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Transgenic maize lines with cell-type specific expression of fluorescent proteins in plastids

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Summary

Plastid number and morphology vary dramatically between cell types and at different developmental stages. Furthermore, in C4 plants such as maize, chloroplast ultrastructure and biochemical functions are specialized in mesophyll and bundle sheath cells, which differentiate acropetally from the proplastid form in the leaf base. To develop visible markers for maize plastids, we have created a series of stable transgenics expressing fluorescent proteins fused to either the maize ubiquitin promoter, the mesophyll-specific phosphoenolpyruvate carboxylase (*PepC*) promoter, or the bundle sheath-specific Rubisco small subunit 1 (*RbcS*) promoter. Multiple independent events were examined and revealed that maize codon-optimized versions of YFP and GFP were particularly well expressed, and that expression was stably inherited. Plants carrying *PepC* promoter constructs exhibit YFP expression in mesophyll plastids and the *RbcS* promoter mediated expression in bundle sheath plastids. The *PepC* and *RbcS* promoter fusions also proved useful for identifying plastids in organs such as epidermis, silks, roots and trichomes. These tools will inform future plastid-related studies of wild-type and mutant maize plants and provide material from which different plastid types may be isolated.

Introduction

Maize has a long history as a model for plastid biology and is of particular importance as a platform for studying the biochemistry and developmental biology of C4 photosynthesis. The successive maturation of chloroplasts from proplastids from leaf base to tip was described nearly 35 years ago (Leech *et al.*, 1973) and the dimorphism, or Kranz anatomy of maize leaf chloroplasts in mesophyll vs. bundle sheath cells was noted several years later (Miranda *et al.*, 1981). The maize plastid gene expression apparatus (Maier *et al.*, 1995) and proteome (Majeran *et al.*, 2005) have also been scrutinized. Two mutants specifically defective in bundle sheath differentiation have been isolated (Langdale and Kidner, 1994; Roth *et al.*, 1996) and later studied at the molecular level (Hall *et al.*, 1998; Brutnell *et al.*, 1999). Despite these advances, the signals that regulate cell type specification and plastid morphology are still poorly understood.

A valuable tool for investigating organelle number and morphology is labelling with fluorescent proteins (FPs). Among numerous applications have been studies of mitochondrial division in yeast (e.g. Tieu *et al.*, 2002), observations of plastids and plastid tubules (stromules) in higher plants (Köhler *et al.*, 1997a; Köhler and Hanson, 2000; Kwok and Hanson, 2004; Hanson and Sattarzadeh, 2008), and identification of mitochondria and mitochondrial mutants in higher plants (Köhler *et al.*, 1997b; Logan *et al.*, 2003). In the case of plastids, the fluorescent tag has been expressed in the nucleus fused to a transit peptide (Köhler *et al.*, 1997b; Primavesi *et al.*, 2008), fused to the entire coding region of lipoxygenase 10 (Mohanty

et al., 2009), or expressed within the organelle following insertion of a cassette into the chloroplast genome (Shiina *et al.*, 2000; Reed *et al.*, 2001).

We commenced a project to test different fluorescent tags for labelling of maize plastids in stable transformants and also to develop lines that labelled specifically either bundle sheath or mesophyll chloroplasts. Three new maize codon-optimized fluorescent protein coding regions were produced. We find that the promoter from a Rubisco small subunit-encoding gene can drive bundle sheath-specific expression and the *PepC* promoter results in expression in mesophyll cells. We were also able to visualize plastids in many organs. These transgenic lines should be useful to maize researchers studying various aspects of plastid development and should faciliate fluorescence-activated cell sorting of bundle sheath and mesophyll plastids.

Results

Development of transformation cassettes

Our initial expression cassettes were constructed with the human codon-optimized green fluorescent protein (GFP) that was shown to be effective for transient expression in maize protoplasts (Chiu *et al.*, 1996). Subsequently, coding regions for three different variants of GFP were synthesized, using preferred maize codons and incorporating appropriate mutations with respect to the original jellyfish GFP sequence. All three variants carry the Q80R mutation originally incorporated into most GFPs by accident, plus additional mutations known to enhance solubility or to cause spectral shifts to yellow or blue (Cormack *et al.*, 1996; Crameri *et al.*, 1996; Davis and Vierstra, 1998; Wachter *et al.*, 1998). The predicted protein sequences of mzGFP (mz; modified zea) and mzBFP are identical to smGFP and smBFP described by Davis and Vierstra (1998). The mzYFP protein sequence is identical to the yellow fluorescent protein (YFP) for which a crystal structure was determined (Wachter *et al.*, 1998). These are detailed in Table 1.

Table 1 Predicted protein sequences of maize codon-optimized fluorescent proteins

Fluorescent protein	Mutations with respect to jellyfish GFP				
mzGFP	Q80R	M99S	M153T	V163A	
mzYFP	S65G	V68L	S72A	Q80	T203Y
mzBFP	Y66H	Q80R	F99S	M153T	V163A

Table 2 Maize transformation summary*

Plasmid name	Transit sequence from	Cassette†	Events‡
pPTN343	Maize cpRNA polymerase	*35S/TP-GFP*	7
pPTN372	Maize cpRNA polymerase	*OsActin/TP-GFP*	14
pPTN442	Pea Rubisco SS	*PepC/TP-GFP*	5
pPTN448	Pea Rubisco SS	*UBi1/TP-mzBFP*	12
pPTN458	Pea Rubisco SS	*Ubi1/TP-mzGFP*	**16**
pPTN469	Pea Rubisco SS	*Ubi1/TP-mzYFP*	**18**
pPTN512	Pea Rubisco SS	*PEPC/TP-mzYFP*	**7**
pPTN533	Pea Rubisco SS	*RbcS1/PsTP-mzYFP*	21
pPTN629	Maize Rubisco SS	*RbcS1/ZmTP-mzYFP*	**7**

*Cassettes in bold are those for which experimental data are presented here.

†35S; CaMV 35S promoter; TP, chloroplast transit peptide; OsActin, rice actin promoter; SS, small subunit; GFP, human codon-optimized GFP; mzFP coding regions, maize codon-optimized FPs as in Table 1.

‡Independent T0 events analysed by microscopy for fluorescent protein expression.

Table 2 shows the series of chimeric genes used for stable transformation of the maize recipient genotype Hi II. Our aim was to develop a cassette driving ubiquitous expression of a chloroplast-targeted GFP on one hand, and tissue-specific cassettes driving mesophyll or bundle sheath chloroplast expression on the other hand. To determine the best promoter for strong general expression, we tested the CaMV 35S and rice actin (Cao *et al.*, 1992) promoters, reasoning that the 35S promoter is widely used for high expression and is known to function in transformed maize callus (Fromm *et al.*, 1986) and regenerated plants (Gordon-Kamm *et al.*, 1990). Although heterologous, the rice actin promoter was also initially tested, as it is reputed to be stronger than the 35S promoter in monocot transformation (discussed in Prakash *et al.*, 2008). We also created a cassette where GFP was driven by the *Phosphoenolpyruvate Carboxylase* (*PepC*) promoter; this gene should express GFP only in mesophyll cells. Conversely, we used the promoter of Rubisco small subunit (*RbcS*) to drive GFP expression specifically in bundle sheath chloroplasts. The cassettes were used to generate an initial set of transgenic lines.

