Understanding Regulatory DNA in Plants Through Molecular Cloning and Transient Transformation

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Cesar Juarez

Abstract

Gene expression in plants (and other eukaryotes) is a very complex process. Gene expression is primarily controlled by proteins and their binding sites near the gene in the chromosomal DNA. More specifically, interactions among transcription factors (DNA-binding proteins) and *cis*-regulatory modules (CRMs-sets of binding sites) control the expression of nearby genes. The Fowler lab has been working towards understanding how CRMs work by using an *in vivo* quantitative method of examination. By establishing a plasmid construct enabled for Golden Gate Cloning with a fluorescent gene reporter, and a method to analyze the CRMs via biolistic transformation, further understanding of how these CRMs work alone or with different combinations can be achieved. Slight variation was found in quantitative data with biolistic transformations from day to day, but the method has promise as a quick and stable way of analyzing sequences of interest. Some validation for the method of examination was accomplished, but further research is needed to characterize the CRMs of interest.

Introduction

The expression of genes in plants (and other eukaryotes) is a complex process that is not completely understood. Eukaryotic cells contain chromosomes, composed of DNA, and encoding genes that provide functions that are crucial for life. The chromosomes, and their associated genes, are arranged in a chromatin complexes that can allow or inhibit transcription at a particular gene, depending on how tightly the DNA is wound. Transcription is the first step in the complex process of gene expression, followed by producing a protein (translation), which can then carry out some function (e.g., an enzymatic activity) to influence an organism's

biology. In transcription, DNA is copied into mRNA (a transcript) by an RNA polymerase. Thus, transcription can not only be influenced by chromatin, but also by factors that more directly regulate RNA polymerase activity (e.g., how rapidly the polymerase is brought to the gene's transcription stat site). The default setting for genes in eukaryotic cells is "off", so many factors such as promoters, transcription factors, binding sites, and other signaling proteins, aid in controlling the expression of accessible genes.

Gene expression is primarily controlled by proteins and their binding sites near the gene in the chromosome's DNA. Transcription factors (TFs) are specific proteins that bind to DNA. The regions of DNA to which the TFs bind are called *cis*-regulatory modules (CRMs) (Lelli *et. al.,* 2012). The interactions among TFs and their CRMs control the expression of nearby genes. CRMs, in general, can serve as promoters, enhancers, silencers, and insulators. CRMs are often upstream from the gene of interest, but the proximity can range from relatively close to a few hundred kb (Jeziorska *et al.,* 2009). Promoters and enhancers are usually associated with positive effects on transcription, whereas silencers have negative effects. Insulators help insure that CRM effects are limited to particular genes (Raab and Kamakaka, 2010). TFs act as the protein messengers, in combination with the binding sites in CRMs. Each TF protein finds its correct, specific binding site, and then, either on its own, or in combination with other nearby TFs, will interact with other proteins (e.g., RNA polymerase) that affect the expression of the nearby gene.

Currently there are relatively few *cis*-regulatory modules that have been characterized in plants. One primary current method of identifying CRMs is by using bioinformatics and computational analysis. Information is obtained though genome sequences, large-scale gene expression studies (so-called "transcriptomics"), protein structures, molecular interactions and functional information of molecular pathways (Jones *et al.,* 2006). While bioinformatics methods have helped discover and classify the putative functions of different, hypothesized CRMs, additional methods are needed to test and validate them.

One goal of the project was to further the understanding of gene regulation and CRMs using an *in vivo* method. The overall goal is to understand how gene expression is controlled through associated regulatory DNA in two grass species, maize (corn) and sorghum. Putative regulatory DNA (i.e., a possible CRM) was previously identified via bioinformatics analysis (Freeling *et al.,* 2015). Our objective was to test these putative CRMs via transient transformation of a reporter construct into plant cells, to validate their ability to influence expression of a reporter gene.

Promoters are defined as molecular DNA regions that initiate transcription of nearby genes. Promoter regions have been able to be tested by linking them to reporter genes (e.g., Park *et al.,* 2010). Reporter genes are visual markers that serve to indicate transcription in regulatory gene studies (Mann *et al.,* 2012; Ruijeter *et al.,* 2003). One type of reporter gene is a protein that has autofluorescence, and can be viewed under a fluorescent microscope.

The reporter genes used in this study are tdTomato-ER and GFP-ER. The reporter tdTomato-ER is an orange fluorescent protein that is directed to the endoplasmic reticulum (ER) (Mann *et al.,* 2012) and fluoresces when excited by green light. Green fluorescent protein (GFP-ER) is characterized by its bright green fluorescence when illuminated by blue light or UV light (Ruijter *et al.,* 2002) and is also directed to the ER. In the method to be tested here, GFP-ER and tdTomato-ER are used in a dual fluorescent reporter system that allows for direct comparison of the fluorescence signal between the two proteins in a single plant cell. The two fluorescent reporter genes are cloned into identical vectors, but with different promoters, and then co-transformed, which allows for ratio analysis of the expression of the reporters (i.e., orange signal to green signal). Ideally, by keeping one reporter plasmid (the internal control) constant across all transformations, the effects of changing the promoter/CRM sequences on the expression of the other fluorescent reporter can be determined. Similar methods have been used to study yeast and mammalian cells (Kainth *et al.,* 2009; Hua *et al.,* 2012), but haven't been used on plants to date.

Promoters and reporter constructs can be made using molecular cloning techniques. Recent drastic improvements are due to the understanding of restriction and ligation enzymes. The main purpose of molecular cloning is to isolate a specific segment of DNA and insert it into a cloning vector, usually a plasmid that bacteria can replicate. After replication, plasmid DNA clones can then be harvested from the bacterium and used for an array of experiments.

Traditional molecular cloning is now simple enough to make it a common practice in many labs attempting to study DNA, genes and gene regulators. However, complications and inconveniences of traditional molecular cloning still exists, including time consumption, errors in DNA fragment addition, and expensive kits. A new method of cloning, called "Golden Gate Cloning" has recently been introduced (Engler *et al.,* 2008). It is advocated as a highly efficient process that has the possibility to work for large-scale cloning projects.

