Reliable Transient Transformation of Intact Maize Leaf Cells for Functional Genomics and Experimental Study

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Maize (Zea mays) transformation routinely produces stable transgenic lines essential for functional genomics; however, transient expression of target proteins in maize cells is not yet routine. Such techniques are critical for rapid testing of transgene constructs and for experimental studies. Here, we report bombardment methods that depend on leaf developmental stage and result in successful expression with broad applications. Fluorescent marker genes were constructed and bombarded into five developmental regions in a growing maize leaf. Expression efficiency was highest in the basal-most 3 cm above the ligule of an approximately 50-cm growing adult leaf. Straightforward dissection procedures provide access to the receptive leaf regions, increasing efficiency from less than one transformant per cm² to over 21 transformants per cm². Successful expression was routine for proteins from full genomic sequences driven by native regulatory regions and from complementary DNA sequences driven by the constitutive maize polyubiquitin promoter and a heterologous terminator. Four tested fusion proteins, maize PROTEIN DISULFIDE ISOMERASE-Yellow Fluorescent Protein, GLOSSY8a-monomeric Red Fluorescent Protein and maize XYLOSYLTRANSFERASE, and maize Rho-of-Plants7-monomeric Teal Fluorescent Protein, localized as predicted in the endoplasmic reticulum, Golgi, and plasma membrane, respectively. Localization patterns were similar between transient and stable modes of expression, and cotransformation was equally successful. Coexpression was also demonstrated by transiently transforming cells in a stable line expressing a second marker protein, thus increasing the utility of a single stable transformant. Given the ease of dissection procedures, this method replaces heterologous expression assays with a more direct, native, and informative system, and the techniques will be useful for localization, colocalization, and functional studies.
multiple insertion events, and transgene silencing occurred (Klein et al., 1988; Vasil, 1994; Kumpatla et al., 1997) until new Agrobacterium vectors were designed for use in grasses (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Tingay et al., 1997; Zhao et al., 2000; Manickavasagam et al., 2004). New binary vectors and other technical progress increased efficiency and reduced the number of transgene insertions; subsequently, Agrobacterium-based transformation became routine in maize, although success was limited to a subset of inbred lines (Zhao et al., 2001; Frame et al., 2002, 2006, 2011; Ishida et al., 2003; Huang and Wei, 2004; Zhao and Ranch, 2006; Vega et al., 2008).

Microprojectile bombardment was initially used for transient expression in whole maize tissue (Oard et al., 1990; Reggiardo et al., 1991; Bansal et al., 1992; Schenk et al., 1998; Ivanchenko et al., 2000), but the methods have not yet become routine. Instead, transient gene expression often relies on transformation of protoplasts isolated from various species (Sheen, 2001; Davey et al., 2005; Hay and Spanswick, 2007; Horie et al., 2011; Isayenkov et al., 2011; Jia et al., 2011; Zhou et al., 2011) or by biolistic transformation of onion epidermal cells (Scott et al., 1999; Böhlenius et al., 2010; Wei et al., 2010; Yang et al., 2011; Zhang et al., 2011; Zou et al., 2011). A particle bombardment system was optimized in wheat (Triticum aestivum; Schweizer et al., 1999) and useful for plant pathogen studies in barley (Hordeum vulgare) and other grasses (Dong et al., 2006), but has not yet been successfully mimicked in maize. Transient assays using Agrobacterium infection were reported for Setaria viridis, an emerging C4 grass model system (Brutnell et al., 2010), which may effectively replace the distant onion epidermis as a transient model for maize. A direct maize system is still needed, and experiments in other grasses (Dong et al., 2006) point to the value of developing a species-specific transient assay system.

We describe here a reliable approach for transient expression in maize leaves, permitting more direct study of protein function and localization at higher efficiency. This system supplements protoplast methods and replaces the use of dicots or onion epidermis as platforms for expression, and it allows direct observation of endogenous transient expression in maize. The method can be used to verify predicted protein expression prior to generating stably transformed lines, to demonstrate protein-protein colocalization, and to provide for rapid overexpression and dominant-negative analyses. Transformation efficiency is distinctly increased due to cell development stage; the method thus has broad application if researchers take into account the cellular and developmental context while planning assay experiments.