Analysis of transgenic plants containing non-codon-optimized green fluorescent protein

A total of 26 independent events were analysed for the first three cassettes shown in Table 2. In all cases, we either did not observe fluorescence above the background contributed by chlorophyll, or the signal was slightly above

background in the cases of some of the transformants from the constitutive promoter-driven cassettes (data not shown). For these plants, subsequent RNA and immunoblot analysis showed low mRNA accumulation and very low GFP accumulation, suggesting that the genes were weakly transcribed or the mRNAs were unstable, and that those RNAs that accumulated were poorly translated (data not shown). Therefore, these transgenic lines were abandoned.

Analysis of transformants expressing codon-optimized cassettes

Because of the unsatisfactory results with the initial constructs, we utilized maize codon-optimized BFP, GFP and YFP (Table 1) in subsequent experiments. We also switched from the rice *Actin* promoter to the maize *Polyubiquitin-1* (*Ubi1*) promoter (Streatfield *et al.*, 2004) for cell type-independent expression. Plants expressing the maize-optimized fluorescent proteins under the control of the *Ubi1* promoter displayed fluorescent protein signals using gain settings where chlorophyll fluorescence was not observed in negative controls (Figure 1). When the images were merged, the fluorescent protein signal colocalized with the chloroplast autofluorescence. In Figure 1, small non-chlorophyll-containing epidermal plastids are visible in panels (b) and (c) because the images contain signals from both the leaf epidermal layer as well as mesophyll tissue.

To develop archival lines, we screened multiple events through the T2 generation (Table S1), seeking lines where transgene expression was not silenced. DNA gel blot

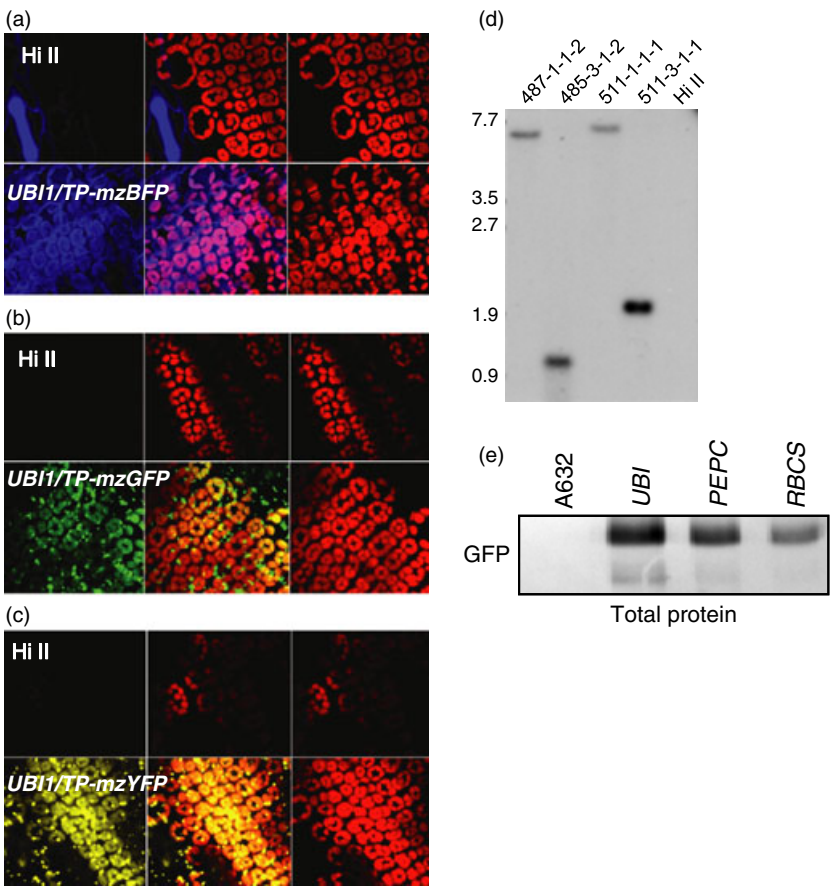

Figure 1 Confocal microscopy images of primary maize transformants expressing the fluorescent protein constructs indicated above each panel. The view is from the top of the leaf. For (a–c), the top row is an untransformed control, and left to right columns are fluorescent protein channel, merged images, and chlorophyll autofluorescence. Constructs used for transformation were (a); pPTN448, event 480-1-1-1; (b) pPTN458, event 485-3-1-2; (c) pPTN469, event 487-1-1-2. (d) Total DNA from the indicated events was digested with BamHI and probed with the mzYFP coding region, which hybridizes with all the fluorescent protein genes. DNA from an untransformed plant (variety Hi II) was a negative control. (e) Equal amounts of total protein of leaves from an untransformed control (A632) and transgenic lines carrying the indicated promoters were analysed using an anti-GFP antibody.

analysis of the events ultimately selected is shown in Figure 1d, showing that each has a single insertion. Therefore, these lines should be appropriate for introgression and other standard manipulations. We noted that some progeny expressing fluorescent proteins at high levels, particularly those with the ubiquitin promoter, sometimes were more susceptible to stress and exhibited slow growth rates (data not shown). Currently T2 lines, which segregate for transgene expression, are being used for experimental material and also to propagate subsequent generations, where it is hoped that homozygous plants with no growth defect can be obtained. The frequency of transgene expression in the T2 generation, however, is sufficient that among a dozen seedlings, some fluorescent progeny were always readily identified for experimental use.

To gain an idea of the overall expression levels of G/YFP in the transgenic lines, total plant protein was analysed by immunoblotting with anti-GFP antiserum, as shown in Figure 1e. As expected, on a total protein basis, the *Ubi1* promoter gave the highest expresion, with *PepC* driving an intermediate level, and *RbcS*-driven expression being slightly lower. It should be noted that because the number of mesophyll cells vs. bundle sheath cells is higher per unit volume, the results reflect not only promoter strength, but also cell-type specificity and the prevalence of a given cell type.

Examination of cell type specificity

Using confocal microscopy, we examined plants where the transgene had been functionally inherited, (i.e. T1 generation and beyond) in more detail. Detailed Z-series images of leaf cells from the plants producing RBCS/TP-mzYFP are shown in Figure 2. RBCS/TP-mzYFP was observed in the merged images not to colocalize with all chlorophyll fluorescence, but instead to surround vascular tissue when viewed in cross-section or longitudinal section. In the