The main attraction of the Golden Gate Cloning method is its efficient and rapid manner of combining multiple DNA fragments in a vector, in order to produce a desired multicomponent construct. Golden Gate Cloning substitutes site specific enzymes for Type IIS restriction enzymes, such as BsaI (Engler *et al.,* 2008; Lampropoulos *et al.,* 2013; Emami *et al.,* 2013). The Type IIS restriction endonucleases cleave the DNA at a single site just outside of their recognition site and create a 5' or 3' overhang. Golden Gate Cloning applies a specific method of cleaving which makes the process a one-way route to the desired vector (Fig. 1). Golden Gate Cloning Type IIS sites (e.g., *BsaI*) are strategically arranged so that when the enzyme cleaves at the recognition site, the inside fragment and the recognition site are cut out. This method of cleaving ensures that the plasmid cannot be re-cut after the desired product is formed.

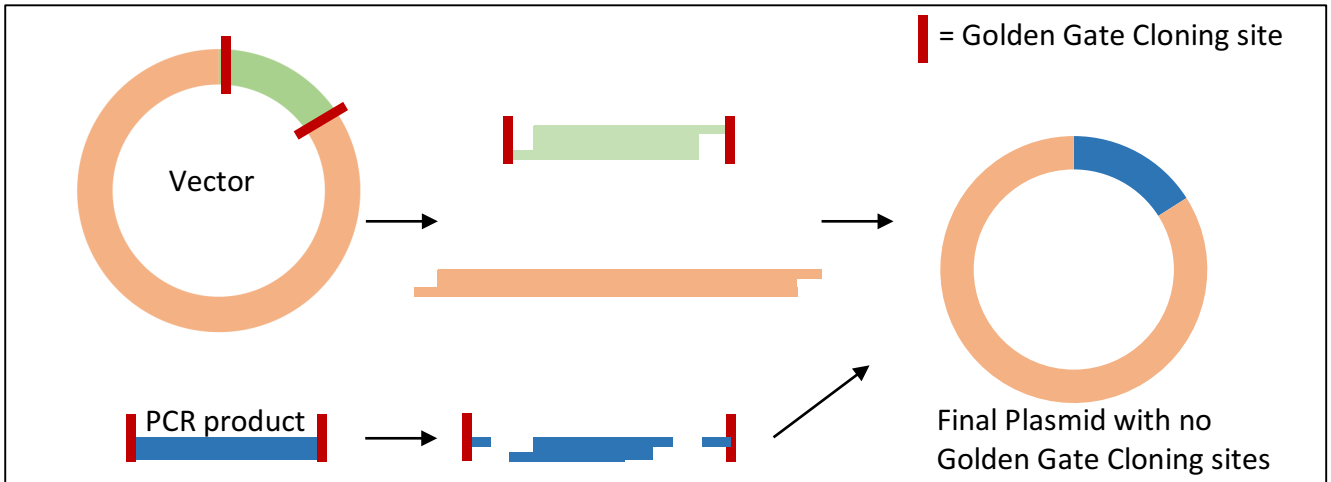

Fig. 1 Schematic representation of Golden Gate Cloning. Type IIs restriction enzymes recognize their appropriate restriction site (red) and cleave DNA, leaving a four base "sticky end" overhang, while simultaneously eliminating the recognition site from the intended sequence. Elimination of recognition sites allows for ligation enzymes and restriction enzymes to be added into the mixture together, which makes the cloning a one-step reaction since the final construct cannot be re-cut. PCR products and destination vectors can be designed to have compatible sites at their ends, and can be applied to multiple fragments for synchronous addition.

Promoter/reporter constructs can be tested by using a transient transformation protocol established for maize cells (Ivanchenko *et al.,* 2000), but are also potentially useful in other plant species (e.g., sorghum). The protocol can have some variation, depending on the type of cell transformed (leaf, root, or pollen), but is ultimately very similar. Positively charged micro-particles attract the negatively charged plasmid DNA constructs and are delivered into plant cells (Heiser, 1992). A gene gun (also called a biolistic delivery system) is used to shoot tungsten particles (coated with DNA) at a high velocity onto exposed immature leaf cells. Successful delivery of DNA into the leaf cells is determined by epifluorescence microscopy, which detects expression of the bombarded fluorescent protein construct. Fluorescent cells are imaged digitally, followed by computational analysis of the signal data. Turnover times between transformations and analysis is very quick (less than 24 hours). Similar techniques have been

applied to studies on regulatory proteins with strawberries and onions (e.g., Angius *et al.,* 2004).

The two main objectives of this project were: (1) to generate a plasmid for quicker and more efficient method of cloning CRMs in the Fowler lab (via Golden Gate Cloning) and (2) to validate the effectiveness of a dual fluorescent reporter system.

Results

Construction of a Golden Gate Cloning Plasmid with a Fluorescent Reporter

The desired Golden Gate Cloning construct included two *BsaI* restriction sites just upstream of fluorescent tdTomatoER reporter, and a *LacZ*α gene in between the two *BsaI* restriction sites (Fig. 2c). In the Golden Gate Cloning Strategy, the location of the *BsaI* sites (which are Type IIS restriction enzyme sites) ensures the removal of the *LacZ*α fragment in conjunction with the addition of the desired CRM sequences upstream of a fluorescent reporter. The inserted *LacZ*α gene gives a quick reference as to how well the cloning worked, as bacterial colonies that turn blue in the presence of the indicator X-gal still contain the *LacZ* gene, whereas bacterial colonies that are white do not (and thus should contain the CRM sequences).

To generate such a construct, an initial three-step cloning scheme was devised, starting from pBS-tdTomatoER, a tdTomatoER plasmid with a2x35S promote. A schematic diagram showing the *BsaI-LacZ-BsaI* component of this strategy is shown in Fig. 2a. This *LacZ*α fragment (Fig. 2a) was obtained as a synthesized double-stranded DNA fragment (Eurofins Genomics), PCR-amplified, and then cloned into the pCR2.1 cloning vector.

