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# Fluorescent protein marker lines in maize: generation and applications

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**ABSTRACT Fluorescent proteins (FP) have significantly impacted the way that we study plants in the past two decades. In the post-genomics era, these FP tools are in higher demand by plant scientists for studying the dynamics of protein localization, function, and interactions, and to translate sequence information to biological knowledge that can benefit humans. Although FP tools have been widely used in the model plant *Arabidopsis*, few FP resources have been developed for maize, one of the most important food crops worldwide, and an ideal species for genetic and developmental biology research. In an effort to provide the maize and cereals research communities with a comprehensive set of FP resources for different purposes of study, we generated more than 100 stable transformed maize FP marker lines, which mark most compartments in maize cells with different FPs. Additionally, we are generating driver and reporter lines, based on the principle of the pOp-LhG4 transactivation system, allowing specific expression or mis-expression of any gene of interest to precisely study protein functions. These marker lines can be used not only for static protein localization studies, but will be useful for studying protein dynamics and interactions using kinetic microscopy methods, such as fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), and fluorescence resonance energy transfer (FRET). All of the constructs and maize marker lines are publicly available through our website, http://maize.jcvi.org/cellgenomics/index.php**

KEY WORDS: *fluorescent protein, FRET, maize, pOp, LhG4*

## Introduction

Maize, one of the most important crops worldwide, has contributed unparalleled value to genetic research. Advances in maize genomics, including a sequenced genome, have further facilitated maize research in recent years. However, a continuing challenge is to convert a sequence catalog into the knowledge required to improve maize as an important crop for human benefit. One of the most effective approaches to achieve this goal is to understand the spatial and temporal complexity of biological pathways, requiring a knowledge of the dynamics of protein localization, functions and interactions. However, studies in these research areas in maize have not advanced as rapidly as in other systems, in part because of the lack of appropriate tools. Traditionally, static visualization procedures, such as immunolocalization and *in situ* hybridization have been applied on a small scale (Jackson, 2002). As the capacity and quality of maize transformation has improved, we are now able to use fluorescent proteins (FPs) as an invaluable tool to study the dynamics of protein expression, thus bridging the gap between the genome sequence and protein function.

FPs have significantly impacted biological research over the past decades, since the first FP, *Aequorea victoria* green fluorescent protein (AvGFP), was discovered in the early 1960s as a companion protein to aequorin from jellyfish (Rizzo *et al.*, 2009, Shimomura *et al.*, 1962). The cDNA of GFP was cloned several decades later in 1992; however, the potential of GFP as a molecular probe in virtually any species was not fully recognized until 1994, when it was demonstrated that GFP driven by a neuron specific promoter in *Caenorhabditis elegans* marked the site of gene expression (Chalfie *et al.*, 1994, Rizzo *et al.*, 2009). GFP has thereafter been

*Abbreviations used in this paper:* FACS, fluorescence activated cell sorting; FCS, fluorescence correlation spectroscopy; FCCS, fluorescence cross-correlation spectroscopy; FLIM, fluorescence-lifetime imaging microscopy; FLIP, fluorescence loss in photobleaching; FP, fluorescent protein; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; FT, flowering locus T; GT1, grassy tillers 1; SBT, spectral bleed through; SPB, squamosa promoter-binding; TSH1, tassel sheath 1; TU1, tunicate 1.

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engineered to produce a vast number of spectrally distinct mutants with improved folding and stability properties. Now, with the identification of FPs from other species, scientists can choose from a broad range of FP variants that spans nearly the entire visible spectrum for many applications (Rizzo *et al.*, 2009).

The use of FPs has revolutionized plant biology just as it has other fields, but plant research faced several specific challenges to use them effectively (Jones *et al.*, 2012). Initially, wild-type GFP expression was problematic in plant cells (Hu and Cheng, 1995, Niedz *et al.*, 1995, Sheen *et al.*, 1995). Haseloff and colleagues reported that the expression of GFP in plant cells was hampered by aberrant splicing of wild-type *gfp* mRNA, due to a cryptic intron between nucleotides 380 and 463 (Haseloff *et al.*, 1997). Thereafter, an optimized GFP variant for plant cells, mGFP5 was produced and widely applied in plant research (Harper *et al.*, 1999, Haseloff *et al.*, 1997). Another challenge for plant applications was the brightness and color of FPs. For example, chlorophyll and stress-induced phenolics autofluoresce are at the same wavelength as many FPs, requiring higher signal intensity or spectral variants (Jones *et al.*, 2012).

In an effort to provide the plant community with a set of common sub-cellular compartment markers with different colors and improved brightness, we have generated a set of maize marker lines containing different proteins with a wide range of codon optimized FPs. In this review, we describe the methods used to generate these stable maize marker lines, and discuss how the lines can help us pursue basic questions in plant biology concerning the dynamic spatial and temporal control of gene expression at a genome-wide level.