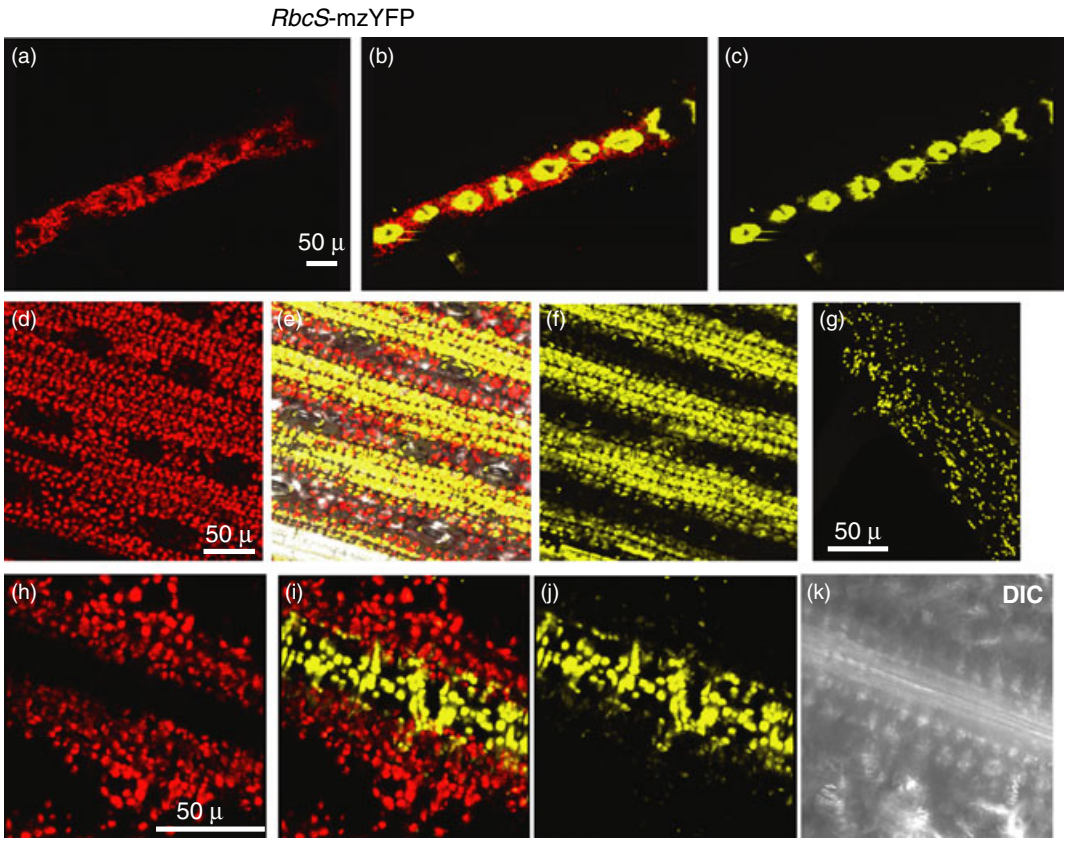

Figure 2 Localization of RBCS/TP-mzYFP in bundle sheath cells of maize leaves. (a–c) Leaf cross-section sliced manually. (a) Chlorophyll autofluorescence; (b) merged chlorophyll and YFP signals; and (c) YFP fluorescence. (d) YFP fluorescence in leaf epidermal plastids (e–g) longitudinal view of leaf: (e) chlorophyll autofluorescence; (f) merged chlorophyll, differential interference contrast (DIC) and YFP signals; and (g) YFP fluorescence; (h) YFP fluorescence in hypocotyl epidermis (i–k) close-up longitudinal view of leaf cells as in (d–f) with (l) DIC image to illustrate location of vein. Each image represents a composite Z-series through mesophyll and bundle sheath cells from the surface of the leaf.

differential interference contrast (DIC) image, the co-localization of the YFP signal cells near the vascular tissue is evident. Small YFP-labelled plastids seen in Figure 2g,h are present in the epidermal layer. Thus, the *RbcS* promoter appears to mediate bundle sheath-specific expression in leaves. In contrast to the RBCS/TP-mzYFP transgenic plants, PEPC/TP-mzYFP was found in the mesophyll cells but apparently absent from the chloroplasts in cells immediately surrounding leaf veins, either when viewed in cross-section (Figures 3a-d), or in a longitudinal view (panels e–g). At this level of analysis, the *PepC* promoter appeared to primarily drive, as desired, mesophyll but not bundle sheath expression of YFP. This was confirmed through an independent approach, described immediately below.

Analysis of fluorescent protein in separated bundle sheath and mesophyll cells

To further examine the location of GFP or YFP, we separated mesophyll protoplasts and bundle sheath strands from transgenic and control plants, and examined the preparations by confocal microscopy, as shown in Figure 4. GFP and YFP fluorescence, resulting from expression from the *Ubi1* and *PepC* promoters, respectively, was clearly visible in mesophyll protoplasts, whereas this was not the case for mzYFP driven by the *RbcS* promoter (left panel). RBCS/TP-mzYFP expression was readily observed in chloroplasts in bundle sheath strands (right panel), again indicating cell type specificity. The *Ubi1* promoter drove some GFP expression in the bundle sheath. While this experiment strongly argues for relatively strong mesophyll and bundle sheath specificity of PEPC/Tp-mzYFP and RBCS/TP-mzYFP, respectively, it does not exclude expression in organs or cell types not present in these two preparations.

Expression of fluorescent protein in additional cell types

We examined a variety of cell types to determine the tissue-specific expression patterns of UBI1-mzG/YFP, RBCS-mzYFP and PEPC-mzYFP in transgenic plants. As expected, the *Ubi1* promoter drove expression in a wide range of tissues, including trichomes, leaf mesophyll, vascular tissue, bundle sheath, leaf epidermis, silks, root hairs and roots (Figure 5). However, not all chloroplasts visualized by chlorophyll autofluorescence in leaf cross-sections (see Figures 5e–g) are labelled with YFP. Abundant stromules could be readily visualized in leaf epidermal cells (panel o). The YFP signal in plastids of root hairs could be distinguished from mitochondria stained with Mitotracker Red (panels q–s). In addition to fluorescent protein signal in plastids of hypocotyl epidermis, we visualized small fluorescent vesicular structures (panel p) that were not seen in wild-type nor in transgenic plants carrying the other two promoter constructs (Figures 6 and 7). These vesicles were smaller than typical hypocotyl plastids and were moving

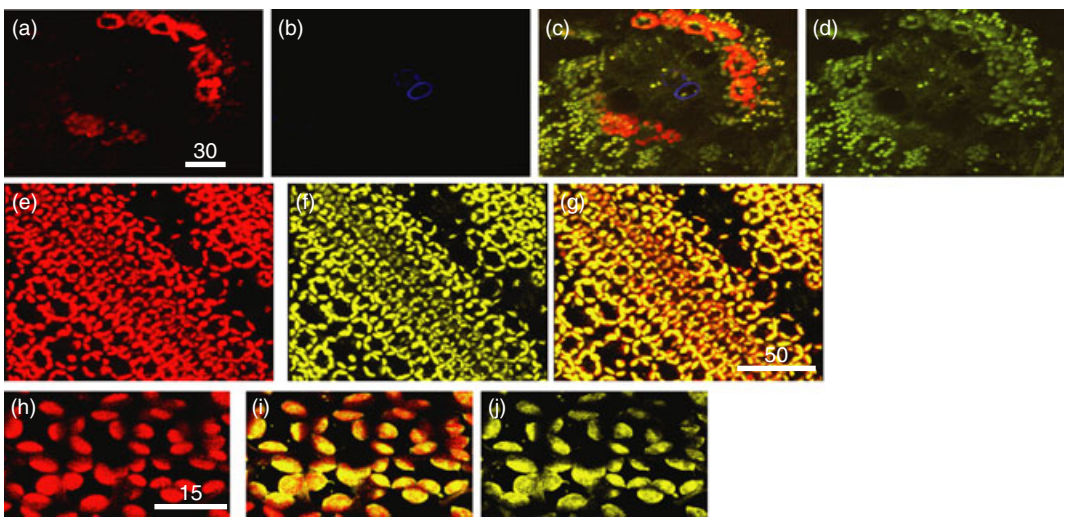

Figure 3 Localization of PEPC/TP-mzYFP in mesophyll cells. (a–d) Leaf cross-section sliced manually. (a) chlorophyll autofluorescence (red) (b) vein autofluoresence (blue) (c) merged chlorophyll, vein autofluoresence and YFP (yellow) signal; and (d) YFP fluorescence. (e–g) Longitudinal view of leaf: (e) chlorophyll autofluorescence; (f) merged chlorophyll and YFP signals; and (g) YFP fluorescence. (h–j) Close-up of plastids in mesophyll cells: (h) chlorophyll autofluorescence; (i) merged chlorophyll and YFP signals with short stromules visible; (j) YFP signals. Each image represents a composite Z-series through mesophyll and bundle sheath cells from the surface of the leaf.