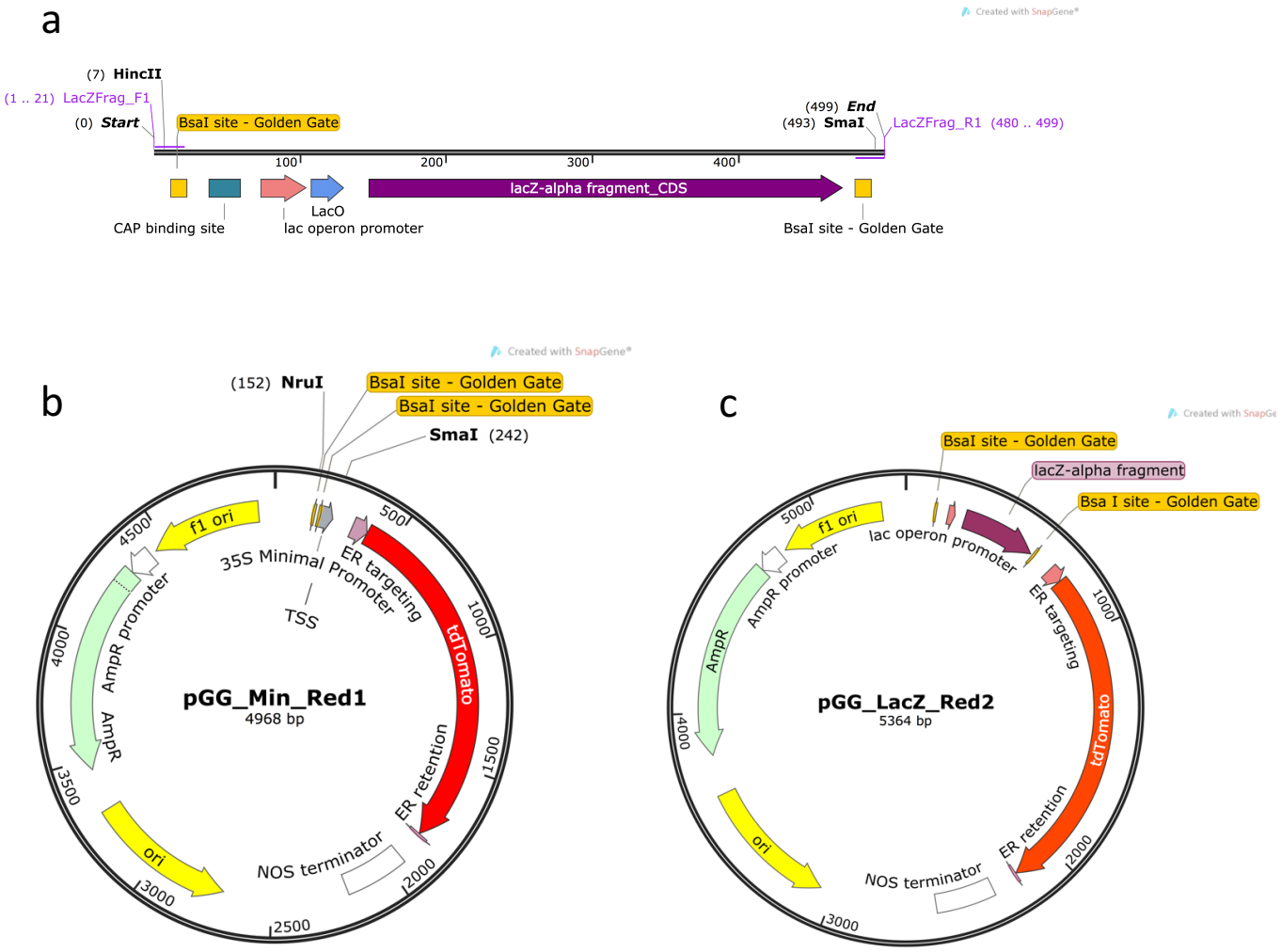

Fig. 2 Schematic diagram of the construction of the desired "Golden Gate Cloning" plasmid. a-c depicts the plasmid construction. (a) The 499 bp *LacZ*/Golden Gate fragment was designed with Golden Gate (*BsaI*) sites on opposite sides of the *LacZ-alpha* gene. (b) pGG_Min_Red1 has Golden Gate sites (*BsaI*) upstream of the 35S minimal promoter, where enhancers and other potential CRMs can be inserted. The 35S minimal promoter serves as an essential sequence required for binding of certain transcription factors that direct transcription initiation at correct location, but it does not lead to robust transcription alone, as it requires additional sequences (e.g., CRMs that act as enhancers). The Transcription Start Site (TSS) notes the start of the tdTomato-ER transcript, and the NOS terminator stops transcription at the end of the tdTomato-ER gene. ER targeting signals are at the N-terminal and C-terminal ends of the tdTomato protein. (c) The final reporter plasmid, pGG_*LacZ*_Red2, with the *BsaI* sites, *LacZ* and tdTomato-ER genes.

The *LacZ-alpha* fragment is 499bp that serves as a reporter gene by turning bacteria blue in the presence of Xgal and *LacZ-omega* counterpart. After an EcoRI digest, a gel electrophoresis confirmed that a fragment of the correct size was successfully cloned into multiple colonies (Fig. 3a). Further confirmation was obtained when these bacteria were streaked on an ampicillin LB plate with Xgal and IPTG, which produced blue cells, thus containing they contained a functional LacZ fragment (Fig. 3b).

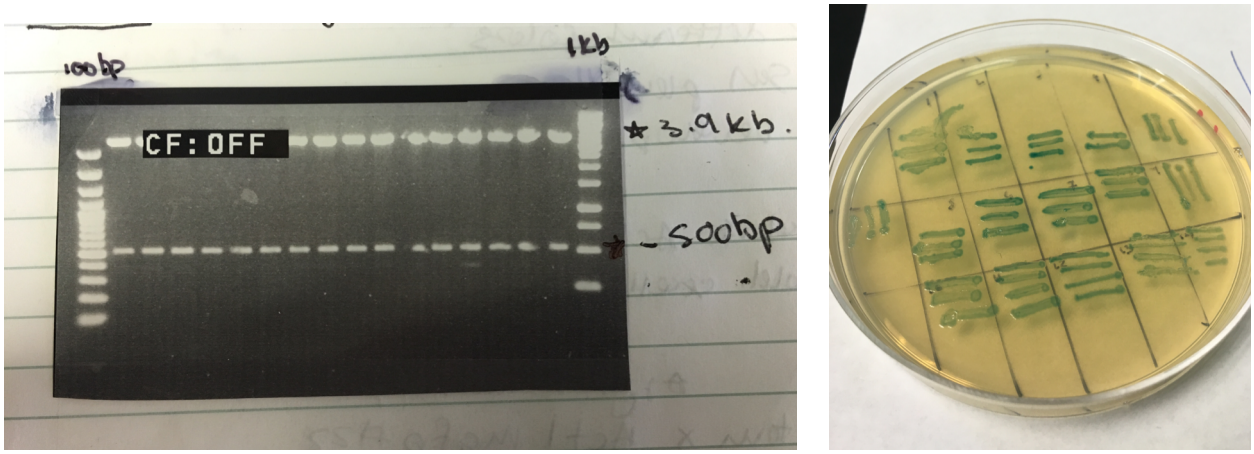

Fig. 3 Images show confirmation of cloning the correct fragment (*LacZ*) into the pCR2.1 vector. Image a shows that the expected band sizes of 3.9kb and 500bp were obtained after an EcoRI digest. Image b shows that the *LacZ* fragment is present in the bacterial DNA by turning the bacterial culture blue after Xgal and IPTG were spread on the plate.