## Generation of fluorescent protein marker lines

### *Generation of protein tagged lines*

We developed a pipeline to generate fusion proteins with different color variants of FPs in the context of each gene's native regulatory elements (Mohanty *et al.*, 2009). All the FP variants are tested to ensure proper expression in maize cells, and some have been maize codon optimized. Table 1 lists the properties of these optimized FP variants used in our maize marker lines. Among the list, the most common FP we used to tag proteins is Citrine FP, a YFP variant which is brighter and more resistant to photobleaching, acidic pH, and other environmental effects than EYFP (Griesbeck *et al.*, 2001). We incorporate significant upstream sequences, including the promoter and associated regulatory regions, introns, and 3' sequences for each FP tagged gene, to promote native expression level and localization in a tissue and developmentally-specific manner. The Multisite Gateway Pro (Invitrogen, Carlsbad, CA, USA) system is used to generate full genomic sequence with the FP insert, which is flanked by linker peptides to minimize folding interference between the FP and tagged protein. The FPs are either fused as N-terminal, C-terminal, or internal tags, according to protein functional domain analysis, to ensure the fusion proteins retain their native functions. After confirming the constructs by sequencing, *Agrobacterium*-mediated transformation of maize is performed at the Iowa State University Plant Transformation Facility using *HiII* embryos (Armstrong *et al.*, 1991).

TABLE 1

**PROPERTIES OF THE FLUORESCENT PROTEIN VARIANTS USED IN MAIZE MARKER LINES**

| Color | FPs | Excitation max. (nm) | Emission max. (nm) | Brightness* | References |
|---|---|---|---|---|---|
| Blue | TagBFP | 402 | 457 | 32.8 | Subach *et al.*, 2008 |
| Cyan | ECFP | 433 | 475 | 13 | Tsien, 1998 |
| | Cerulean | 433 | 475 | 26.7 | Rizzo *et al.*, 2004 |
| | mTFP1 | 462 | 492 | 54.0 | Ai *et al.*, 2006 |
| Green | EGFP | 488 | 507 | 33.6 | Cormack *et al.*, 1996 |
| Yellow | Citrine YFP | 516 | 529 | 58.5 | Griesbeck *et al.*, 2001 |
| Orange | TdTomato | 554 | 581 | 95.2 | Shaner *et al.*, 2004 |
| | TagRFP-T | 555 | 584 | 33.2 | Shaner *et al.*, 2008 |
| Red | mRFP1 | 584 | 607 | 6.27 | Campbell *et al.*, 2002 |
| | mCherry | 587 | 610 | 15.8 | Shaner *et al.*, 2004 |

*Brightness is the product of extinction coefficient and quantum yield, divided by 1000.

### *Generation of promoter marker lines*

Our work with protein fusions in a genomic construct allowed us to learn about the complexity of maize promoters. The maize genome is large, often with nested transposable element arrays between genes, and it was not clear whether the 2 or 3 kb of sequence immediately upstream of the coding region would be sufficient to drive the correct expression pattern. We have tested this for a number of genes, and determined in several cases a 2 or 3 kb region is sufficient. We routinely take all of the 5' sequence up to the first retrotransposon, because PCR amplification within a transposon repeat is usually not feasible. Examples of promoters driving tissue or cell specific expression are shown in Fig. 1.

### *Generation of pOp-LhG4 transactivation marker lines*

The pOp-LhG4 transactivation system has been used for cellular and developmental studies in different species such as *Arabidopsis*, tobacco and tomato, and has also been used in maize (Craft *et al.*, 2005, Fernandez *et al.*, 2009, Rutherford *et al.*, 2005, Segal *et al.*, 2003). The pOp-LhG4 system includes 1) a chimeric promoter, pOp, that consists of *lac* operators cloned upstream of a minimal CaMV promoter and 2) a transcription activator, LhG4, which is a fusion between a high-affinity DNA-binding mutant of lac repressor, $LacI^{His17}$, and transcription-activation-domain-II of GAL4 from *Saccaromyces cerevisiae* (Samalova *et al.*, 2005). The pOp promoter is not activated in the reporter lines until crossed with activator lines that express LhG4 driven by appropriate tissue-specific promoters (Samalova *et al.*, 2005). Thus, the pOp-LhG4 transactivation system allows specific expression or mis-expression of any gene of interest. Using our experience with maize promoters described above, we are now developing such tools in maize to allow precise experimental intervention to study gene function (Fernandez *et al.*, 2009, Gardner *et al.*, 2009, Goll *et al.*, 2009, Jones, 2009, Moore *et al.*, 2006).