RESULTS AND DISCUSSION

Maize leaves and roots grow in a predictable gradient with localized regions of cell division, expansion, and differentiation (Fig. 1; after Reynolds et al., 1998; Mitkovski and Sylvester, 2003; Brutnell et al., 2010). The experiments here were based on the assumption that successful transient expression would depend on cellular, developmental, and physiological traits of the targeted tissues. Cellular features along the leaf gradient suggest that the basal region would be most receptive to successful transformation: epidermal cells at the base of expanding leaves are densely cytoplasmic, and they divide and expand rapidly with newly formed primary cell walls (base region; Fig. 1). Transformation would be more efficient due to the high proportion of cytoplasm, early-stage cell walls, and/or the stage of the cell cycle, which may influence proper transcription and translation of the transgene. Farther up the leaf gradient, cells increase in size and contain large central vacuoles, with the cytoplasm and nucleus compressed against the more advanced primary cell wall (middle and tip regions; Fig. 1).

Developing Adult Leaves Are Useful for Transient Assays

Diverse maize tissues were first tested for reliable transient expression by bombardment. Mature juvenile and immature adult leaves were compared with root tips to determine the most effective tissue for establishing a routine procedure. Expression was not observed reliably in nongrowing juvenile leaves or roots during initial experiments, but further study of these tissues is warranted. Since expression was consistently observed after bombardment of still-expanding adult leaves, we focused our subsequent efforts solely on this material (for further description, see “Materials and Methods”). By restricting all transient assays to the same leaf stage, possible variations in cellular development that may influence receptivity to bombardment were minimized.

Figure 1. Developmental gradients in a maize leaf. A maize leaf (at left; not to scale) was divided into five regions: two adjacent regions in the base, a middle region, and two nonadjacent regions at the tip. The distances from the ligule for each region represent different stages in the developmental gradient of the leaf (at right), including a site of cell division immediately above the ligule (left triangle), a gradient of cell expansion (middle triangle), and a gradient of cell differentiation (right triangle).
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Genes Selected for Transient Expression Tests

Several fluorescently labeled proteins (FPs) were selected to develop optimal transient assays in maize tissue. The FPs were prioritized based on confirmed stable expression of the FP in a predicted subcellular compartment, such as the endoplasmic reticulum (ER), Golgi, or plasma membrane (Mohanty et al., 2009a, 2009b). Four FPs were generated and used to compare stable and transient expression in maize: (1) a protein disulfide isomerase tagged with mCitrine (ZmPDI-YFP), biochemically localized in the ER; (2) a β-1,2-xylosyltransferase tagged with monomeric Red Fluorescent Protein (mRFP; ZmXYLT-mRFP), predicted to localize to the Golgi; (3) a β-ketoacyl reductase identified as GLOSSY8a tagged with mRFP (GL8a-mRFP), biochemically associated with the ER; and (4) a small GTPase of the Rho-of-Plants family, ZmROP7, tagged with a monomeric Teal Fluorescent Protein (mTFP; ZmROP7-mTFP), predicted to be localized to the plasma membrane (for further details, see “Materials and Methods”). These proteins were prioritized because they represent a range of both well-confirmed (ZmPDI-YFP and GL8a-mRFP) and predicted (ZmROP7-mTFP and ZmXYLT-mRFP) localization patterns in diverse membrane compartments, as described more fully here.

The ER compartment was marked by ZmPDI-YFP, a well-characterized protein common to all eukaryotes, and GL8a-mRFP, a protein conserved across evolutionarily diverse taxa (Xu et al., 1997). In all systems studied, including plants (Boston et al., 1996; Gupta and Tuteja, 2011), PDIs localize to the ER and catalyze reactions associated with disulfide bond formation, promoting the normal folding of substrate proteins. Due to its conservation of location and function, ZmPDI-YFP was selected for direct comparison between stable and transient expression patterns in maize. GL8a-mRFP functions as a β-ketoacyl reductase responsible for the biosynthesis of the epicuticular waxes found on maize leaves and likely other grasses, a function that is restricted to plants, although the protein has homologs in many eukaryotes (Xu et al., 1997, 2002; Dietrich et al., 2005). Biochemical evidence has shown an association between GL8a and the ER (Xu et al., 2002). Thus, ZmPDI-YFP and GL8a-mRFP can be used to observe the compartmental localization of distinct ER markers.