Cloning attempts in the second step of the initial three-step strategy with pBS-tdTomato-ER generated plasmids with unexpected restriction patterns (data not shown). Sequencing of these plasmids revealed a mistake: the pBS-tdTomato-ER reporter construct had been generated in pBluescript KS, not pBluescript SK as had been recorded. This incorrect plasmid designation and the resulting reversal of the orientation of the tdTomato-ER reporter eliminated the initial

cloning strategy, and therefore a secondary strategy was devised, resulting in the pGG_Min_Red1 plasmid (Fig. 2b). This plasmid contains two *BsaI* sites (for Golden Gate Cloning), tdTomato-ER reporter gene, NOS terminator and ampicillin resistance gene. pGG_Min_Red1 plasmid was devised to serve as the backbone for insertion of the *LacZ* fragment, intended to form the final construct, pGG_*LacZ*_Red2 (Fig. 2c).

Biolistic Transformation

In order to use the intended CRM reporter constructs, there needs to be an effective method in place to measure transcriptional activities of CRMs of interest. Biolistic transformations can be used to identify and characterize genomic sequences. An understanding of how CRMs function can be obtained by transforming two different reporter constructs into maize leaves.

Test constructs made by using either Golden Gate tdTomato-ER constructs (Fig. 2b and c) can be tested against control constructs expressing green fluorescence when introduced into plant cells, which will allow comparison between the two reporters in the same cell. Red and green signals can be imaged and quantified separately which allows for comparisons between ratios.

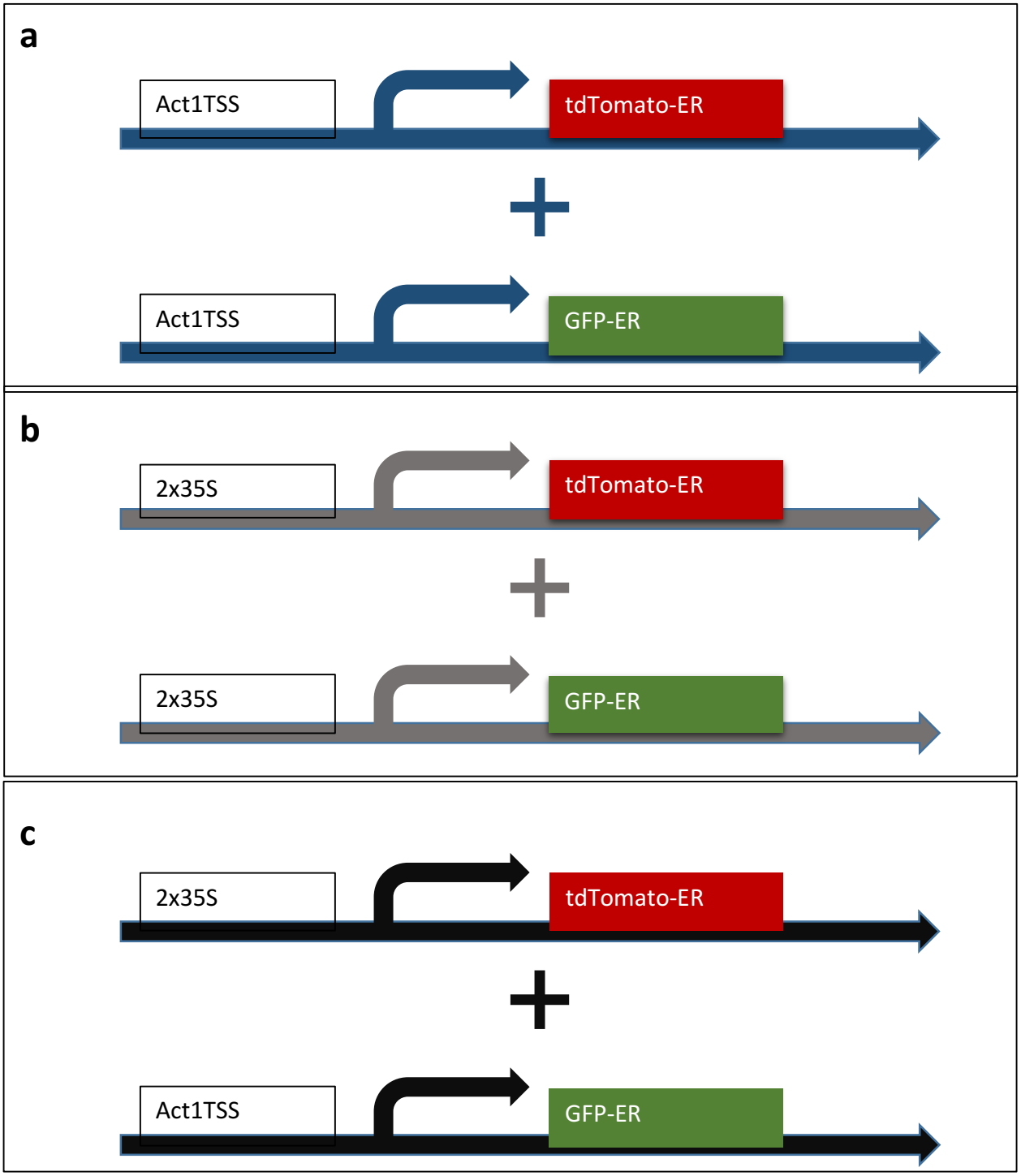

Fig. 4 Three combinations of constructs used in co-bombardments. Diagrams a-c show the promoters and their associated fluorescent protein reporters are depicted on a line, representing plasmid DNA. The black boxes identify promoter sequences, and the color boxes (red and green) represent which reporter gene was used. (a) Act1TSS promoter with both tdTomato-ER and GFP-ER reporter genes. (b) 2x35S promoter with both tdTomato-ER and GFP-ER reporter genes. (c) 2x35S promoter with the tdTomato-ER reporter, and Act1TSS promoter with GFP-ER reporter.