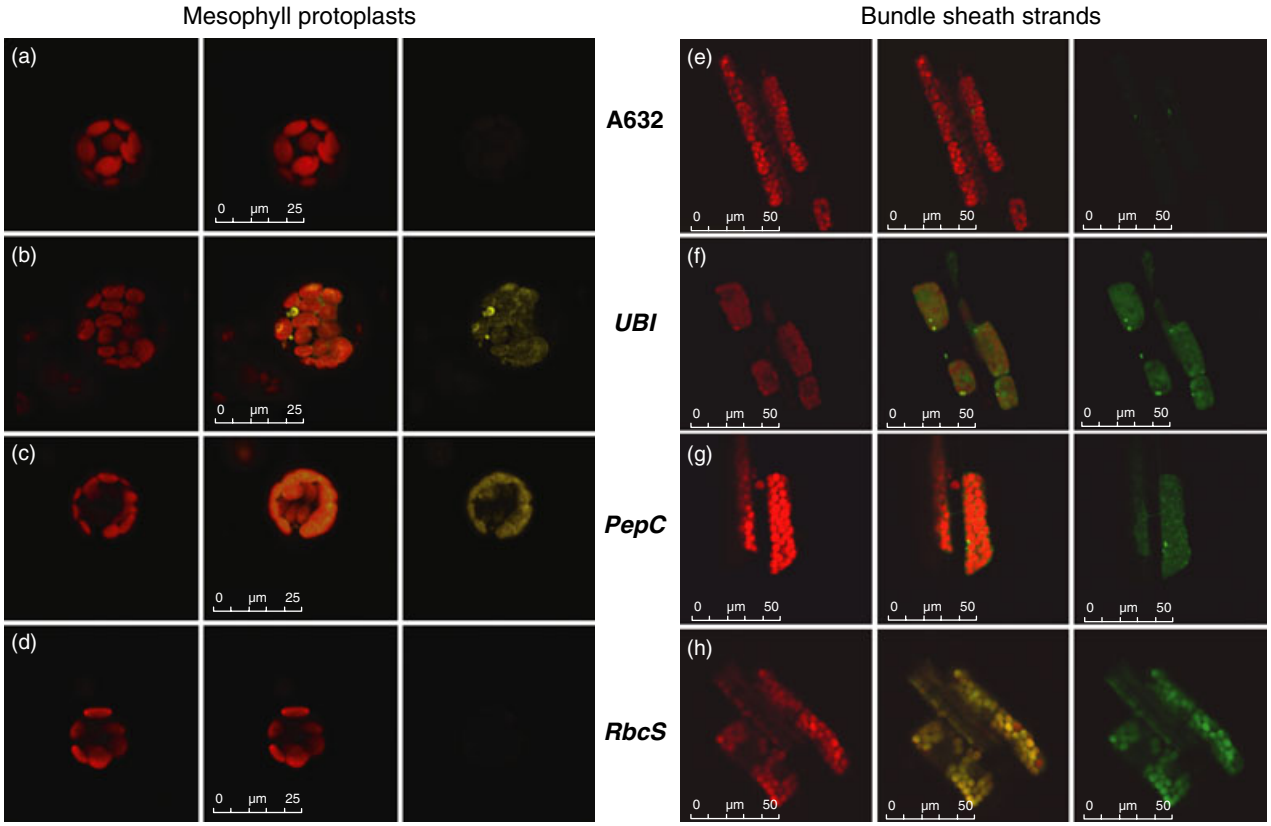

Figure 4 Analysis of fluorescent protein expression in mesophyll protoplasts and bundle sheath strands prepared from leaves of (a,e) wild-type; (b,f) UBI1/TPmzYFP plants; (c,g) PEPC/TPmzYFP plants; and (d,h) RBCS/TPmzYFP plants. Each set of columns, left to right: chlorophyll fluorescence, merged images, and fluorescent proteins. In panel b, YFP flurorescence not overlapping with chlorophyll fluorescence may represent immature plastids lacking thylakoid membranes.

more rapidly (Movie S1). Movies of tobacco hypocotyl plastids are available for comparison (Kwok and Hanson, 2003).

In addition to bundle sheath chloroplasts (Figure 2), plants carrying the *RbcS* promoter contained YFP in plastids of leaf epidermis, guard cells, trichomes, roots and silks (Figure 6). The PEPC/TP-mzYFP plants exhibited YFP signals in plastids of trichomes, leaf epidermis, roots and root hairs (Figure 7). No PEPC/TP-mzYFP was evident in silks. An example of a leaf region lacking YFP fluorescence in guard cell plastids of PEPC/TP-mzYFP is shown in Figure 7g–j, but we also sometimes did observe fluorescent plastids in stomatal cells (data not shown).

RbcS/TP-mzYFP expression in the *bsd2* mutant background

One possible use for organelle-labelled transgenic lines is to examine the morphology of chloroplasts and other plastid types in mutant backgrounds. Because expression of the *RbcS/TP-mzYFP* construct appeared to be specific to bundle sheath cells, we decided to use it as a test case by crossing the transgene into the *bundle sheath defective2* background (Brutnell *et al.*, 1999). The *bsd2* mutant is pale-green and seedling-lethal because it does not accumulate Rubisco, and exhibits abnormal chloroplast morphology in light-exposed tissues that are biochemically C4 (Roth *et al.*, 1996). The major gross morphological difference is that bundle sheath chloroplasts appear to be swollen; by electron microscopy, altered membrane structure can be observed (Roth *et al.*, 1996). Figure 8 compares WT (panels a–c) and *bsd2/RBCS-YFP* (panels d–f) in a cross-section from a part of the leaf that would normally exhibit Kranz anatomy. In the mutant background, the bundle sheath cells were smaller in number and irregularly shaped, in contrast to the regular shape and spacing observed in a wild-type background (Figure 2). Thus, use of the fluorescent protein allows visualization of cell morphology in *bsd2* which would normally require more arduous techniques.

