matched set of binary vectors created to express native proteins, GFP- or DsRed-fusions in plant cells following agroinfiltration. The multiple cloning site incorporated into the vectors permits parallel subcloning of the genes under investigation into all three vectors. Using four proteins encoded by the plant nucleorhabdovirus *Sonchus yellow net virus*, we have conducted extensive expression experiments to determine the practicality of using DsRed as a vital marker alone or in conjunction with GFP in co-localization studies.

These experiments have allowed us to overcome a number of potential drawbacks to the use of DsRed as a vital marker in plants. First, chlorophyll emits autofluores-

cence between 660 nm and 800 nm (Strasser and Buttler, 1977) and thus may interfere with the visualization of a red fluorescent protein. However, this does not pose a problem in confocal microscopy and the fusions also are useful for epifluorescence microscopy, particularly in epidermal cells where autofluorescence interference with Ds-Red fluorescence can be reduced to minimal levels with the use of appropriate filter sets (Dietrich and Mais, 2002). Second, the native form of DsRed has the capacity to form oligomers that might affect the correct localization of fusion proteins (Baird *et al.*, 2000). Since the native DsRed is prone to aggregation and hence might result in anomalies in the fusion protein, a mutant form of DsRed, called DsRed2, that has a substantially reduced propensity to oligomerize relative to the wild-type protein was used as the autofluroscence protein fusion partner. Third, DsRed is slow to form the mature protein conformation (Baird *et al.*, 2000), and the immature form has an emission maximum similar to that of GFP that might interfere with co-localization experiments conducted with GFP. However, our experiments indicated that emission from the immature form of DsRed2 is negligible relative to that of GFP after two or more days post agroinfiltration. Although the accumulation of DsRed was sensitive to the fusion partner, similar sensitivities are also expected to arise with various GFP derivative fusions, depending on the fusion partner. In the case of the GFP and DsRed fusions to the P protein very similar patterns of localization were observed although there was a propensity of DsRed:P to form aggregates that appeared to reside within or near the periphery of nuclei of transfected cells. A more substantial difference in fusion protein expression was noted when the M protein was used as a fusion partner. Although bright GFP:M fluorescence expressed from the pGDG vector emanated from the entire nucleus, we were unable to detect DsRed:M fusions from the pGDR vector. This differential result may reflect a synergistic consequence of the tendency of both DsRed and the M protein to aggregate. We have reported previously that the SYNV P protein can form homo-oligomers (Goodin *et al.*, 2001), and this may partly account for the unusual peripheral nuclear localization patterns observed with the DsRed:P fusions. Although the levels of accumulation of GFP:M and DsRed:M have not been compared directly using Western blotting, the hydrophobic matrix protein is expected to have a strong tendency to aggregate, and therefore DsRed:M fusions may fail to mature into stable forms exhibiting intense DsRed fluorescence. Given these results, it is evident that the physicochemical properties of the fusion partner can affect the detection of some DsRed fusions, and one might also anticipate similar results with a variety of GFP fusion interactions. In any case, the identical multiple cloning sites incorporated into pGDR and pGDG provides an opportunity to construct both GFP and DsRed fusion derivatives for comparisons in expression experiments. Once compatible fusions are found, the pGD vector series provides a powerful system for co-expression and co-localizaton studies.

In order to determine the utility of DsRed in co-expression with GFP, we wanted to determine whether the overlapping and emission maxima of GFP and the immature form of DsRed (Gross *et al.*, 2000) would interfere in co-localization experiments. The results of experiments in which DsRed:P/N was co-expressed with the *Arabidopsis* nucleolar marker Fib1:GFP revealed the presence of two distinct subnuclear foci corresponding to the foci formed when Fib1:GFP and DsRed:P/N, respectively, were expressed alone. Moreover, examination of the DsRed signal in the green channel revealed essentially no green fluorescence in the area occupied by DsRed. These results therefore provide confidence that in these autofluorescent protein examples, the immature form of DsRed does not accumulate to an abundance sufficient to interfere with GFP visualization.