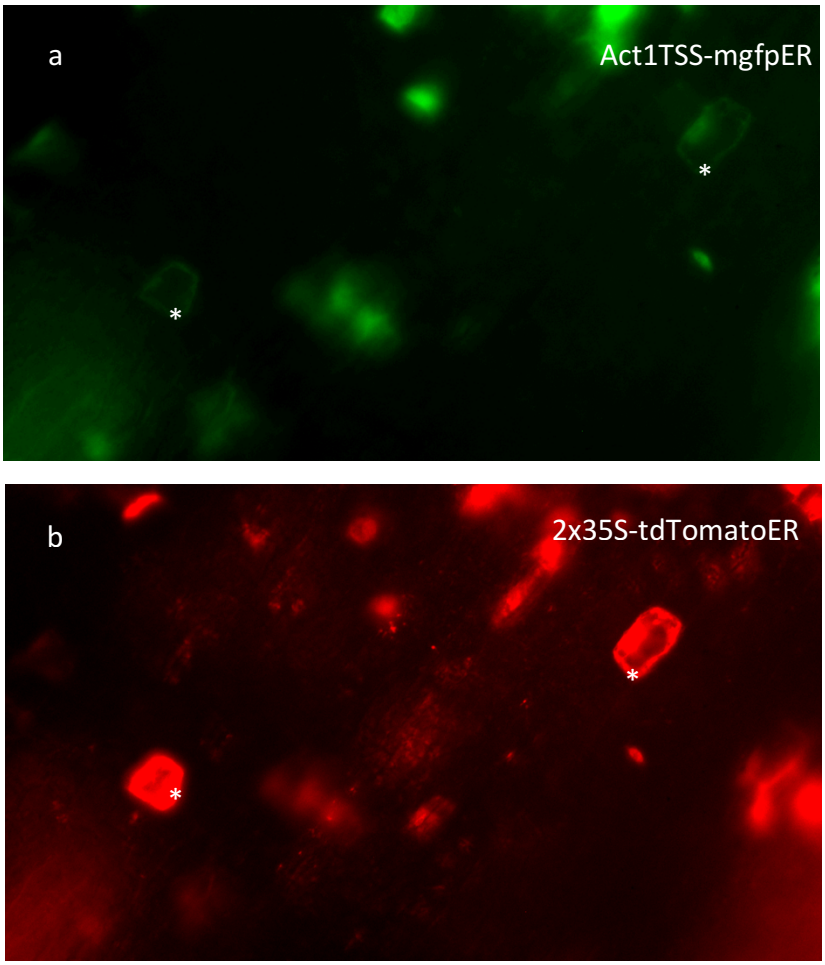

Fig. 5 Representative images showing results from a biolistic bombardment. (a) Green and (b) red fluorescent imaging of the same area of leaf tissue. Asterisks identify two cells that were transformed and analyzed, each showing green and red fluorescence due to expression of their corresponding protein (mgfpER or tdTomatoER). Both show a fluorescence pattern within the cell consistent with ER localization, which helps differentiate them from cells auto-fluorescing due to bombardment damage. Minimum, Maximum, and Mean fluorescence signal quantities were collected from the two imaged cells.

As an initial test of the methodology, existing plasmids were used, with two different, well-understood strong promoters (2x35S and Act1TSS) (Fig. 4). The 2x35S promoter, which functions in monocot leaves, contains two direct repeats of the entire cauliflower mosaic virus 35S promoter (Mann *et al.,* 2012). The Act1TSS promoter contains the entire regulatory region upstream of the transcription start site of the rice *Actin1* gene, which, like the 2x35S promoter, confers strong expression in monocot cells (McElroy *et al.,* 1990). In general, biolistic transformation of immature maize leaf cells resulted in zero to fifty fluorescing cells per leaf

primordium bombarded (data not shown). Successfully transformed cells could be identified by the pattern of fluorescence in the cell, due to the reporter directing the fluorescent proteins to the endoplasmic reticulum, which lies within the plasma membrane and in strands around the nucleus and throughout the cell (Fig. 5). The fluorescent signals from two independent experiments were analyzed quantitatively, and the mean ratios of at least three different transformed cells from five different leaves were used for these comparisons.

The initial biolistic transformations were used to compared the ratio of red brightness intensity over green brightness intensity for two co-bombardments, in this case with two different strong promoters driving expression of mgfpER (Fig. 6). The two co-bombardments evaluated were: 2x35S-tdTomatoER vs 2x35S-mgfpER and 2x35S-tdTomatoER vs Act1TSS-mgfpER. One plasmid, with the 2x35S-tdTomatoER reporter, was used in both co-bombardments and served as an internal control. The idea was that the co-bombardment would allow the possibility to detect differences between a control ratio (both red and green fluorescence driven by the 2x35S promoter), and a test ratio (the 2x35S promoter driving the red fluorescence, compared to the Act1TSS promoter driving green fluorescence. The expectation for this experiment was that these two co-bombardments would produce a significant difference in red/green ratios, given a likely difference in the transcriptional activity from the 2x35S and Act1TSS promoters. Although the resultant mean ratios were 2.95 and 2.02 respectively, suggesting that the two promoters have different activity levels, the difference between these two co-bombardments was not significant ($p=0.076$).

Results for the second biolistic transformation again compared the ratio of red brightness intensity over green brightness intensity. Two co-bombardments were assessed: 2x35S-tdTomatoER + 2x35S-mgfpER and Act1TSS-tdTomatoER + Act1TSS-mgfpER (Fig. 7). The expectation was that the two co-bombardments would produce similar ratios, because the same promoter was used to drive both the red and green fluorescence in each co-bombardment, with the two different promoters in separate bombardments. However, the mean ratios for these bombardments were 1.19 and 0.49 respectively, indicating a significant difference ($p=0.006$).