We are generating cell, tissue or developmental-stage specific promoter transactivation lines based in part on sequence information learned from our translational fusions. Each selected promoter is amplified and Gateway cloned into a derivative of our standard maize binary vector, upstream of the LhG4 gene. The same backbone vector includes a pOp reporter to allow for screening of driver constructs in the T0 generation without the need to cross to reporter lines, which is time consuming. The pOp sequences can drive expression bi-directionally (Fig. 2), and can therefore transactivate two reporters simultaneously. We took advantage of this by making a GUS<< pOp >>NLS-TagRFP-T (Shaner *et al.*, 2008) reporter (Fig. 2). "NLS" refers to tandem nuclear localiza-

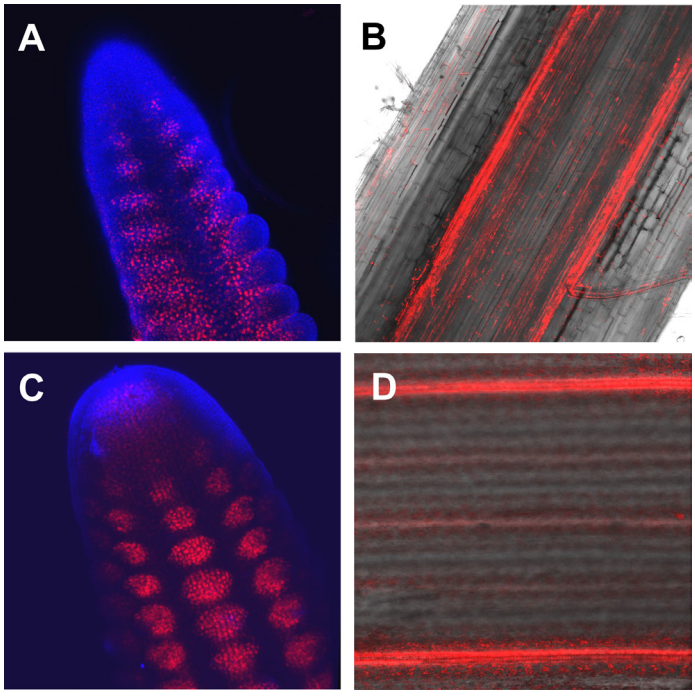


**Fig. 1. Images show tissue specific expressions of some maize promoters.** *Expression of pRAMOSA3::NLS-TagRFP-T at the base of spikelet meristems* **(A)**, *pSUCROSE TRANSPORTER 1 (SUT1)::RFP in root vascular tissue* **(B)**, *pWUSCHEL::NLS-TagRFP-T in spikelet meristems* **(C)**, *and pSUCROSE TRANSPORTER 1 (SUT1)::RFP in leaf vascular tissue* **(D)**.

tion sequences that were added to ensure cell autonomy of the TagRFP-T protein (Goldshmidt *et al.*, 2008). The two reporters will be inherited in *cis*, so that either GUS or NLS-TagRFP-T expression can be used to verify driver specificity. GUS staining can be used for imaging in tissues that are highly auto-fluorescent (eg. mature tissues), whereas NLS-TagRFP-T can be used for non-invasive imaging of living tissues. Use of TagRFP-T further distinguishes chlorophyll auto-fluorescence, because its emission peak of ~620 nm (Shaner *et al.*, 2008) will facilitate separation from chlorophyll autofluorescence, at ~650-680nm (Billinton and Knight, 2001). Another advantage of using TagRFP-T is the potential to distinguish it from our lines already tagged with mRFP1 or mCherry (Shaner *et al.*, 2005). Even though the driver lines always have the TagRFP-T reporter, users will still be able to observe expression of a distinct FP fusion in the reporter lines using a contrasting color FP tag.

Besides the driver lines, we are also generating a set of reporter constructs for transactivaton, driven by a multimeric pOp sequence. These reporters will enhance the utility of the promoter driver lines by allowing us to confirm their specificity. For example, we generated a maize pOp:: *ZCN8*, a *FLOWERING LOCUS T (FT)-LIKE* gene in maize (Meng *et al.*, 2011), to permit flexible control of the expression of ZCN8 and studying the maize flowering process. Similarly we are generating a responder for the *LIGULELESS1* gene that encodes a novel protein with a domain similar to a SQUAMOSA PROMOTER-BINDING (SPB) protein (Moreno *et al.*, 1997) and a pOp line that drives an auxin biosynthesis gene. More promoter and driver lines are being constructed for diverse uses, including complementation tests of mutants to test for cell autonomy, tissue specific knockouts for genes that are lethal as nulls, cell/ tissue ablation, and fluorescence activated cell sorting (Birnbaum *et al.*, 2005). All the constructs, seed stocks and images are publicaly avaible through our website http://maize.jcvi.org/cellgenomics/index.php.

## Application of fluorescent protein marker lines

### *Application of fluorescent protein marker lines in protein localization studies*

FPs have become an indispensable tool for studying protein localization in living organisms, tissues, and cells because their ability to be cloned into any open reading frame enables real time *in vivo* imaging of tissue specificity, sub-cellular localization and dynamics. In the past, using FPs to study protein localization relied on transient expression in protoplasts, in *N. benthamiana* leaves or onion epidermal cells, due to the laborious nature of stable transformation. However, the physiological and developmental condition of cells used in transient assays in other organisms can differ significantly from their native cells or tissues in the whole plant, thus potentially influencing protein localization and dynamics and heterologous expression reduces the reliability of the results. To advance use of our FP resource, we developed a robust transient expression system in maize that reduces complications from heterologous expression in these systems (Kirienko *et al.*, 2012). A practical advantage of maize transient expression for FP studies is the ability to test constructs prior to stable transformation. Further, co-bombardment and use of transient assays in stable lines using contrasting color markers, and experimental studies significantly advance the FP resource.