The Golgi compartment was marked with ZmXYLT-mRFP, a β-1,2-xylosyltransferase that transfers Xyl to the core of N-linked glycoproteins (Bondili et al., 2006). The Arabidopsis (Arabidopsis thaliana) homolog, AtXYLT, which shows 58% identity to ZmXYLT, localizes to the Golgi in transformed, cultured BY2 tobacco (Nicotiana tabacum) cells (Pagny et al., 2003). Golgi in plants is a dynamic and multifunctional organelle system involved in biosynthesis of cell wall carbohydrates and sorting of cargo in the endomembrane system (Törmäkangas et al., 2001; Neumann et al., 2003; Jurgens, 2004). Many fundamental questions about plant Golgi function are unanswered, including mechanisms of trafficking for soluble and membrane proteins and the functional relationship with the ER (Faso et al., 2009); transient assay methods will be particularly useful to answer these questions.

A third membrane compartment, the plasma membrane, was observed using ZmROP7, a member of the plant-specific ROP family of the Ras protein superfamily. ROP proteins have been implicated in pollen tube growth, cell death, and cell wall synthesis (Delmer et al., 1995; Lin et al., 1996; Kawasaki et al., 1999; Yang and Fu, 2007) and were specifically localized to the plasma membrane in maize cells (Ivanchenko et al., 2000). A stable line expressing an mTFP-tagged ZmROP7 (ZmROP7-mTFP) was recently produced as a color complement to other related proteins, and this reporter line is now being studied extensively in leaf tissue, where it shows predicted plasma membrane location of ROP7 in the leaf, in contrast to prior punctate localization observed in the root (Mohanty et al., 2009a, 2009b). These contrasting observations in leaves and roots emphasize that transient expression can be useful for studying localization differences in diverse tissues.

Efficiency of Transient Transformation Is Highest in the Base of Immature, Adult Leaves

Transformation efficiency was studied along the leaf gradient by analyzing transformation success in five discontinuous segments of expanding adult leaves. Each leaf was approximately 50 cm in length with a basal sheath length of only 1 cm or less. The developing leaf was divided into five parts: two adjacent sectors at the base, one sector in the middle, and two sectors at the leaf tip (Fig. 1). Transformation efficiency is an important measure of the reliability of the method and is defined here as the number of successful transformed cells per piece of leaf tissue of a given size, in this case an area of approximately 0.25 cm². The leaf pieces were bombarded with polyubiquitin:ZmXYLT-mRFP, and the number of transformed cells was counted 24 h after bombardment (for details, see “Materials and Methods”).

Transformation was most efficient in the basal region, 0 to 3 cm from the ligule, and decreased up the developmental gradient of the leaf, with a nonsignificant increase at 12 to 18 cm from the ligule (Fig. 2A). At the base, where the transformation frequency is the highest, a median value of 21.5 transformed cells per cm² was calculated, but the values range between 6.25 and 91.67 transformed cells per cm² (Fig. 2A). As little as 3 cm farther along the leaf blade, in the region from 3 to 6 cm, transformation dropped to a median value of less than 1 cell per cm², a level typical for the remainder of the leaf. Although transformation efficiency varied, expression was observed throughout the leaf, albeit at low frequencies in the upper regions (Fig. 2).

Increased transformation efficiency of basal cells can be explained by their developmental stage: cells in the base of leaves are smaller and more cytoplasmically dense with less well-developed primary cell walls and more rapid cell cycling. To determine if cell size
correlates with efficiency, cell areas were measured for each bombarded sample and graphed against the number of transiently transformed cells (Fig. 2B). The average cell size varied in these regions, from approximately 260 \( \mu \text{m}^2 \) in the base to approximately 1,500 \( \mu \text{m}^2 \) by the middle of the leaf. The correlation between transformation frequency and leaf blade location was stronger than the correlation between cell size and transformation frequency (Supplemental Fig. S1), suggesting that leaf blade position is a better predictor of transformation efficiency than cell size alone. This could be explained by other factors such as cellular differentiation, differences in cell wall structure, and/or stage of the cell cycle.