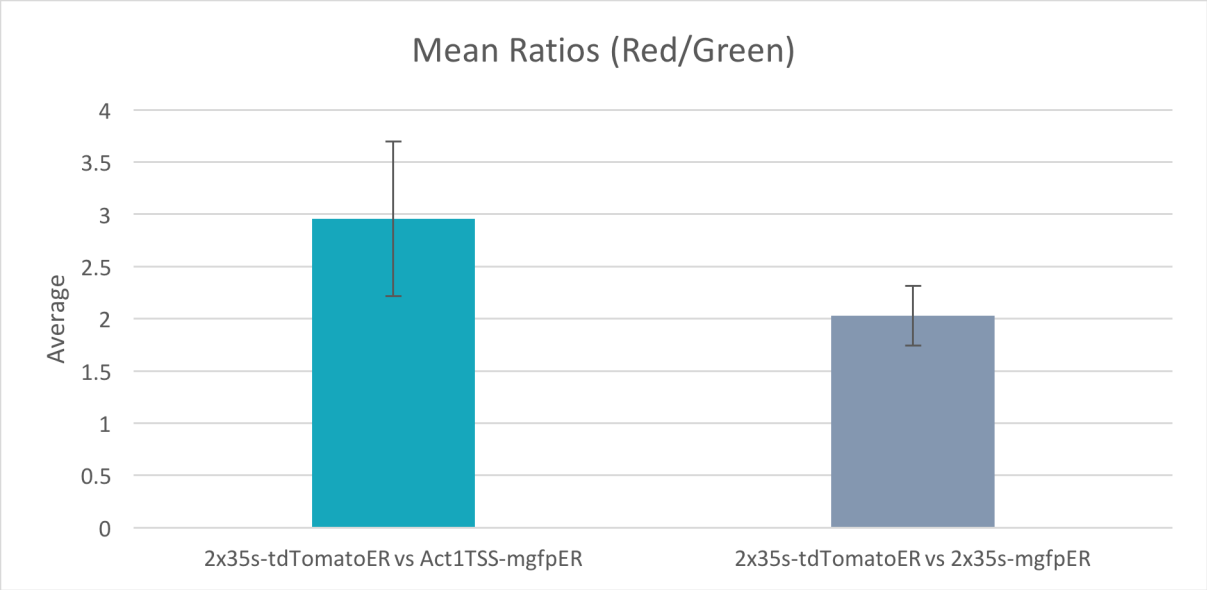

Fig. 6 Graph shows the results from two co-bombardment experiments. Promoter construct 2x35S served as a control. We wanted to see the difference between two reporter constructs, 2x35S-tdTomatoER and Act1TSS-mgfpER. The ratios resulted in 3.79 for the test bombardment, 2x35S-tdTomatoER vs Act1TSS-mgfpER, and 2.05 for the control bombardment, 2x35S-tdTomato vs 2x35S-mgfp.

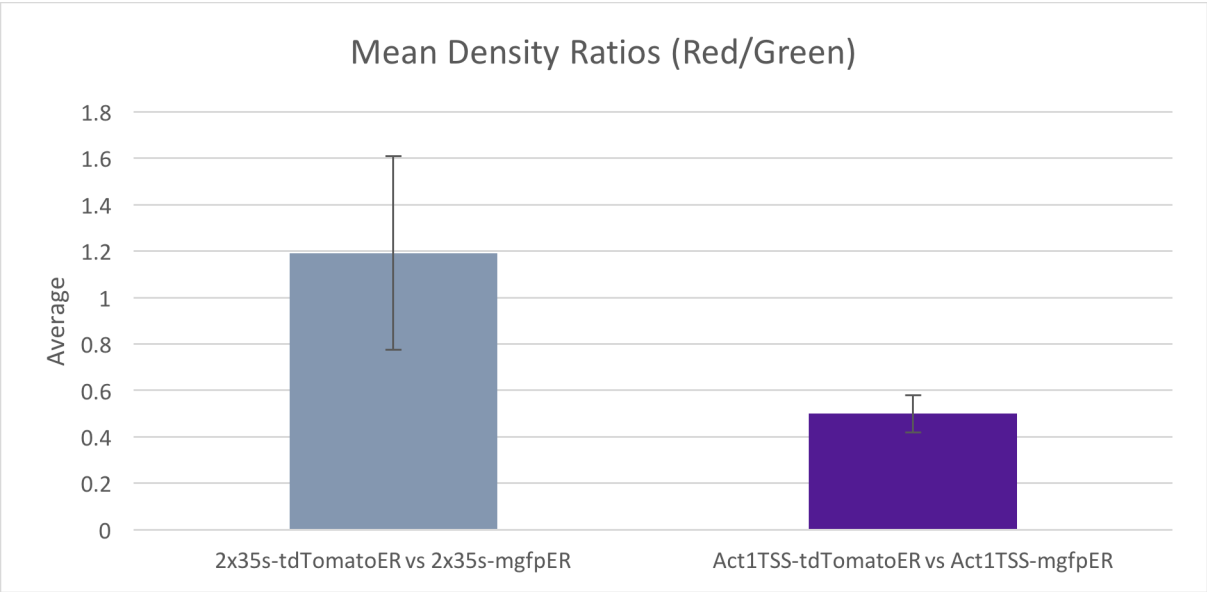

Fig. 7 Graph shows the results from two co-bombardment assays done with promoters 2x35S and Act1. They were tested against their promoter counterpart with different reporter gene sequences (tdTomatoER and mgfpER). Ratios resulted in 1.47 and 0.48 for the 2x35S and Act1TSS assays respectively

Finally, a comparison between the two bombardments that used the exact same plasmids (2x35S-tdTomatoER vs 2x35S-mgfpER) on different days was determined, to test whether ratios differed significantly depending on the day. The hope was that the ratio values would be fairly similar, but ratios were 2.03 and 1.19 (Fig. 8), with a significant difference (p=0.014) despite the use of the same plasmid mixture.