The transient assay system in maize expands the uses of our

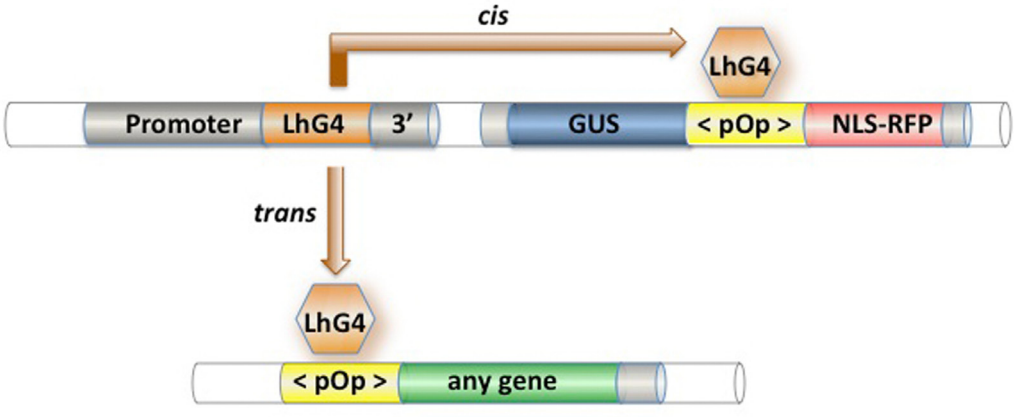


**Fig. 2. pOp-LhG4 transactivation system in maize.** *A tissue specific promoter drives expression of the LhG4 transcription factor, which binds to pOp sequences and activates expression of GUS and NLS-tagRFP-T in the same cells. This line can also be crossed to any other pOp reporter line, to activate any gene* in trans.

FP lines; however stable transformation remains the gold standard for studying protein function *in vivo*. As an example, Bommert *et al.* (2013) showed that a functional YFP tagged maize G alpha protein is highly expressed in shoot meristems, and using the YFP tag for immunoprecipitation could demonstrate an interaction between G alpha and the CLAVATA LRR receptor FASCIATED EAR2. In a second example, by tagging a YFP to the C-terminus of the protein, Whipple *et al.,* (2011) showed that GRASSY TILLERS1 (GT1), a class I HD-Zip that controls lateral branching in maize, localizes to the nucleus and expresses in the leaves of axillary buds (Whipple *et al.*, 2011). In addition, using RFP and YFP tags, Han *et al.,* (2012) showed nuclear localization of TUNICATE1 (TU1), a MADS box transcription factor, in leaf primordia. In the dominant *Tu1* mutant, expression was also observed at the base of spikelet pair meristems, which helps explain the dominant phenotype. In fact, use of the FP fusions was instrumental in proving that the candidate MADS box gene was responsible for the *Tu1* mutation (Han *et al.*, 2012). In another example, Whipple *et al.,* (2010) showed that a YFP-tagged TASSEL SHEATH 1 (TSH1), which is a zinc-finger protein controlling maize bract growth, displays the strongest fluorescence in the basal-most bracts subtending tassel branch promordia, matching the localization pattern of the *Tsh1* transcript (Whipple *et al.*, 2010). This result indicates that unlike some developmental transcription factors, the TSH1 protein probably does not traffic from cell to cell (Whipple *et al.*, 2010). In other examples, stably transformed maize lines expressing FP fusion proteins have also been used to study biosynthetic proteins (Christensen *et al.*, 2013, Falcone Ferreyra *et al.*, 2012) and proteins that regulate asymmetric cell divisions during leaf development (Humphries *et al.*, 2011, Zhang *et al.*, 2012). Transcriptional fusions have also been used to make hormone responsive reporters (Lee *et al.*, 2009). All of these studies suggest that FP reporters are powerful tools to study protein localization and tissue specificity, especially after being stably transformed into maize plants.

In order to provide the maize community with resources for protein localization and tissue specificity studies, we have generated a set of FP tagged marker lines which mark most compartments in maize cells with different FPs using the pipline described above. Fig. 3 shows several images from different compartment markers in maize. All the constructs and seeds are available by request through our website at http://maize.jcvi.org/cellgenomics/index.php.

### *Application of fluorescent protein marker lines in cell specific gene expression studies using fluorescence activated cell sorting (FACS)*

Cell-specific resolution of gene activity is critical to understand specific developmental events (Birnbaum *et al.*, 2005). However, such high resolution data have been difficult to obtain at a genomic level because specific types of cell need to be isolated (Birnbaum *et al.*, 2005). One of the most efficient ways to isolate specific cells of interest from neighboring cells in the same organ or tissue is FACS, which sorts FP-labeled protoplasted cells on the basis of their fluorescence (Afonso *et al.*, 1985). The isolated cells can be used for subsequent genomic analsis. The FACS-based genomic approach has been sucessfully applied for developmental research in *Arabidopsis*. For example, Benfey and colleagues have mapped gene expression profiles of specific root zones, corresponding to various cell types and tissues at different developmental stages, using a combination of FACS and microarray analysis (Birnbaum *et al.*, 2003). Further discussion about the application of FACS in plants can be found in the review by Carter *et al.,* in this issue (Carter *et al.*, 2013).