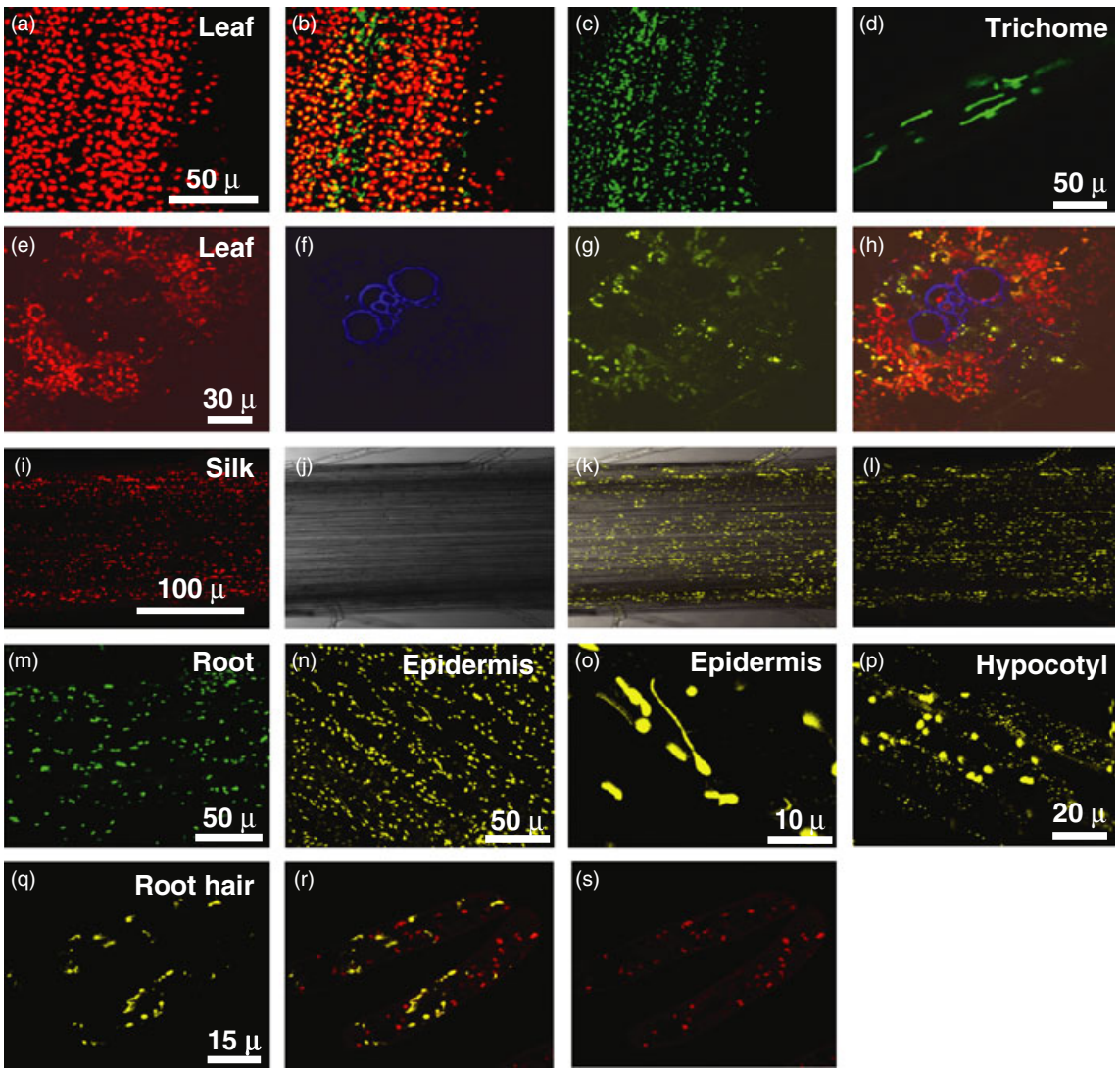

Figure 5 Expression of UBI1/TP-mzG/YFP in various cell types. (a–c) Longitudinal view of leaf: (a) chlorophyll autofluorescence; (b) merge of chlorophyll, vein autofluorescence and YFP signal; (c) GFP fluorescence; (d) trichome with GFP fluorescence in plastid body and stromules. (e–g), cross-section of mature leaf: (e) chlorophyll autofluorescence; (f) vein fluorescence (g) YFP fluorescence; (h) merge of chlorophyll, vein, and YFP fluorescence (i–l), Longitudinal view of silk: (i) chlorophyll autofluorescence; (j) DIC; (k) merged images of YFP and DIC; (l) YFP fluorescence. Other tissues are (m) root, GFP; (n) leaf epidermis, YFP; (o) leaf epidermal cell close-up with stromules; and (p) light-grown hypocotyl, YFP fluorescence in plastids and small vesicles. (q–s), root hair: (q) YFP fluorescence (r) merge of YFP fluorescence and Mitotracker Red stain; (s) Mitotracker Red stain. Sizes of bars are as shown.

Discussion

We have demonstrated the efficacy of three different maize codon-optimized fluorescent protein coding regions in transgenes controlled by three different maize promoters and Rubisco plastid transit sequences from either pea or maize. These codon-optimized fluorescent protein ORFs are potentially useful in constructs designed to target BFP, GFP or YFP to other subcellular locations in maize and other grasses. Previously, a synthetic GFP coding region was expressed in transgenic maize under the control of the maize *Ubi1* promoter and brightly fluorescent cells could be visualized, however imaging of GFP at the cellular level was problematic because of autofluorescence emanating from cell walls, which often masked GFP fluorescence (van der Geest and Petolino, 1998). The synthetic coding region constructed by van der Geest and Petolino (1998) contained the same four mutations with respect to jellyfish GFP as our mzGFP construct (Table 1), but differed in the particular codon alterations that were made.

A synthetic GFP coding region optimized for human codon usage and carrying a S65T mutation was found to

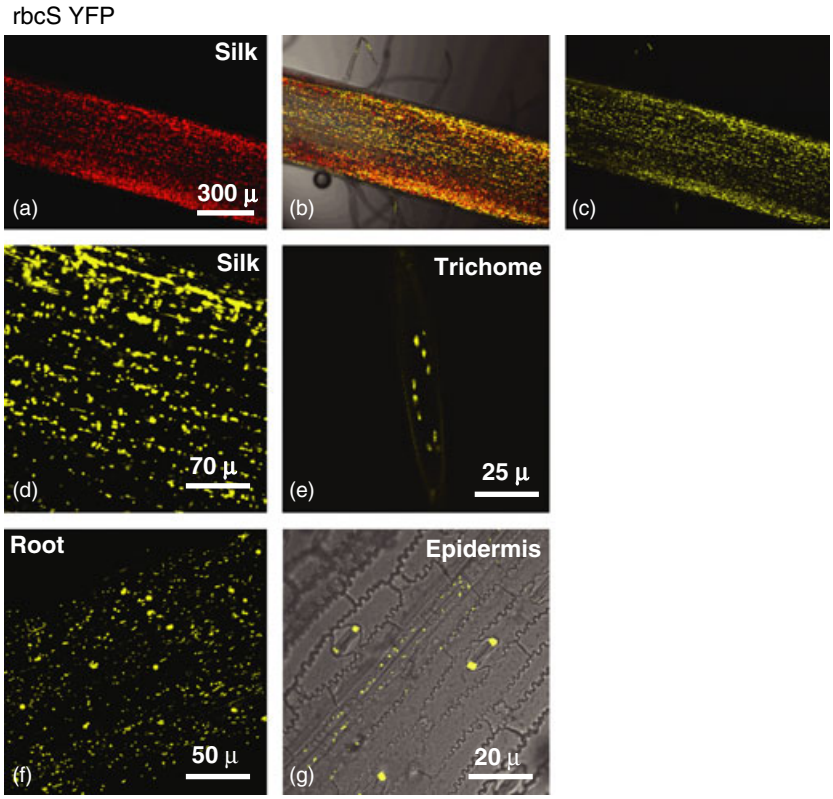

Figure 6 Expression of RBCS/TP-mzYFP in various cell types. (a–c) silk: (a) chlorophyll autofluorescence; (b) merged chlorophyll, DIC and YFP; (c) YFP fluorescence. (d) YFP fluorescence from silk at higher magnification. (e–f) YFP fluorescence from trichome and root, respectively; (g) leaf epidermis, merged YFP and DIC.

be a useful reporter for transient expression in transfected maize protoplasts (Chiu *et al.*, 1996). The synthetic GFP coding region used by Chiu *et al.* (1996) was also effective for obtaining GFP expression in rice transgenic plants when placed under the control of the rice *ubiquitin* promoter (Kumar *et al.*, 2005; Lu *et al.*, 2008), in transgenic sorghum or transgenic barley fused to the maize *Ubi1* promoter (Murray *et al.*, 2004; Gao *et al.*, 2005), and in transgenic bentgrass plants when under the control of a chimeric promoter (Yu *et al.*, 2000). However, when we attempted to utilize this GFP coding region in our constructs, we were unable to obtain plants expressing GFP at high levels, even though a rice actin promoter/human codon-optimized GFP gene was expressed in transgenic maize following shoot meristem transformation (Sairam *et al.*, 2003). We have not explored the reasons for our failure to obtain maize transformants expressing this GFP at high level under the control of the three different promoters and two transit sequences we tested.