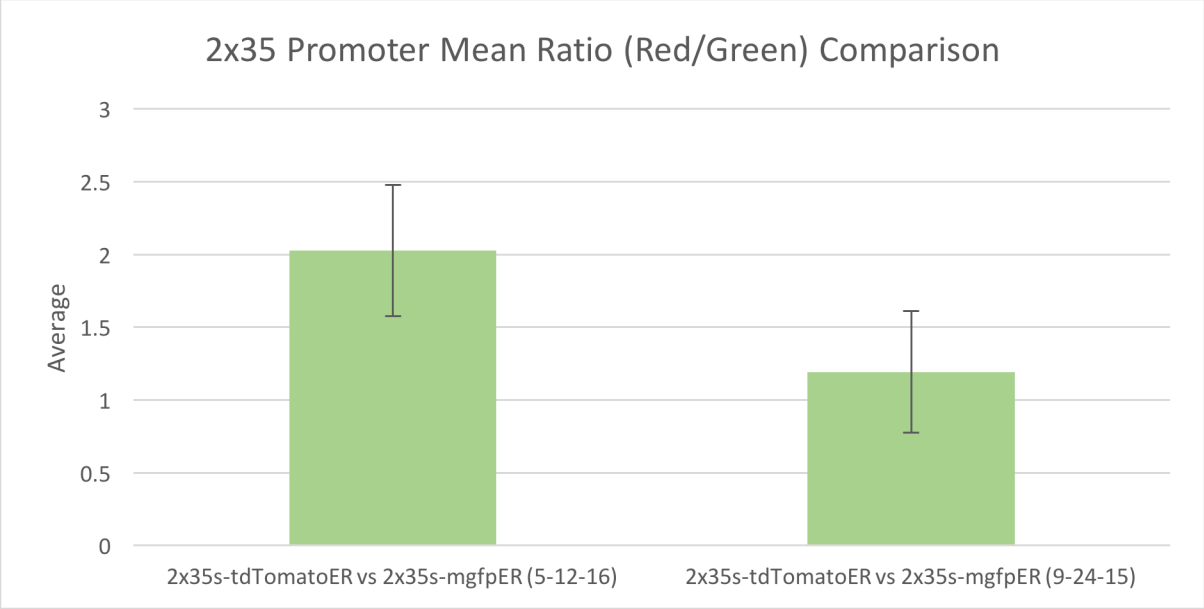

Fig. 8 Comparison between two co-bombardments, performed on different days, which utilized promoter 2x35S for both tdTomatoER and mgfpER. Results between the two varied, with differences in ratios of 0.83 and 0.0319 in 95% confidence intervals.

Discussion

This project explored the use of biolistic transformation with dual fluorescent reporters as a method of measuring transcriptional activity, and it helps inform the development of this methodology. First, successful transformations were apparent because of the number of cells

that fluoresced within a leaf, and the localization of fluorescence. The reporters used protein signals to direct the fluorescence the endoplasmic reticulum of leaf cells, which results in clear localized fluorescence inside of the cell but excluded from the nucleus and the tonoplast, an example of which is found in Fig. 5. We also suspect that stress can have an effect on the success of the procedure, because we found that stressed plants had leaf cells that fluoresced green at an unusually high level when damaged. During one experiment (data not shown), examination of bombarded leaf cells under the fluorescent microscope revealed a high amount of damaged areas, which prevented accurate quantification of fluorescence from transformed cells. Because the seedlings from which these leaves were dissected appeared to be somewhat unhealthy and were producing pigments, I believe that plant stress could have played a role in the increased damage. Thus, I would want to ensure that any future experiments utilize healthy maize plants in order to avoid unwanted fluorescence.

Another unexpected complication that was found during the analysis process is that too much fluorescence of a cell can lead to skewed data. The digital camera used (Qimaging) can only distinguish up to 4096 levels of light, and anything brighter maxed out at that number. Data for cells that reached this maximum had to be discarded in order to obtain a correct interpretation of brightness intensity ratios. If cells that reached the saturation point were left in the data, the outliers would change the mean, and thus it would be less representative of the true value of the cell. Future imaging should be more discriminating when selecting cells. By selecting cells that don't saturate the digital camera, we can get a better interpretation of cell fluorescence.

Analysis from the biolistic transformations indicate that there are significant differences between two of the three comparisons. Most notably, the p-value of 0.006 indicates that there is a significant difference between the ratios obtained in the 2x35S-tdTomatoER + 2x35S-mgfpER and Act1TSS-tdTomatoER + Act1TSS-mgfpER experiment. This difference (more than 2-fold) was unexpected, as we assumed that since we were testing identical promoters against each other, there would not be a strong difference. One possibility is that there might be some unexpected interactions between promoter and reporter genes, leading to unforeseen results.

We were not sure whether the ratios derived from the same promoter combination (2x35S-tdTomatoER + 2x35S-mgfpER) on separate days would be different. However, the statistical analysis resulted in a p-value of 0.014, which supports the idea that these ratios can be different from day to day and experiment to experiment. These differences could be due to slight variation of materials or procedure during each day (e.g., health of plants, materials used, shelf-life of DNA). The data indicates that there could be some discrepancies from day to day transformations, but more trials need to be done in order to reach a clear conclusion.

Constructing the Golden Gate Cloning plasmid is still underway. The success in cloning the *LacZ* fragment into pCR2.1 allows us to move on to the second step of cloning the *LacZ* fragment into pGG_Min_Red1. This will create the final Golden Gate (pGG_LacZ_Red2) construct and will allow us to test its efficiency and usefulness in the biolistic transformation assays. It will also be important to test both the base pGG_Min_Red1 and pGG_LacZ_Red2 constructs in the biolistic assay, as neither should lead to expression of significant red fluorescence. It is predicted that

having these two final constructs will ease the addition of different CRM sequences to the reporter. Alongside the improvements to the methodology based on my work, this will hopefully allow robust testing of a number of putative CRMs.

Experimental Procedures

DNA Fragment Design and Molecular Cloning

A 499 bp *LacZ* fragment (Fig. 2a) was synthesized with flanking *BsaI* sites (as in Emami *et al.,* 2013), and synthesized as a double-stranded DNA fragment (Eurofins Genomics). This fragment was amplified via PCR with Platinum Taq, and then cloned into the pCR®2.1 plasmid, using premade vector from the TA Cloning®kit with the manufacturer's protocol (Thermo Fischer). Transformed cells were plated on ampicillin LB plates and incubated overnight. For the pGG_Min_Red1 construct, a tdTomato-ER reporter gene (Mann *et al.,* 2012) with the 35S CaMV minimal promoter (-46 to +6 bp) and a pair of *BsaI* sites upstream of the gene was designed. This 'Golden Gate'/promoter/reporter fragment was inserted into the pSKB- plasmid, a pBluescript derivative in which the endogenous *BsaI* site had been eliminated (Volohon*sky et al.,* 2015), and later validated via sequencing (Custom DNA Constructs, University Heights, OH).