Although FACS has been succesfully used in *Arabidopsis*, a lack of tissue or cell specific promoters has minimized the use of FACS in maize. Until recently, there were only a few specific promoters defined in maize (Cao *et al.*, 2007, Sattarzadeh *et al.*, 2009, Srilunchang *et al.*, 2010, Zhang *et al.*, 2009). We generated a set of promoter-FP direct fusion marker lines, which can be used for FACS experiments. For example, we generated pWUSCHEL::NLS-RFP and pRAMOSA3::NLS-RFP lines, and these direct fusions will facilitate gene profiling of the maize shoot by marking different regions of the meristem. In addition, we are currently generating more LhG4 driver lines, which can also be useful for FACS applications.

### *Live cell imaging using fluorescent protein markers*

Live cell imaging is a powerful tool for observational and experimental studies of plant development in real time and in 3D. Such studies trace back historically to 3D reconstructions of fixed images at sequential time points, which were used to understand basic subcellular architecture in plants (Donohoe *et al.*, 2013, Staehelin, 1997). Live imaging of FP lines now permits experimental studies of whole organ growth, interactions among neighboring cells, behavior of individual cytoskeletal elements and the ability to trace movement of proteins between cells (Grossmann *et al.*, 2012, Gutierrez *et al.*, 2009, Krebs *et al.*, 2012, Lindeboom *et al.*, 2013, Mathur *et al.*, 2012, Sampathkumar *et al.*, 2011, Teh *et al.*, 2013). With the advent of microfluidic devices, FP markers can be used as biosensors for hormone induction studies during whole root or shoot growth. Computational modeling of plant growth responses has become more powerful and informative because simulations can be guided by *in vivo* observations, and then the modeled outcomes further tested *in vivo* for validation (Robinson *et al.*, 2011). In some cases, the FP provides an important cellular marker regardless of its specific function in the cell (Robinson *et al.*, 2011). In other cases, the FP tagged protein is studied functionally during live cell imaging (Cunha *et al.*, 2012). In one example, responses of microtubule arrays to deformation or damage of neighboring cells was studied *in vivo* by live cell imaging over time, and reconstructions showed reorientations of microtubule cortical arrays upon perturbation (Heisler *et al.*, 2010). For maize, visual FP tools will be a dynamic tool for cell biology studies through live cell imaging. Furthermore, study of single molecule function demands the use of live cell imaging techniques best accomplished using FPs, as described in the next section.

### *Application of fluorescent protein marker lines in protein dynamics studies*

In addition to live cell imaging of growth, FP technology enables an investigation of protein dynamics in living cells and single molecule studies. Several quantitative fluorescence spectro-microscopy approaches such as fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and Fluorescence correlation spectroscopy (FCS) have been used for protein dynamic studies in plants (Field *et al.*, 2010, Grossmann *et al.*, 2012, Harter *et al.*, 2012, Hoover *et al.*, 2010). Other techniques such as dual-color fluorescence cross-correlation spectroscopy (FCCS), and fluorescence resonance energy transfer (FRET) have

been used for protein-protein interaction studies.

FRAP is a powerful technique to quantify the average rate of protein translocation within living cells (Reddy *et al.*, 2007). In a FRAP experiment, fluorescently tagged proteins in an area of the cell are irreversibly photobleached with a high-intensity laser pulse. The movement of fluorescently tagged proteins from surrounding areas into the bleached area is recorded by time-lapse microscopy to estimate protein mobility. FRAP is useful for studying organelle dynamics, protein diffusion within membranes or organelles, or protein turnover in complexes (Fang *et al.*, 2004, Fricker *et al.*, 2006, Kwok and Hanson, 2004, Runions *et al.*, 2006). For example, the factors that influence protein traffic from the ER to Golgi apparatus have been studied using FRAP (Brandizzi *et al.*, 2002, DaSilva *et al.*, 2004, Yang *et al.*, 2005). In addition, Luu and colleagues recently used FRAP to estimate the cycling of aquaporin between intracellular compartments and the cell surface under salt stress conditions (Luu *et al.*, 2012). The results showed that the recovery of fluorescence of GFP tagged aquaporin increased 1.3-1.5 fold under salt stress as compared with the values for control, suggesting that salt treatment enhances the cycling of aquaporin (Luu *et al.*, 2012). All of these studies demonstrate that FRAP is a powerful tool to investigate protein dynamics. However, if the goal of the experiment is to determine total connectivity within an extended membrane system, such as the ER, a similar technique termed FLIP is generally chosen (Fricker *et al.*, 2006, Ward and Brandizzi, 2004). FLIP is performed by continuous photobleaching FPs in one area of the cell to drain signal from all connected compartments. By monitoring the fluorescence in the non-photobleached regions, the mobility and connectivity of a fluorescently tagged protein within the membrane can readily be observed (Lippincott-Schwartz and Patterson, 2003, Nehls *et al.*, 2000). FLIP successfully demonstrated protein movement from one plastid to another via long tubular extensions referred to as stromules (Hanson *et al.*, 2007).