Several other GFP coding regions have also been successfully expressed in grasses. Rice transgenic plants could be obtained that express a partially synthetic GFP coding region modified to remove cryptic splice sites and carrying an ER retention signal (mGFP5-ER) (Johnson *et al.*, 2005). The mGFP5-ER protein also carries Q80R V163A S175G I167T (Siemering *et al.*, 1996); two of these amino acid substitutions are not present in the mzGFP coding region we utilized (Table 2). Transient expression of mGFP5 in plastids of barley, sugarcane and wheat was observed when either a tobacco *RbcS* or tomato transit sequence from *Defective Chloroplasts and Leaves* gene was fused to the fluorescent protein ORF (Gnanasambandam *et al.*, 2007). For plastid labelling of wheat, the human codon-optimized S65T GFP was mutagenized further to enhance fluorescence by incorporating S65G and S72A mutations (Cormack *et al.*, 1996), previously found to be useful for chloroplast imaging (Reed *et al.*, 2001). Constructs carrying the rice *actin 1* promoter and intron with transit sequences from either wheat RbcS, maize *ferredoxin 3*, or rice FtsZ resulted in healthy transgenic wheat plants expressing GFP in plastids in a variety of cell types (Primavesi *et al.*, 2008).

Our data indicates that the promoter sequences from the maize *RbcS* and *PepC* genes that were used in our

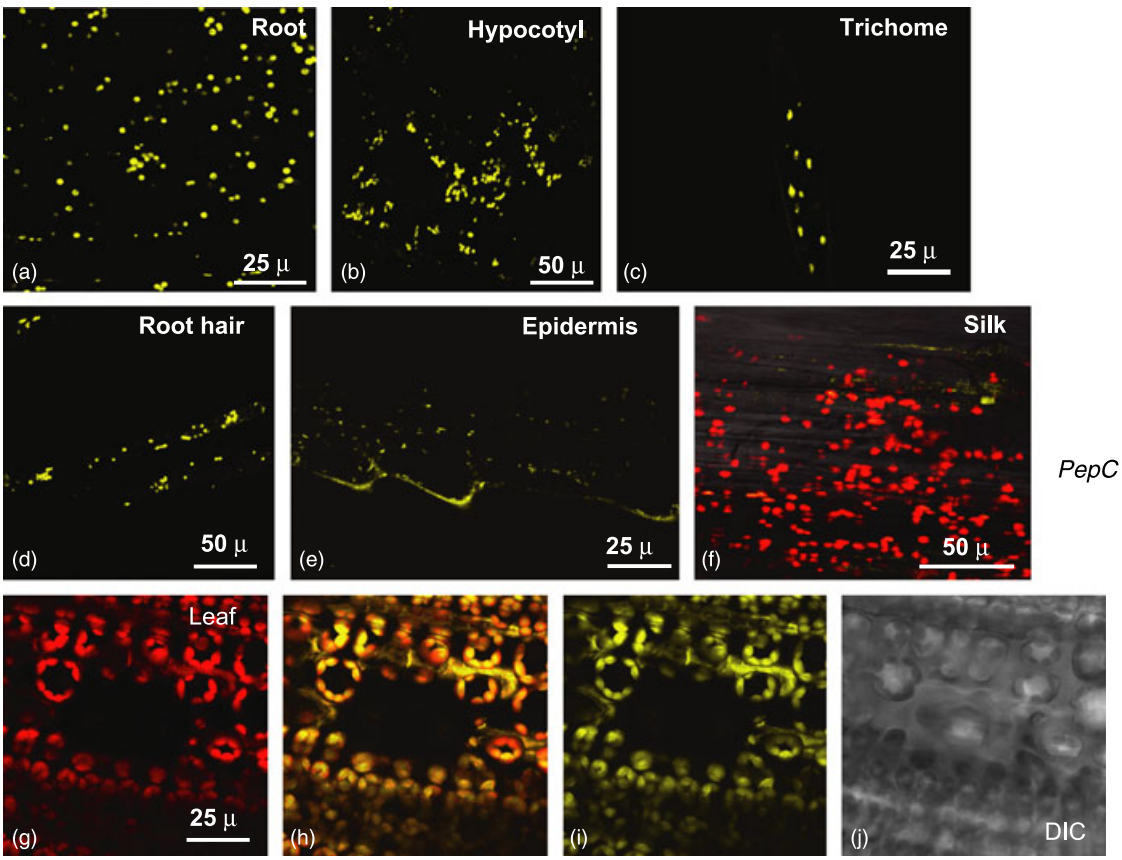

Figure 7 Expression of PEPC/TP-mzYFP in various tissues. (a–e) YFP fluorescence in (a) root; (b) light-grown hypocotyl; (c) trichome; (d) root hair; (e) leaf epidermis; (f) silk, merged chlorophyll autofluorescence and YFP; (g–j) leaf near guard cell: (g) chlorophyll autofluorescence; (h) merged chlorophyll and YFP; (i) YFP fluorescence and (j) DIC image reveals location of guard cell.

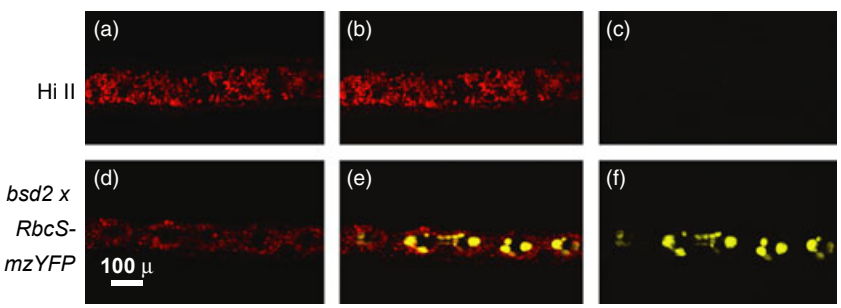

Figure 8 Effect of the *bsd2* mutation on bundle sheath cell morphology. Confocal images of leaf cross-sections of a plant expressing *RbcS/TP-mzYFP* (a–c) or a homozygous *bsd2* progeny plant carrying the *RbcS/TP-mzYFP* cassette following crossing to event 511-3-1-1 (d–f) Columns from top to bottom are chlorophyll autofluorescence, YFP fluorescence and merged images.

fluorescent protein cassettes were sufficient for preferential expression in bundle sheath vs. mesophyll chloroplasts. Indeed, our *RbcS*-specific cassette comprises all the elements identified by Viret *et al.* (1994) required for photostimulation in bundle sheath cells on the one hand, and mesophyll repression on the other hand. These elements, defined from the analysis of the *RbcS1* gene (also called *RbcS-m3*), comprise a region in the promoter region extending into the coding sequence present in pPTN629, but not pPTN533, and distal regions in the promoter where an upstream silencer was identified (also seen by Schaffner and Sheen, 1991), and in the terminator region, justifying our use of the *RbcS* terminator region for this construct instead of the standard *Nos* terminator.