Although laser-scanning microscopy provides clearly superior images, epifluorescence microscopy also was useful for determining the subcellular localization of the autofluorescence protein fusions when appropriate filter sets were used to evaluate epidermal cells. Under these conditions, epifluorescence microscopy can be used to provide high resolution analysis of the subcellular distribution of DsRed without excessive interference from chlorophyll autofluorescence. From these results, the use of the pGD vector series provides a battery of new reagents that extends our ability to evaluate localization of proteins in plant leaf cells.

Experimental procedures

Plant material, growth conditions and agroinfiltration procedures

N. benthamiana plants grown in the greenhouse under conditions described by Martins *et al.* (1998) were used for all agroinfiltration experiments. Leaves were infiltrated with *Agrobacterium tumefaciens* strain C58C1 essentially as described by Bendahmane *et al.* (2000) with the exception that bacterial suspensions for infiltration were derived from fresh (1- to 2-day-old) cultures grown on Petri plates containing LB media amended with appropriate antibiotics. Stain C58C1 was maintained on LB agar containing rifampicin (100 μg ml^{-1}) and tetracycline (5 μg ml^{-1}). This medium was modified by adding kanamycin to a final concentration of 100 μg ml^{-1} in order to maintain the transformed C58C1 agrobacteria carrying the binary pGD vectors. Plasmids were transformed into C58C1 using the freeze-thaw method of An *et al.* (1988).

For 'agroinfiltration', suspensions of transformed C58C1 bacteria were adjusted to an OD_{600} of 0.6 in MES buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6), and acetosyringone was added to a final concentration of 150 μM. Bacterial suspensions were then maintained at room temperature for 2–3 h. For co-infiltration of different *Agrobacterium* transformants, equal volumes of each

culture suspension were mixed prior to infiltration. Infiltrations were conducted by gently appressing a 1-ml disposable syringe to the abaxial surface of fully expanded leaves that were approximately 2.5 cm wide at the midleaf and slowly depressing the plunger. A sufficient amount of bacterial suspension was used to completely infiltrate the leaves and give a water-soaked appearance. This typically required 1–4 infiltration sites per leaf. Following agroinfiltration, plants were maintained in the laboratory under continuous fluorescent lighting for at least 24 h. Plants kept for longer periods were maintained in a growth chamber at 22°C with a 16 h/8 h. light/dark photoperiod. Leaves were examined by microscopy between 40 h and 90 h post-infiltration.

Cloning of polymerase chain reaction products

Polymerase chain reactions (PCR) were used to introduce restriction sites into full-length clones of SYNV genes to facilitate directional cloning into the pGD vectors. PCR was performed using the high-fidelity DynazymeEXT polymerase (Finnzymes, Finland). Existing plasmids containing SYNV gene inserts known to have the correct nucleotide sequence were used as templates for PCR. PCR products were cloned directly from reaction mixtures using topoisomerase-mediated cloning into the cloning vector pTOPOII (Invitrogen, Carlsbad, CA, USA). Following verification by DNA sequencing, full-length clones of the SYNV *N*, *P*, *sc4* and *M* genes were inserted into the pGD derivatives as indicated below.