FCS is another sensitive fluorescence technique used for the study of diffusion rate of fluorescent molecules, and can also determine if proteins are in a complex (Hink *et al.*, 2002). FCS measures fluorescent intensity fluctuations due to movement of single fluorescent molecules in and out of a small defined focal volume over short periods of time (Goedhart *et al.*, 2000). This technique has been routinely used to obtain information of a protein's diffusion coefficient, binding constant, and concentrations within a living cell by correlating the fluorescent fluctuations over time (Reddy *et al.*,

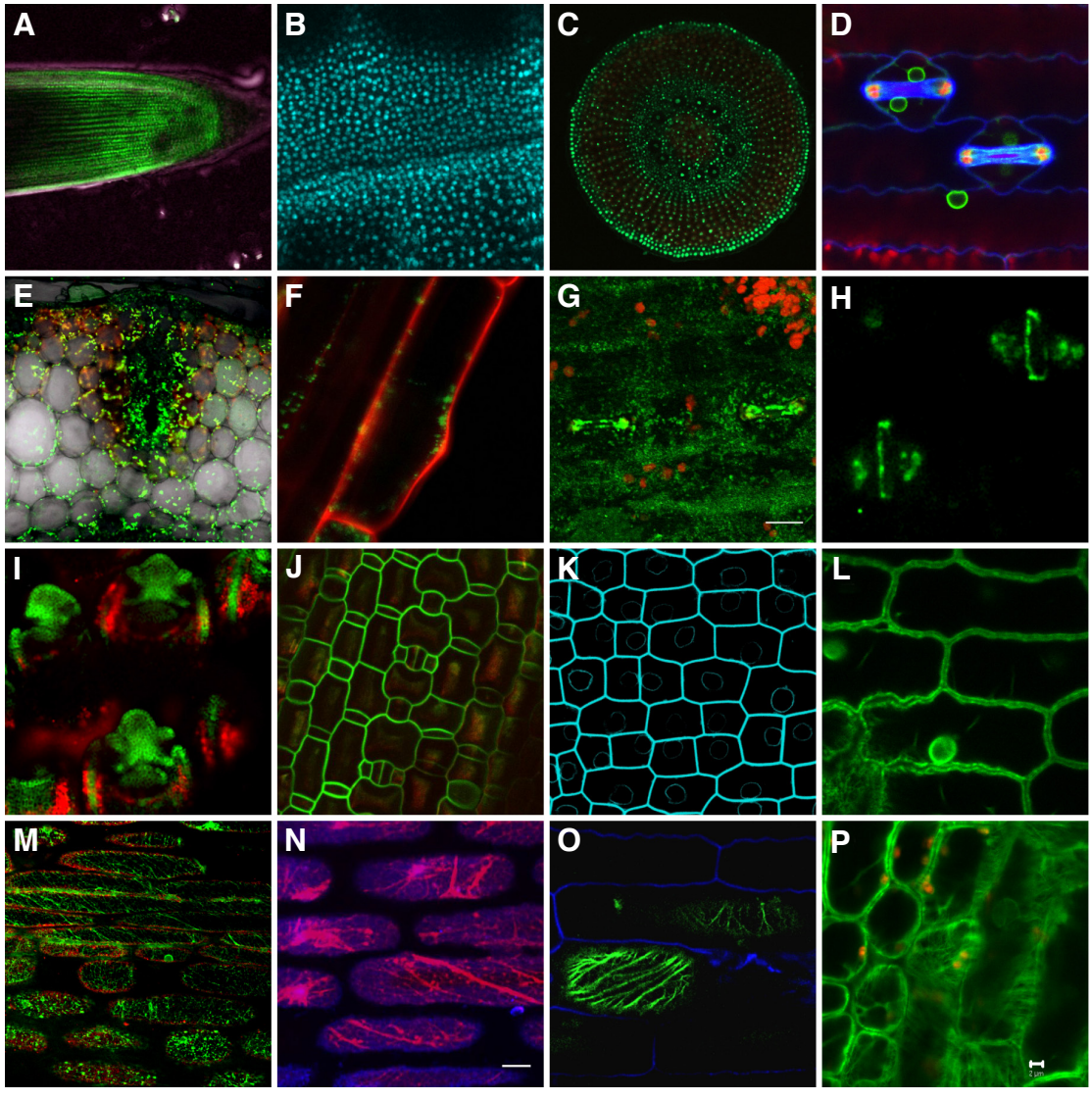


**Fig. 3. Subcellular localization of expressed FP-tagged maize proteins. (A)** *DECREASE IN DNA METHYLATION 1-YFP, nuclei, root.* **(B)** *LIGULELESS 2-mTFP, nuclei, leaf.* **(C)** *HISTONE H1-YFP, nuclei, root transverse section.* **(D)** *RANGAP-YFP, nuclear envelope, leaf epidermis.* **(E)** *MALATE DEHYDROGENASE-YFP, mesophyll chloroplasts, leaf.* **(F)** *PEROXIN11-YFP, peroxisome, root.* **(G)** *Heat Shock Protein22-YFP; mitochondria, leaf.* **(H)** *TANGLED1-YFP, preprophase band, leaf.* **(I)** *PINFORMED1-YFP, plasma membrane, ear floral meristem.* **(J)** *PINFORMED1-YFP, plasma membrane, leaf.* **(K)** *PIP2-1-CFP, plasma membrane, leaf.* **(L)** *TONOPLAST INTRINSIC PROTEIN1-YFP, vacuole membrane and ER, leaf.* **(M)** *PROTEIN DISULFIDE ISOMERASE-YFP, ER, leaf.* **(N)** *FLAVONOL SYNTHASE1-RFP, ER, leaf.* **(O)** *FIMBRIN ACTIN BINDING DOMAIN-YFP, actin cytoskeleton, leaf.* **(P)** *α-TUBULIN-YFP, microtubules, leaf.*

2007). For example, using FCS, Goedhart *et al.,* (2000) examined the diffusion of fluorescent Nod factor analogues on living *Vicia sativa* root hairs (Goedhart *et al.*, 2000). The results suggest that the fluorescently labeled Nod factor have a > 1000-fold reduction of mobility upon binding to the cell wall, indicating the molecules are tightly bound to a specific receptor located in the cell wall (Goedhart *et al.*, 2000). FCS can also be used in living cells to study diffusion velocity. For example, Kohler *et al.,* (2007) showed that the diffusion velocity of GFP within plastid tubules is 50 times slower than within the cytosol (Kohler *et al.*, 2000).