Whether the *RbcS* terminator is an absolute requirement for cell type-specific expression *in vivo* is still unclear. Viret *et al.* (1994) identified this element using an *in situ* transient assay, and subsequent work (Xu *et al.*, 2001) led to the hypothesis that this region is a binding site for TRM1, a transcriptional repressor of the YY1 family. On the other hand, Nomura *et al.* (2000) observed bundle sheath localization for a GUS reporter gene driven only by the extended maize *RbcS* promoter in transgenic maize, and little localization in mesophyll cells. As the experimental systems and reporter genes differ, it is neither possible to draw a firm conclusion regarding the requirement for the *RbcS* terminator, nor of the precise regulatory mechanism involved. While Xu *et al.* (2001) suggested that the terminator was required for transcriptional repression in mesophyll cells, studies in the C4 dicot *Flaveria bidentis* revealed that the 5′ and 3′ regions of the *RbcS* genes are required for post-trancriptional regulation and bundle sheath localization (Patel *et al.*, 2006). In any event, the studies reported here show that the full combination of these elements lead to bundle sheath-restricted expression of the YFP transgene.

With the PEPC cassette our results were comparable to those of Kausch *et al.* (2001) using the same 1.7 kb *PepC* promoter region, which is sufficient to drive C4 mesophyll-specific expression of chloroplast-targeted YFP (our results) as well as of a GUS reporter gene in transgenic maize (Kausch *et al.*, 2001). It should be noted that a shorter promoter region of 0.6 kb was also shown to be sufficient for mesophyll-specific expression of a GUS transgene (Taniguchi *et al.*, 2000).

The maize *RbcS* and *PepC* promoters were also tested in rice (Matsuoka *et al.*, 1994). While the maize *PepC* promoter did confer mesophyll expression in rice, it was also found to be much stronger than the endogenous *PepC* promoter. The maize *RbcS* promoter, on the other hand, reversed its cell type specificity, being expressed in mesophyll but not bundle sheath cells. This observation, combined with the reciprocal observation in maize, contributed to a model for *RbcS* expression in maize, in which the *RbcS* promoter contains *cis* elements for both bundle sheath and mesophyll expression, but mesophyll expression is repressed by a *trans* factor. Both cassettes also resulted in expression in leaf epidermal cells and trichomes, as well as in root cells (Figures 6,7), at variance with previously published observations using PEPC-GUS (Taniguchi *et al.*, 2000; Kausch *et al.*, 2001) or RBCS-GUS (Nomura *et al.*, 2000) fusions, which showed little or no expression in roots, and restricted expression of the transgene in guard cells but not epidermal cells. This discrepancy might be due to differences in transgene dosage and/or position effects. In comparison to the *RbcS* and *PepC* promoters, the *Ubi1* promoter resulted in the greatest range of expression (Figure 5). Stromules were less often seen in plants carrying the RBCS-mzYFP and PEPC-mzYFP constructs, but were quite evident in leaf epidermal cells containing the *Ubi1* promoter. Because stromules are narrow, high-level expression of fluorescent proteins is necessary to visualize them. Stromules in maize have been visualized previously following transient expression of a synthetic GFP gene in maize protoplasts under the control of an aminoacyl tRNA synthetase promoter (Rokov-Plavec *et al.*, 2008).

We have produced four fluorescent protein-expressing maize lines that will be useful in further studies of plastid biogenesis and composition. Although some progeny of plants expressing fluorescent proteins at high levels exhibited some abnormalities in growth and development, maize plants with mzYFP in bundle sheath or mesophyll plastids can be grown to flowering and produce progeny expressing the fluorescent protein. Plants carrying the *mzYFP* transgenes can be crossed to maize mutants to study the effect of mutations on plastid number or morphology and on bundle sheath or mesophyll cell development, as demonstrated by plants carrying the *bsd2* mutation in combination with YFP expressed in bundle sheath cells. Plants carrying the *mzBFP* or *mzGFP* constructs could be crossed to the large set of transgenic maize plants being labelled by citrine-YFP in many different locations (Mohanty *et al.*, 2009) to visualize interactions of plastids with other subcellular structures. Furthermore, these plants could be useful in separations of bundle sheath and mesophyll plastids. For example, plastids carrying either the RBCS-mzYFP or PEPC-mzYFP construct could be prepared from leaves and further purified by flow cytometry before analysis of RNA or protein contents. Flow cytometry parameters may need to be adjusted to select for organelles expressing levels of fluorescent protein that are sufficient to discriminate between bundle sheath and mesophyll plastids, given that some level of cross-expression of fluorescent protein in RBCS-mzYFP and PEPC-mzYFP plants is likely to occur. Even if some expression is not strictly cell-type specific, cells can be selected that exhibit high levels of one fluorescent protein, thus eliminating cells with lower expression levels. Further considerations in separating bundle sheath from mesophyll cells have been reviewed in detail by Edwards *et al.* (2001). We note that while both of the constructs

label leaf plastids outside of bundle sheath or mesophyll (for example, in leaf epidermis), most of the plastids in other leaf cell types are either smaller or do not contain chlorophyll and therefore most could potentially be sorted away by size or chlorophyll content from either mesophyll or bundle sheath chloroplasts.

Experimental procedures

Maize transformation

Maize transformations were carried out via an *Agrobacterium*-mediated transformation protocol. Immature ears, genotype Hi II (Armstrong *et al.*, 1991), were harvested approximately 12 days post-pollination. Whole ears were surfaced sterilized by applying 70% ethanol spray and allowing to air dry within a laminar flow hood. Immature embryos were isolated and placed immediately in liquid isolation medium composed of 1/2 MS salts with full strength MS vitamins 115 mg/L proline, 6.9% sucrose, 3.6% glucose, 200 μM acetosyringone buffered with 10 mM MES (pH 5.4). Following the isolation of 100 immature embryos, the isolation medium was replaced with inoculation medium, *A. tumefaciens* transconjugant suspended in isolation medium to an OD_{660} of 0.3–0.5. The embryos were inoculated for 5 min, after which they were transferred, scutellum side up, to co-cultivation medium solidified with 0.6% low EEO agarose. Co-cultivation medium consisted of 1/2 MS salts, full strength MS vitamins, 0.5 mg/L thiamine, 1 mg/L 2,4-D, 115 mg/L proline, 1% glucose, 2% sucrose, 20 μM AgNO3, and 200 μM acetosyringone. The medium was buffered with 20 mM MES (pH 5.4). The embryos were co-cultivated for 2 days at 24 °C.

Following the co-cultivation step the embryos were transferred to delay medium composed of N6 salts (Chu *et al.*, 1975), Eriksson's vitamins (Eriksson, 1965), with 1 mg/L 2,4-D, 25 mM proline, 100 mg/L casamino acids, 2% sucrose, 1.7 mg/L $AgNO_3$, and 250 mg/L carbenicillin. The medium was solidified with 0.7% phytagar and buffered with 3 mM MES (pH 5.8). The delay step was carried out for 5 days in the dark at 28 °C, after which developing coleoptiles were removed from the embryos and the explants transferred to selection medium.