Vector construction

All binary vectors used in this study were derived from pCAMBIA1301 (GenBank accession AF234297), which is based upon the pPZP vectors described by Hajdukiewicz *et al*. (1994). Genes encoding the autofluorescent proteins DsRed2 and GFP were obtained from plasmids pDsRed2-C1 and pRSGFP-C1, respectively (Clontech, Palo Alto, CA, USA). All vector sequence from pCAMBIA1301 that was extraneous for transient expression needs was removed. Digestion with *Xho*I and *Eco*RI eliminated the double 35S promoter and the hygromycin resistance gene. The resulting intermediate, pCAMBIAΔ*Xho*I/*Eco*RI, was then digested with *Hin*dIII and *Sna*BI to remove a second 35S promoter and most of the associated GUS reporter gene, to yield pCAMBIAΔ2. A single 35S promoter excised from pBS316 (Goodin *et al.*, 2001) as a *Sac*I/*Bgl*II fragment was subsequently ligated into pCAMBIAΔ2 to create pCAMBIAΔ2 : 35S. DsRed2 was excised as a *Nhe*I/*Hpa*I fragment from pDsRed2-C1 and ligated into pCAMBIAΔ2 : 35S that had been digested with *Xba*I and *Pml*I, to yield the pGDR plasmid for DsRed expression. Similarly, a partial digest of pRSGFP-C1 with *Nhe*I/*Hpa*I yielded a GFP-containing fragment that was ligated into *Xba*I/*Pml*I digested pCAMBIAΔ2 : 35S to yield pGDG for GFP fusions. To create an expression vector with a multiple cloning site matching that of pGDR and pGDG, a triple ligation was conducted by digesting pCAMBIAΔ2 : 35S with *Sac*I/*Pml*I, and ligating this with a *Bgl*II/*Hpa*I fragment from pDsRed2-C1 and a 35S-containing *Sac*I/*Bgl*II fragment from pBS316 to yield pGD. All sequences in pGD, pGDR and pGDG that are relevant to *Agrobacterium*-mediated transient expression in plants are shown in Figure 1.

The *Arabidopsis thaliana* Fib1:GFP nucleolar marker used in this study was excised as a *Pst*I-35S-Fib1:GFP-*Pst*I fragment from pPK100-Fib1:GFP (Barneche *et al.*, 2000) and ligated into *Pst*I-digested pCAMBIAΔ2.

Immunoblot analysis of proteins expressed in agroinfiltrated leaves

Three 9 mm diameter disks from agroinfiltrated leaves were ground in 100 μl of extraction buffer, boiled for 5 min and quenched on ice. Proteins from these crude extracts were separated by electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulphate (Laemmli, 1970), and electroblotted to nitrocellulose membranes. The membranes were blocked with TBS containing 5% skimmed milk powder and 2% bovine serum albumin and reacted with polyclonal antisera against the P protein (Heaton *et al.*, 1987), or with a monoclonal antibody specific for GFP (Clontech). Bound alkaline phosphatase antirabbit (Sigma, St Louis, MO, USA) or antimouse (Bio-Rad, Richmond, CA, USA) conjugate was detected using X-omat X-ray film (Kodak, Rochester, NY, USA) following incubation with CDP-Star chemiluminescent substrate containing Nitro-Block II (Tropix Applied Biosystems, Bedford, MA, USA).

Laser-scanning confocal microscopy

Confocal microscopy was performed using a Zeiss LSM 510 confocal laser scanning microscope equipped with helium/neon lasers and multitracking. GFP was excited at 488 nm, and the resulting fluorescence filtered through a primary dichroic (UV/488/543/633), 570 nm secondary dichroic, and BP505-550 nm emission filters to the photomultiplier tube (PMT) detector. DsRed was excited at 543 nm, and the emission was passed through the same primary and secondary dichroic mirrors, and through a LP570 nm emission filter to the PMT detector. Images were captured using Zeiss's LSM 510 software, converted to TIFF for export, and processed in Adobe Photoshop 5.0.

Epifluorescence microscopy

Epifluorescence microscopy was conducted using a Zeiss Axiophot microscope. A Rhodamine-X (Chroma) filter set that consisted of HQ 570/20X excitation, Q 585 LP dichroic and HQ 620/60 nm emission filters, was used to view epidermal cells of water mounted leaf pieces at approximately 40 h post-infiltration. Images were captured using NIH image as modified by Scion and renamed Scion Image 1.60 for CG-7. All subsequent image manipulations and figure preparations were carried out in Photoshop 5.0 (Adobe Systems Incorporated, San Jose, CA, USA) and Canvas 7 (Deneba Software, Miami FL, USA).

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