***Application of fluorescent protein marker lines in protein interaction studies***

Although FCS is a powerful technique to study protein dynamics, it is difficult to discriminate small mass changes of fluorescent molecules (Hink *et al.*, 2002). For example, Meseth and colleagues have pointed out that the resolving power of FCS is a four-fold mass increase, indicating that a homo-dimer cannot be detected by FCS (Meseth *et al.*, 1999). FCCS is a technique that overcomes the limitation of FCS, by tagging candidate proteins with two different fluorophores (Schwille *et al.*, 1997). FCCS can evaluate the molecular interaction between two spectrally distinct molecules in a quantitative manner. If two labeled molecules bind together, the cross-correlation between the fluctuations in fluorescence intensity of the two molecules would be strong. FCCS has been used to study the interaction between *Arabidopsis* auxin response factors and their Aux/IAA repressors in a quantitative manner by transiently expressing the fluorescently tagged proteins in HeLa cells (Muto *et al.*, 2006). One advantage over FRET methods is that FCCS is not affected by the relative orientation between the donor and acceptor fluorophores, or by the distance between them (Muto *et al.*, 2009). However, some other factors, such as cellular autofluorescence, photobleaching of the dye, cell damage, and reduced signal-to noise ratios have limited the application of FCS and FCCS in living cells, especially in plant cells (Hink *et al.*, 2003). These problems can be partially resolved by developing FPs with high brightness and photostability. FCCS holds great promise for analyzing protein-protein interaction and protein dynamics in living plant cells.

