Visualization of intracellular structures using fluorescent proteins in tobacco BY-2 cells

We have established several intracellular structure-visualized cell lines of tobacco BY-2 using fluorescent proteins. GFP-tubulin, GFP-AtVAM3, GFP-ABD2 and histone H2B-RFP visualized microtubules, vacuolar membrane, actin microfilaments and chromosomes/ cell nuclei, respectively (Kumagai et al. 2001, Kutsuna and Hasezawa 2002, Sano et al. 2005). In this protocol, we will introduce the procedure used to establish these cell lines.

I. Construction of fusion genes

Plasmid construction was carried out in a modified pUC18 vector which contained the CaMV 35S promoter and the nopaline synthase terminator sequence from pBI101. The constructed region was subsequently subcloned into a binary vector. Molecular cloning was carried out by the standard method and only the sequence information is shown below.

I-1. Fluorescent proteins

GFP: sGFP(S65T), Niwa et al. 1999
RFP: tandem-dimer Tomato (tdTomato), Shaner et al. 2004, R Tsien
http://www.tsienlab.ucsd.edu/

Although we used sGFP(S65T) as GFP for every construct, EGFP (BD Biosciences Clontech, http://www.clontech.com/) can be used as well.

Because the commercially available DsReds of DsRed (now off the market), DsRed2 and DsRed-Express form tetramer that often aggregates in living cells when fused with interested proteins, monomeric RFPs are recommended (Campbell et al. 2001). Thanks to the energetic efforts of R. Tsien's lab in California, monomeric forms of RFP were developed and improved. The first, mRFP1, was improved from the DsRed (Campbell et al. 2001). Although we succeeded in visualizing some of the intracellular structures of microtubules and actin microfilaments in tobacco BY-2 cells, because of the low fluorescent quantum yield of 0.25 they were unsuitable for time-sequential observations over a long period or for obtaining images with a very short exposure time. Thanks to the improvement of the monomeric RFPs, monomeric red, orange and yellow fluorescent proteins have been developed (Shaner et al. 2004). We used tandem-dimer Tomato (tdTomato), whichforms a dimmer intra-molecularly, and succeeded in

visualizing chromosomes in tobacco BY-2 cells by preparing fusion proteins with histone H2B (manuscript submitted). However, with some proteins, such as membrane proteins, the fusion proteins with tdTomato did not show proper intracellular localization, possibly because of problems with the three-dimensional structures of the fusion proteins or because of the intra-molecular dimerization of tdTomato.

I-2. Intracellular proteins

Tobacco α-tubulin: Accession number AB052822 (Kumagai et al. 2001)
Arabidopsis AtVam3: Accession number TU88045 (Sato et al. 1997)
Arabidopsis AtFim1: Accession number U66424 (McCurdy and Kim et al. 1998)
Tobacco histone H2B: clone number: 35066r1 in the TAB (Transcriptome Analysis of BY-2) database (http://mrg.psc.riken.jp/strc/index.htm).

I-3. Choice of N- or C-terminus tags and linkers

Tobacco α-tubulin, AtVAM3 and the actin binding domain (ABD) of AtFim1 visualized the intracellular structures with N-terminus tags of fluorescent proteins (ex. GFP-tubulin). In contrast, tobacco histone H2B visualized chromosomes with a C-terminus tag of the fluorescent protein (histone H2B-RFP). In the reported literature, membrane proteins often worked with C-terminus fluorescent tags.

Between the fluorescent protein and the protein of interest, some liker sequence of several amino acids is often required to keep the three-dimensional conformation of both the fluorescent protein and the protein of interest. We inserted two and eleven amino acids of GA (glycine-alanine) or GAGAGAGAGAG to prepare GFP-tubulin and GFP-ABD2, respectively.

I-4. Binary vectors

pBI series (pBI101, pBI121): Clontech (out of the market)
pCAMBIA series: CAMBIA (http://www.cambia.org/daisy/cambia/home.html)
pGWB series: Nakagawa et al. (unpublished,
http://bio2.ipc.shimane-u.ac.jp/nakagawalab.htm)

pCAMBIA vectors were developed to overcome the difficulties of handling the existing binary vectors. pCAMBIA vectors are relatively small (about 9 kbp), have a high copy number in *E.coli* and several selectable markers for bacteria and plants.

pGWB vectors are designed to enable gene fusion by using Gateway cloning technology and cloning efficiency has been dramatically improved (Nakagawa et al.

unpublished result). We believe the pGWB vectors in combination with the Gateway technology are the most efficient available at this moment, though there are still some points to be attended to. The pGWB vectors provided 14 (pGWB5) or 9 amino acids (pGWB6) between the GFP and proteins of interest by recombination and the relatively long linker sequences may sometimes affect GFP fluorescence. Since the original pGWB vectors have two selective markers against kanamycin and hygromycin for plants, dual transformation will be difficult when the pGWB vectors are chosen for the first transformation. Although we have not yet tried them, new pGWB vectors have been developed to overcome these disadvantages (Nakagawa et al. in press).

II. Transformation into BY-2 cells

The fusion protein-construct in a binary vector was transformed into *Agrobacterium tumefaciens* strain, LBA4404. A 5-ml aliquot of 2-d old BY-2 cells was incubated with 100 μl of an overnight culture of the transformed *A. tumefaciens*. After 2-d incubation at 27°C, the BY-2 cells were washed 4 times in a 10 ml liquid medium and then plated onto solid medium containing 30 to 50 mg liter^{-1} of kanmycin or hygromycin to select transformants and 250 to 500 mg liter^{-1} carbenicillin or claforan to remove bacteria. Calluses, which appeared after 3 to 4 weeks (2-5 mm in diameter), were transferred onto new solid plates and cultured independently until they reached approximately 1 cm in diameter. To prepare liquid cultures, pore 0.1 to 1 g callus (about 0.5 to 1 cm in diameter) in a small amount of fresh liquid medium (5-10 ml);when they're fully grown , add fresh medium (10-20 ml). The repetition of this cycle increases the culture volume. After 2-3 days, transfer the calluses into liquid medium.Gentle pipetting of the calluses will accelerate their growth.

To dual-visualize intracellular structures, two binary-vector constructs were sequentially transformed into tobacco BY-2 cells. For example, to dual-visualize microtubules and chromosomes, the binary-vector construct of histone H2B-RFP was transformed into the established BY-2 cell line that visualized microtubules with GFP-tubulin.

References

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