Bacterial Transformation and Culture

For standard molecular cloning experiments, 1μl ligation mixtures were added to 50μl of *Escherichia Coli* (E. coli) cells prepared in the Fowler lab. Bacterial cell mixtures were transferred to electroporation cuvettes and electroporated at 2500V, 200Ω, and 25uF. One milliliter of SOC media was added to the cuvette and then transferred to a 2mL microtubule for

shaking at 200RPM for 1hr at 37°C. Afterwards the mixture was plated onto ampicillin LB plates and incubated at 37°C for 16 hours. Plasmid DNA was extracted from AmpR bacterial strains following overnight growth at 37°C growth with 300RPM shaking in 2mL SOB media containing ampicillin. The QIAGEN Mini Prep kit protocol was used isolate DNA plasmids in order to assess clones via restriction digests. Standard restriction digest, PCR, and gel electrophoresis protocols were used for initial analysis of clones; select clones were sequences via the Sanger method at the OSU center for Genome Research and Biocomputing (cgrb.oregonstate.edu/core/sanger-sequencing). The QIAGEN Midi Prep kit was used to extract larger quantities of DNA for biolistic transformation.

Biolistic transformation

Plant extraction and transformation protocols were modeled after Ivanchenko (Ivanchenko *et al.,* 2000). Using aseptic technique, one month old, inbreed maize plants (W22 line) were dissected, and immature leaves were obtained from the base of the plant, directly above the apical meristem. Leaf sizes ranged from 5mm-15mm and were placed on MS (Murashige and Skoog) plates (about 10 per plate). Culture leaves were subjected to an osmotic treatment for 2-3 hours for increased transformation success (Vain *et al.,* 1993). A PDS-100 helium biolistic system (Bio-Rad, Hercules, CA, US) was used to perform transformations. Bio-Rad protocols were followed with the use of tungsten 17 micro particles and helium pressure of 1100ps.

Biolistic transformations that were performed consisted of three co-bombardments using plasmids created in the Fowler lab by Zuzana Vejlupkova (Fig. 4).

1. Act1TSS-tdTomato + Act1TSS-GFP (fig. 4a)
2. 2x35S-tdTomato + 2x35S-GFP (fig. 4b)
3. 2x35S-tdTomato + Act1TSS-GFP (fig. 4c)

Analyzing fluorescence

Sixteen to twenty hours after biolistic transformations the bombarded leaves were examined with a Zeiss Axiovert S100 fluorescent microscope. 15 cells per transformation (three cells from five different leaves) were imaged for red and green fluorescence (Fig. 5). Images were taken by a Qimaging camera and analyzed computationally by Image-pro Plus (Media Cybernetics, Rockville, MD), a computer program that characterized each individual fluorescent cell based on fluorescent intensity.

REFERENCES

Agius, F., Amaya, I., Botella, M.A. and Valpuesta, V. (2005) Functional analysis of homologous and heterologous promoters in strawberry fruits using transient expression. J Exp Bot, 56, 37–46.

Emami, S., Yee, M.-C. and Dinneny, J.R. (2013) A robust family of Golden Gate Agrobacterium vectors for plant synthetic biology. Front Plant Sci, 4, 339.

Engler, C., Kandzia, R. and Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS ONE, 3, e3647.

Freeling, M., Scanlon, M.J. and Fowler, J.E. (2015) Fractionation and subfunctionalization following genome duplications: mechanisms that drive gene content and their consequences. Curr Opin Genet Dev, 35, 110–118.

Heiser, W. (2000) Optimization of biolistic transformation using the helium-driven PDS-1000/He system, Bio-Rad Laboratories.

Hua, L., Li, M., Sun, X., Wang, J., Li, Z., Xu, Y., Hu, S. and Chen, H. (2012) A dual color fluorescent reporter system for the real time detection of promoter activity. Biotechnol Lett, 34, 823–830.

Ivanchenko, M., Vejlupkova, Z., Quatrano, R.S. and Fowler, J.E. (2000) Maize ROP7 GTPase contains a unique, CaaX box-independent plasma membrane targeting signal. Plant J, 24, 79–90.

Jeziorska, D.M., Jordan, K.W. and Vance, K.W. (2009) A systems biology approach to understanding cis-regulatory module function. Semin Cell Dev Biol, 20, 856–862.

Jones, D.T., Sternberg, M.J.E. and Thornton, J.M. (2006) Introduction. Bioinformatics: from molecules to systems. Philos. Trans. R. Soc. Lond., B, Biol. Sci., 361, 389–391.

Kainth, P., Sassi, H.E., Peña-Castillo, L., Chua, G., Hughes, T.R. and Andrews, B. (2009) Comprehensive genetic analysis of transcription factor pathways using a dual reporter gene system in budding yeast. Methods, 48, 258–264.

Lampropoulos, A., Sutikovic, Z., Wenzl, C., Maegele, I., Lohmann, J.U. and Forner, J. (2013) GreenGate - A Novel, Versatile, and Efficient Cloning System for Plant Transgenesis. PLoS ONE, 8, e83043.

Lelli, K.M., Slattery, M. and Mann, R.S. (2012) Disentangling the many layers of eukaryotic transcriptional regulation. Annu Rev Genet, 46, 43–68.

Mann, D.G.J., Abercrombie, L.L., Rudis, M.R., Millwood, R.J., Dunlap, J.R. and Stewart, C.N. (2012) Very bright orange fluorescent plants: endoplasmic reticulum targeting of orange fluorescent proteins as visual reporters in transgenic plants. BMC Biotechnol., 12, 17.

McElroy, D., Zhang, W., Cao, J. and Wu, R. (1990) Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell, 2, 163–171.

Park, S.H., Yi, N., Kim, Y.S., Jeong, M.-H., Bang, S.-W., Choi, Y.-D. and Kim, J.-K. (2010) Analysis of five novel putative constitutive gene promoters in transgenic rice plants. J Exp Bot, 61, 2459–2467.

Raab, J.R. and Kamakaka, R.T. (2010) Insulators and promoters: closer than we think. Nat Rev Genet, 11, 439–446.

Ruijter, N.C.A., Verhees, J., Leeuwen, W. and Krol, A.R. (2003) Evaluation and Comparison of the GUS, LUC and GFP Reporter System for Gene Expression Studies in Plants. Plant Biol (Stuttg), 5, 103–115.

Vain, P., McMullen, M.D. and Finer, J.J. (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep, 12, 84–88.

Volohonsky, G., Terenzi, O., Soichot, J., et al. (2015) Tools for Anopheles gambiae Transgenesis. G3 (Bethesda), 5, 1151–1163.