### Transient Expression in Basal and Tip Sectors of a Developing Maize Leaf

Several different constructs were developed, each tailored to a specific experimental purpose. The polyubiquitin::ZmXYLT-mRFP construct was used to test whether a strong, constitutive promoter and an efficient terminator would allow the expression of a complementary DNA (cDNA). All of the other constructs use native regulatory regions to drive the normal expression of genes of interest. Transient expression of polyubiquitin::ZmXYLT-mRFP demonstrates a pattern of small, scattered punctate fluorescence throughout the cytoplasm, with an increased concentration at the cell cortex (Fig. 3A) in a pattern consistent with Golgi colocalization (Satiat-Jeunemaitre et al., 1999). No other identifiable subcellular compartments were apparent in transiently transformed cells, suggesting that this nonendogenous promoter/terminator combination can be used to express this protein as predicted. Figure 3A also shows the use of autofluorescence as a useful counterpoint to observe transiently expressed fusion proteins: the punctate red fluorescence of the transgene is highlighted by the blue autofluorescence of the cell walls. In Figure 3A, the blue autofluorescent guard cells also contain chloroplasts (normally exhibiting red autofluorescence) that appear pink due to the blue wall/red chloroplast merge. Thus, counterstaining of cell walls, and subsequent effects on cell viability, can be avoided when autofluorescence can be used to identify cellular landmarks.

ZmROP7-TFP was bombarded into the base of immature, adult B73 leaves to verify successful transient expression in additional subcellular compartments (Fig. 3B). Fluorescence localized to the cell periphery, consistent with a predicted plasma membrane location from previous reports (Ivanchenko et al., 2000). Figure 3B highlights the use of bright-field observation in cases where blue wall autofluorescence may overlap in wavelength with a fluorophore. In this example, plasma membrane localization cannot be distinguished from cytoplasmic, but specific experimental manipulations such as plasmolysis could be confirmatory.

### Transient ZmPDI-YFP Expression Matches Stable Transformation

One concern with transient expression is the introduction of localization artifacts caused by cell damage during bombardment or by misexpression. To confirm normal localization, stable and transient expression patterns for ZmPDI-YFP were compared. The same binary vector plasmid used for creating the stably transformed line was bombarded into nontransgenic B73 plants, and expression was compared with its...
comparable, stably transformed line. A maximum projection through a partial scan of blade epidermal cells of the stable transformant is shown for comparison (Fig. 3C; as this is a partial scan, the surfaces of all cells are not shown). Here, ZmPDI-YFP appears as a reticulate network localized in the cortical region of the cell (Fig. 3C), consistent with observations of ER structure in plant cells (Satiat-Jeunemaître et al., 1999). In transiently transformed cells, ZmPDI-YFP also appears as a reticulate network in the cortex of adaxial epidermal cells (Fig. 3D). A maximum intensity projection through 16 Z planes of a cell demonstrates that this reticulate pattern is pronounced in the cell cortex (Fig. 3D). In the noncortical regions of the cell, expression is noted in cytoplasmic strands and the perinuclear region (Fig. 3E, arrow). Cell damage and/or autofluorescence are not detected in neighboring cells. The similarity of the fluorescence patterns in stably and transiently transformed cells suggests, for this ER marker protein, that localization was not affected by transient expression.