FRET is another widely used technique to detect dynamic protein-protein interactions *in vivo*. The principle of FRET is that energy from an excited donor molecule is transferred to an acceptor molecule, when the donor fluorescence overlaps with the absorption spectrum of the acceptor, and when both molecules are in close proximity (between 2 and 10 nm) (Zelazny *et al.*, 2007). There are two major FRET measurement methods, intensity-based and life-time based (Dixit *et al.*, 2006). The most commonly used intensity-based method is the sensitized emission measurement (Sun *et al.*, 2009). This approach requires the identification and removal of the spectral bleed through (SBT) components from FRET signals (Sun *et al.*, 2009), and the FRET efficiency is calculated using total donor and corrected FRET (Wallrabe and Periasamy, 2005). Thus, the results using sensitized methods are affected by the relative fluorescent intensities of donor and receptor (Hink *et al.*, 2002). In contrast, the life-time based method, fluorescence-lifetime imaging microscopy (FLIM), is independent of the concentration of both the acceptor and the donor and insensitive to photobleaching, and is becoming more popular in plant research (Miyawaki, 2011). In FRET-FLIM experiments, the interaction of two proteins can be confirmed by monitoring the reduction of fluorescence lifetime of the donor, because the excited lifetime of the donor is attenuated through transferring energy to the acceptor (Dixit *et al.*, 2006). FRET-FLIM has been used to quantitatively and dynamically detect protein-protein interactions in living plant cells. For example, Immink *et al.,* (2002) applied FRET-FLIM to study the interaction of MADS box transcription factors in petunia protoplasts (Immink *et al.*, 2002). All petunia MADS box heterodimers identified in yeast two-hybrid systems have been confirmed using FRET-FLIM. Additionally, homodimerization of three petunia MADS box proteins has been identified in this study (Immink *et al.*, 2002). Another very interesting research approach using FRET-FLIM characterized the interaction between two receptor kinases, CLAVATA 1 (CLV1) and *ARABIDOPSIS* CRINKLY4 (ACR4), in the plasma membrane (PM) and plasmodesmata (PD) (Stahl *et al.*, 2013). Their FRET-FLIM analyses demonstrated that ACR4 and CLV1 form homomeric and heteromeric complexes with distinct stoichiometries or conformations, depending on their locations in PM or PD (Stahl *et al.*, 2013). FRET-FLIM has also been applied in maize research using maize protoplasts. Zelazny *et al.,* (2007) showed that the maize aquaporin ZmPIP1 is retained at the endoplasmic reticulum; however, it relocalizes to the plasma membrane in the presence of another aquaporin, ZmPIP2 (Zelazny *et al.*, 2007). The FRET-FLIM result suggests that ZmPIP1 and ZmPIP2 relocalization results from their physical interaction (Zelazny *et al.*, 2007). All of these experiments are elegantly designed; however, it will be more convincing to conduct FRET experiments using the native tissues that express fluorescently tagged proteins driven by their endogenous promoters. Potential artifacts can arise due to overexpression, as well as the physiological condition of protoplasts and differences that can arise from heterologous expression in unrelated species. This may influence signaling cascades, and hence protein-protein interactions (Hink *et al.*, 2003). However, factors such as the autofluorescence from the cell wall, or the relative lower expression caused by use of a native promoter, may hamper the use of FRET experiments in stable lines.

Most disadvantages of FRET in stable lines can be circumvented by improvement of the quality of FP tags. CFP and YFP are the most commonly used fluorescent protein pair for FRET experiments. However, this pair is far from ideal, because the quantum yield of CFP is low, and thus requires high excitation intensity, causing cell damage (Bayle *et al.*, 2008). Additionally, YFP originally had a slow maturation rate and high sensitivity to pH. Some of the drawbacks of YFP and CFP were partially resolved by the emergence of improved variants. For example, the Citrine variant of YFP is brighter and more resistant to photobleaching and acidic pH (Griesbeck *et al.*, 2001), whereas Cerulean, a CFP variant, is twice as bright as CFP (Rizzo *et al.*, 2004). With the growing palette of bright FPs, a variety of new FP combinations for use in FRET experiments have become available (Davidson and Campbell, 2009). For example, mTFP1, a monomeric teal-colored FP from coral, which exhibits higher brightness, acid insensitivity, and photostability than any of the cyan *A. victoria* variants (Ai *et al.*, 2006) has been used for FRET with Citrine (Ai *et al.*, 2008). Some other FRET pairs, such as mAmetrine and tdTomato (Ai *et al.*, 2008), and tSapphire and mOrange (Bayle *et al.*, 2008), have been developed recently. A more comprehensive list of FRET pairs can be found in Day and Davidson's review paper (Day and Davidson, 2012). We have developed a set of codon optimized FPs, such as Cerulean, Citrine,

mTFP1, mCherry, and stable transformed maize marker lines expressing these FPs to facilitate the investigation of dynamic protein-protein interactions using FRET.

## Conclusions

Use of GFP and its derivatives has revolutionized the way that we study plants. FPs have been extensively engineered to improve their host range and spectral characteristics (Berg and Beachy, 2008, Shaner *et al*., 2005, Tsien, 2009). Their ability to be cloned into any open reading frame makes them an ideal choice for localization studies on a genomic scale, and enables real time *in vivo* imaging of tissue specificity, sub-cellular localization and dynamics. When such reporters are combined with a transactivation system to allow specific expression or mis-expression of any gene of interest, it allows for precise experimental intervention to study gene function (Fernandez *et al*., 2009, Gardner *et al*., 2009, Goll *et al*., 2009, Jones, 2009, Moore *et al*., 2006). We developed a pipeline to generate FP marker lines and transactivation driver and reporter lines in maize. This pipeline could be easily applied to other plants, for example, the new $C_4$ photosynthesis model *Setaria viridis* (Brutnell *et al.,* 2010), providing a broad-based cell biological resource to the plant biology community. All the information about the constructs, seeds and images can be found in our website http://maize.jcvi.org/cellgenomics/index.php. Currently, the seeds from all direct promoter fusions and most protein tagged lines are available by requesting via our website. The T2 generation seeds of the transactivation system will also be available to the research community in the near future. In addition, all the constructs listed in our website are available to facilitate research in other species, or use in maize transient assays.

These tools in maize will facilitate research of diverse programs. Besides the traditional applications, like protein localization or tissue specificity studies, the stable transformed maize marker lines also hold promise for live imaging microscopy to investigate protein dynamics and interactions in a real-time manner.

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