The selection phase was carried in a stepwise fashion using the delay medium supplemented with 25 mg/L paramomycin for 3 weeks, followed by a transfer to 50 mg/L paramomycin for 3 weeks, and finally 100 mg/L paramomycin. Embryogenic tissue was subcultured three times on to fresh 100 mg/L selection regime until the proliferating embryogenic culture mass reached approximately 2 cm in diameter.

Paromomycin-tolerant embryogenic tissue was regenerated in a three-step process. The first step was carried out in the dark at 28 °C for a period up to 14 days, where the tissue was cultured on medium composed of MS salts, Fromm vitamins (Fromm *et al.*, 1990), 0.1 mg/L 2,4-D, 100 μM abscisic acid (ABA), 2% sucrose, 50 mg/L paromomycin, and 250 mg/L carbenicillin. The medium was solidified 0.7% phytagar and buffered with 3 mM MES (pH 5.8). The second stage of regeneration involved culturing embryos for a period up to 14 days in the dark at 28 °C on N6 salts, Eriksson's vitamins, 6% sucrose, 50 mg/L paromomycin and 250 mg/L carbenicillin. The embryo conversion step was carried out in culture vessels under an 18 h light regime on medium with MS salts, Fromm vitamins, 100 mg/L inositol, 150 mg/L asparagine, 2% maltose, 1% glucose, and 50 mg/L paromomycin. The conversion medium was solidified with 0.7% phytagar and buffered with 3 mM MES (pH 5.8).

Fluorescent marker gene cassettes

Three cassettes that harboured a non-maize codon-optimized version (Chiu *et al.*, 1996) were assembled under the control of either the 35S CaMV (Benfey and Chua, 1990), rice actin promoter, coupled with a 5′-intron (Zhong *et al.*, 1996) or the 1.7 kb maize C4 *PepC* promoter. (Yanagisawa and Izui, 1989) (*ZmPpc1*) The respective promoters were fused with translational enhancer element from the maize PPDK-A gene (Sheen, 1993), and GFP was targeted to plastids via the maize chloroplast RNA polymerase RpoTp transit peptide (Chang *et al.*, 1999) for the 35S CaMV and rice actin cassettes, or the pea *RBCS1* transit peptide (Van den Broeck *et al.*, 1985; von Heijne *et al.*, 1991) for the *PEPC* cassette. The non-codon-optimized GFP cassettes were subcloned into either the binary plasmid pPZP211 or pPZP212 (Hajdukiewicz *et al.*, 1994), and the resultant vectors were referred to as pPTN343, pPTN372 and pPTN442, for the 35S, rice actin and *PEPC* promoters, respectively.

A set of maize codon-optimized fluorescent marker genes encoding blue (mzBFP), green (mzGP) or yellow (mzYFP) were commercially synthesized (Genscript Corporation, Piscataway, NJ, USA). The respective markers were assembled in plastid-targeted expression cassettes. The binary vector pPTN448 carries mzBFP ORF under the control of the 1.9 kb *PstI* fragment directly upstream the ATG of the maize *ubiquitin1* promoter coupled with its first intron (Christensen *et al.*, 1992). The mzBFP peptide is targeted to plastid via the pea Rubisco small subunit transit peptide (von Heijne *et al.*, 1991). The binary vectors pPTN458 and pPTN469 are identical to pPTN448, except they harbour the mzGFP and mzYFP ORFs, respectively. Sequences are available through Genbank under accession numbers 1218408 (mzYFP), 1218425 (mzGFP) and 1218426 (mzBFP).

The binary vector designated pPTN512 has the mzYFP ORF under the control of the 1.7 kb maize C4 *PepC* promoter (Yanagisawa and Izui, 1989) coupled with the pea SSU transit peptide. The binary plasmid pPTN533 has the mzYFP ORF under the control of a 0.9 kb region upstream of the initiation codon comprising the maize *RBCS1* promoter (Lebrun *et al.*, 1987; Viret *et al.*, 1994), and is plastid localized via the pea SSU transit peptide, and has the terminator region from the *RBCS1* gene believed to contribute to cell-type specificity (Viret *et al.*, 1994). Finally, the binary vector pPTN629 is identical to pPTN533, except it utilizes the maize *RBCS1* transit peptide (Lebrun *et al.*, 1987). All but the pPTN533 and PTN629 constructs use the standard *Nos* terminator.

Preparation of bundle sheath strands and mesophyll protoplasts

Mesophyll protoplasts were prepared from third and fourth leaf blades after digestion of their cell walls, and bundle sheath

strands were mechanically isolated, as previously described (Markelz *et al.*, 2003).

Confocal microscopy

Confocal laser scanning microscopy (CLSM) for Figure 1 was conducted with an Olympus FV500 (Olympus America Inc. Center Valley, PA USA). GFP and YFP were excited at 488 nm and images detected with a 505–525 nm filter. A 405 nm wavelength was used to excite BFP and images detected with a 430–460 nm filter. Images were captured and recorded using the FluoView 4.3 (Olympus America Inc. Center Valley, PA USA) version software. CLSM for Figures 2,6 and 7 was performed on a Leica microscope equipped with a TCS-SP2 confocal scanning head (Leica Microsystems, Heidelberg, Germany). The 488 and 514 nm lines of an argon laser was used to excite G/YFP and chlorophyll, respectively. Images were recorded and processed using the LCS software 2.5 (Leica Microsystems). For staining with the MitoTracker Red CMXRos (Molecular Probes, Invitrogen, Carlsbad, CA, USA) the root hairs of transgenic maize were immersed for 10 min in a solution of 0.01% Silwet and 10 μM MitoTracker Red. Leaf sections of 3 to 4-week-old plants were prepared manually with a razor blade. CLSM for Figure 1 was performed with an Olympus FV500, at the University of Nebraska's Imaging Core Research Facility (http://biotech.unl.edu/Core%20Facilities/Microscopy/Services/MicroscopyServices.html). CLSM for the leaf sections in Figures 3 and 5 was performed with an Olympus FV1000. The 405, 488 and 515 nm lines of laser were used to excite vascular tissue autofluorescence, chlorophyll autofluoresence and YFP, respectively. The images in Figures 4 and 8 were obtained at the BTI Plant Cell Imaging Center (http://bti.cornell.edu/facilitiesServicesPlantCellImagingCenter-Equipment.php) on a Leica SP5 instrument, described in detail at that web site, following excitation at 458 nm.

DNA and protein blots

DNA gel blots were performed as previously published (Howe *et al.*, 2006). Immunoblotting was performed as described by Wostrikoff and Stern (2007)**.** Antibodies against RbcL and GFP were obtained from Agrisera (Agrisera AB, Vännäs, Sweden) and Roche (F. Hoffmann-La Roche Ltd, Basel, Switzerland) respectively, and HRP coupled secondary antibodies directed against rabbit and mouse were purchased from Promega (Promega Corporation, Madison, WI USA) and Roche (F. Hoffmann-La Roche Ltd, Basel, Switzerland). The PEPC antibody was a kind gift of Dr Michael Salvucci (USDA-ARS, Maricopa, AZ, USA). Antibodies against RbcL and PEPC were used at a 1 : 20 000 dilution, GFP antibody at a 1 : 10 000 dilution, while secondary antibodies were used at a 1 : 10 000 dilution.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Movie S1 Small vesicular structures within a hypocotyl epidermal cell of UBI/TP-mzYFP plants are highly mobile. Time-lapse series of 10 frames, 3 s intervals. Time indicated in seconds.

Table S1 Details of transgenic line analysis.

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