The maximum intensity projections shown in Figure 3E are useful for simultaneously viewing fluorescence patterns across multiple Z planes. However, localization of the same protein to more than one subcellular region can complicate the interpretation of fluorescence patterns. For example, in Figure 3E, the cortical, reticulate network and the perinuclear and cytoplasmic fluorescence patterns for ZmPDI-YFP are not readily distinguishable. To simplify the visualization of these separate patterns, fluorescence from the same FP, ZmPDI-YFP, was differentially false colored, based on Z location. For example, medial Z planes were colored magenta, while cortical planes near the epidermal surface were shaded green. Thus, the three-dimensional depth of ZmPDI-YFP is emphasized while still being represented in a single image (Fig. 3F). The imaging technique makes it clear that the cortical ZmPDI-YFP at non-epidermal cell surfaces demonstrates a reticulate nature similar to that at the epidermal surface (Fig. 3, compare E and F). Imaging methods such as these optimize viewing of the three-dimensionally rendered object.

Transient Cotransformation to Localize Multiple Proteins

Simultaneous localization of multiple proteins can be used to study protein function and to test for potential interactions. Transient transformation of two proteins together can make the experimental study more efficient. Coexpression from two separate vectors can be accomplished in two ways: first, two or more tagged proteins can be simultaneously transiently expressed by cobombardment of their encoding plasmids; second, a tagged protein can be transiently expressed in a stably transformed maize line expressing a fusion protein.

ZmXYLT-mRFP and ZmPDI-YFP were coprecipitated onto microcarriers and bombarded together into tissue sections from the base of a growing adult B73 leaf (Fig. 4, A–C). ZmPDI-YFP demonstrates the ER-specific reticulate pattern previously observed (compare Fig. 3, C and D, with Fig. 4A). ZmXYLT-mRFP
shows a punctate distribution concentrated at the periphery of the cell, as was described previously (compare Figs. 3A and 4B). The merged image demonstrates that the two proteins are transiently coexpressed in the same cell (Fig. 4C). Notably, the localization of each transiently expressed protein remains independent and apparently correct, reinforcing the conclusion that localization patterns are not altered during simultaneous expression of more than one construct.

Transient Expression in a Stably Expressed Line for Colocalization Study

An important application for a reliable transient expression system is to localize proteins rapidly in a plant that reproduces slowly, such as maize. In addition to the cobombardment, it would be an advantage to colocalize transiently expressed proteins in already stable lines. The stably transformed GL8a-RFP ER marker line confirmed the feasibility of this approach (Fig. 4, D–F). The tagged protein was first stably expressed in maize (Fig. 4E) using methods described previously (Mohanty et al., 2009a, 2009b). A second ER marker, ZmPDI-YFP, as described above, was transiently expressed in the GL8a-mRFP marker line to coexpress two ER markers in the same plant and to test for colocalization. Transiently expressed ZmPDI-YFP showed the expected cortical reticulate networks and perinuclear fluorescence (Fig. 4D). Interestingly, GL8a-mRFP localized in a diffuse pattern through the cytoplasm, with minimal reticulation into networks (Fig. 4, E and F). There are at least two explanations to explain the localization pattern of GL8a-mRFP. First, protein localization may be different during adult leaf development because GL8a functions when juvenile epicuticular wax is being synthesized (Bianchi et al., 1985; Xu et al., 1997). Second, GL8a and ZmPDI may localize to different ER subcompartments, a phenomenon that has been described previously (Staehelin, 1997). Notably, none of the coexpression analyses performed using bombardment have exhibited signs of mislocalization when compared with stably transformed lines.

Further evaluation of the ER localization patterns of the two markers suggests colocalization in the perinuclear and dispersed cortical regions (merging to white or lime-green regions in Fig. 4F). The merged image also shows that the reticulate network at the adaxial epidermal surface shows only ZmPDI-YFP (Fig. 4F). The partial colocalization suggests that the two proteins function or are transported through both distinct and overlapping domains of the ER, but additional work will verify this possibility. These observations support the subcompartmentation hypothesis for the ER and point to the value of using transient bombardment in plant lines that have already been engineered to stably express an FP.

The observation of partial colocalization is intriguing. Further study is needed and can be accomplished by transient expression assays. For example, once GL8a-RFP is proven to be localized and functioning correctly, by complementation tests with a gl8a mutant, further experiments could test whether GL8a-mRFP localizes in a distinct ER domain. This could be a consequence of different biological functions. GL8a is an enzyme involved in wax synthesis (Xu et al., 1997, 2002), whereas PDI is predicted to be a protein chaperone, as in other systems (Boston et al., 1996; Li and Larkins, 1996; Gupta and Tuteja, 2011). Thus, the localization data would clarify if unique ER domains are associated with very-long-chain fatty acids. Up to 16 different functional regions of the ER have been predicted in plants (Staehelin, 1997), and the extent of functional overlap may be resolved in vivo using transient assay methods.

CONCLUSION

Microprojectile bombardment for transient expression assays in maize has been reported previously, but
the methods are not yet routine. Prior attempts may have used only the easily accessible leaf middle and tip regions, where transformation efficiency is low, averaging less than one transformed cell per cm² (Fig. 2A). Using the more efficient basal tissues, transformation success is greatly improved. Differences in transformation efficiency may be due to the developmental state of the cell, which influences various factors including cell wall composition and cell division/cell expansion profiles. For example, wall lignification begins approximately 8 to 14 cm from the ligule, increasing the strength and the rigidity of the cell walls (Vincent et al., 2005). Alternatively, comparatively higher levels of β1,3-β1,4 mixed linkage glucans are found in base tissue (Carpita et al., 2001). The hydrolysis of these compounds is necessary for cell growth (Hoson et al., 1992; Inouhe and Nevins, 1998); these wall features are indicators of the developmental state of cells.

Basal leaf tissue is ideal for the optimal expression of proteins of standard or constitutive expression levels, as shown here. Proteins expressed at low levels, in very specific subsets of cell types, or at distinct phases of the cell cycle would require selected tissue sampling. Transient expression is successful either using a gene's endogenous promoter, terminator, and introns or a strong promoter-terminator combination to drive a cDNA construct, as in the case of ZmXYLT-mRFP. Previous reports show that transient expression in cultured maize cells is improved using an intron for high levels of expression (Callis et al., 1987): the 5′ untranslated region of the polyubiquitin gene contains an endogenous intron (Christensen et al., 1992), making this promoter ideal for expressing cDNA sequences via microprojectile bombardment in the intact maize leaf. Promoter studies are also feasible, by engineering a minimal expression cassette similar to that developed for ZmXYLT-mRFP, but with the entire coding region for the gene of interest. Placing a multiple cloning site upstream of the start codon would facilitate promoter swaps and truncations, yielding information on promoter function in maize and other grasses. Gateway cloning, as used here for ZmPDI-YFP and ZmROP7-mTFP, further increases versatility. Although the overexpression of secreted proteins can alter localization patterns (Brandizzi et al., 2003; Lisenbee et al., 2003), particularly if setting pathways are saturable, ZmXYLT-mRFP appeared to be localized as predicted.

We report here a reliable method for robust and efficient transient expression of fluorescently tagged proteins in intact maize leaf cells of the correct age and size. The method can be used to express genes with their native regulatory sequences, including introns, or as cDNAs using a constitutive promoter and a heterologous terminator. Transient expression is consistent with observations of stably transformed lines and can be used for localization, colocalization, and functional studies. These approaches will complement newly emerging capabilities for functional genomics studies in maize (Mohanty et al., 2009a, 2009b; Penning et al., 2009; Li et al., 2010; Sekhon et al., 2011; Cook et al., 2012). The reliability and utility of the transient assay methods described here are thus essential to exploit fully the maize genome for systems biology study, and the focus on developmental state will have broad application to other plants that may be resistant to transient expression.

MATERIALS AND METHODS

Plant Growth Procedures

Maize (Zea mays) plants from the B73 inbred line were used for transient procedures that did not involve stably transformed lines. For experiments involving stably transformed lines, maize transformation was accomplished by the Plant Transformation Facility at Iowa State University using Agrobacterium tumefaciens-mediated transformation. T0 seedlings were outcrossed at least two generations into a B73 inbred line. Single and stable transgene inserts were confirmed by 50% segregation of the transgene in T1 and T2 generation progeny. Inbred and transgenic seeds were planted in standard fertilized potting soil and grown in a controlled environment at 25°C with a 16-h-light/8-h-dark photoperiod for 2 to 4 weeks to the appropriate L9 stage and L3 stages.

Plasmid Construction

pUBI1-XylTmRFP-Vsp encodes a C-terminally, mRFP-tagged, Golgi-resident β-1,2-xylosyltransferase whose expression is driven by a maize polyubiquitin promoter fragment (Christensen et al., 1992) with the terminator from soybean (Glycine max) vegetative storage protein (Mason et al., 1988). This plasmid was created as follows. pUC-mRFP was generated by inserting a PCR-amplified mRFP into the Xhol and SplI sites of pUC19. pUC-XylTmRFP was generated by subcloning a PCR-amplified β-1,2-xylosyltransferase (GRMZm2G19267; Bondilí et al., 2006) open reading frame from B73 cDNA into the KpnI and Xhol sites of pUC-mRFP. pUC-Vsp was created by inserting a PCR-amplified soybean Vsp terminator sequence from pTF101.1 (generously provided by Dr. Kan Wang) into the SplI and HindIII sites of pUC19. pUBI1-Vsp was generated by subcloning the PCR-amplified polyubiquitin promoter from pAH25 (generously provided by Dr. Kan Wang) into the Saci and KpnI sites of pUC-Vsp. The final construct, pUBI1-XylTmRFP-Vsp, was generated by inserting a KpnI-SplI fragment from pUC-XylTmRFP into pUBI1-Vsp. pTF101.1-gb-mRFP was generated by triple-template PCR methods as described previously (Mohanty et al., 2009a, 2009b) using CjLsa (Xu et al., 1997) and the mRFP fluorescent protein genes (Campbell et al., 2002). pTF101.1-ZmPDI-YFP was generated using a MultiSite Gateway three-way cloning method (Invitrogen) with the ZmPDI1-1 (GRMZm2G091485; Houston, et al., 2005) and mCitrine fluorescent protein genes (Heikal et al., 2000), as was pTF101.1-ZmROP7-mTFP, which used a modified cyan fluorescent protein derivative (Ai et al., 2006). Additional details and protocols can be found at http://maize.jcvi.org/cellgenomics/protocol.shtml.

All plasmids were sequence verified after steps involving PCR or by restriction mapping after steps involving ligation or propagation. All final constructs were sequence verified.

Preparation of Tissue Samples for Microprojectile Bombardment

Approximately 0.25-cm² tissue samples were cut from plant leaves for each experiment. With the exception of experiments testing differences in transformation efficiency based on tissue maturity, all tissue tested was within 3 cm of the ligule and not yet greened. Tissue was obtained as follows. First, freshly grown, age-appropriate (between six and 13 leaves emerged), greenhouse-grown plants were cut transversely through the stalk at the level of the soil. Outer leaves were sequentially removed until the final emergent leaf was discarded. Once nonemergent leaves were obtained, the first of these leaves was generally discarded and the subsequent nonemergent leaf was selected for use, based on the position of the ligule. Attention was focused on a developing leaf that contained 1 cm of sheath tissue and a partly developed ligule fringe.

The 1 cm of sheath tissue was discarded. Material within 3 cm of the developing ligule was preferentially used for all assays, except in quantitative
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experiments testing for the efficiency of transformation as a function of age. Dissections were conducted in a small pool of water to prevent dehydration of the tissue. After being cut into approximately 0.25-cm² pieces, leaf samples were distributed densely, adaxial side up, on Murashige and Skoog agar plates (4.4%, w/v; Sigma-Aldrich). Plates were bombarded within 1 h of dissection. For experiments testing the effect of developmental stage on transformation efficiency, the same procedure was used except that samples of 0.25 cm² were cut at the positions described in Figure 1. Tissue was separated by developmental stage, placed on Murashige and Skoog agar plates, and bombarded within 1 h of dissection. Experiments using particular cell types for transformation would require harvesting tissue from age-appropriate times and locations.

Microprojectile Bombardment

DNA was prepared by commercially available column purification in either miniprep or maxiprep formats (Qagen) or by classical alkaline lysis followed by cesium chloride-ethidium bromide isopycnic gradient purification (Sambrook et al., 1989). Equal concentrations of DNA, as determined by both spectroscopy and analytical gels, showed no difference in transformation efficiency (Supplemental Fig. S2).

All bombardments were performed with Bio-Rad Tungsten M-10 microcarriers using 1 μg of DNA precipitated onto mirocarriers (Bio-Rad; max 107 microcarriers) per bombardment. Procedures, with slight modifications to the manufacturer’s instructions, were as follows. First, 20 mg of microcarriers was measured in 1.5-mL microcentrifuge tubes, washed for 5 min with vigorous mixing in 1 mL of 70% ethanol, soaked for 15 min, and then pelleted by microcentrifugation for 10 s. The pelleted microcarriers were washed for 1 min with vigorous mixing in 1 mL of sterile water three times and then resuspended in 1 mL of sterile 50% glycerol. Microcarriers were stored at –20°C until use.

Prepared microcarriers were vortexed vigorously for 5 min prior to dividing into aliquots to break up any agglomerates; 50 μL of prepared microcarriers was then transferred to a fresh microcentrifuge tube and mixed continuously using a platform vortexer with the following added sequentially: 6 μL of DNA at a concentration of 1.0 mg mL⁻¹ and 50 μL of 25 mM CaCl₂, with each addition mixed for 1 min. Finally, 20 μL of 0.1 M spermidine was added and allowed to mix for 5 min. Microcarriers and DNA were centrifuged for 15 s, and the pellet was resuspended and washed in 140 μL of 70% HPLC-grade ethanol twice using 100% HPLC-grade ethanol during the final stage. The microcarriers were resuspended in 50 μL of 100% HPLC-grade ethanol, and 8 μL was transferred to macrocarriers (Bio-Rad) for subsequent bombardment. Macrocarriers were covered to prevent the accumulation of dust while ethanol evaporated and then used for bombardment.

All bombardments were performed with a PDS-1000/He system (Bio-Rad). Rupture discs (1.350 p.s.i.; Bio-Rad) were used to build system pressure. Tissue samples were situated 9.7 cm from the macrocarrier stopping screen, and no other sample screen was used. Helium pressure at the tank was regulated at 1,600 p.s.i. Vacuum pressure within the chamber was allowed to reach at least 20 inches of mercury prior to firing. After bombardment, tissue was allowed to recover for 16 to 24 h at 22°C in the dark (to limit tissue greening) prior to analysis.

Confocal Microscopy and Image Analysis

Bombarded tissue samples were mounted in water and imaged using a 63×1.4 numerical aperture objective on a Zeiss LSM710 microscope using 514- and 561-nm laser lines with ZEN 2010 software (Zeiss). Normal emission spectra of FFs and autofluorescence were narrowed using the ZEN 2010 software function to autoselect emission range, thus avoiding overlapping emission. The normal emission of cell wall autofluorescence ranges between 350 and 550 nm. This spectrum was narrowed to 425 to 500 nm to avoid coemission with the lower emitting wavelengths of mYFP, which normally emits from 500 to 625 nm. The mYFP fluorescence emission spectrum was also narrowed to 525 to 560 nm to ensure no overlap with the cell wall. mTFP emission, normally from 450 to 600 nm, was narrowed to avoid cell wall autofluorescence. The normal emission spectrum for mRFP is from 560 to 750 nm. Chlorophyll autofluorescence, which normally emits between 620 and 800 nm, was avoided by narrowing the emission for mRFP to 570 to 615 nm. Images were analyzed and manipulated using MetaMorph Software (Molecular Devices) or ImageJ software (National Institutes of Health).

Data Analysis

Statistical analysis and final data presentation were prepared using Microsoft Excel and the R Statistical Programming Language (R Foundation for Statistical Computing). Regression values were calculated using a least-squares method in Microsoft Excel. Student’s t tests were performed on transformation frequency data using R, and outliers were defined as data greater than 1.5 times the inner quartile range as determined by the graphing function.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Tissue location correlates with transformation efficiency.

Supplemental Figure S2. Transformation efficiency is independent of DNA purification method.

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