

Plant-derived insulator-like sequences for control of transgene expression

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Abstract

Stable and consistent transgene expression is necessary to advance plant biotechnology. Stable expression can be achieved by incorporating enhancer-blocking insulators, which are *cis*-regulatory elements that reduce enhancer interference in gene expression, into transgene constructs. Sufficient insulators for plant use are not available, and their discovery has remained elusive. In this work, we computationally mined the compact genome of *Utricularia gibba* for insulator sequences and identified short (<1 kb) sequences with potential insulator activity. Based on *in vivo* tests, three of these effectively mitigate the ectopic transgene expression caused by the Cauliflower Mosaic Virus 35S promoter and do so better than previously reported plant insulators. However, all sequences with apparent insulator activity also decrease the effectiveness of the CaMV 35S promoter, and thus may be more accurately classified as silencers. However, since the insulator effect is proportionately much higher than the silencing effect, these sequences are still useful for plant transformation.

Main

Insulators are *cis*-regulatory elements that reduce interference in gene expression. Such cross-interference with transgenes is problematic for crop development as the demand for crops with multiple transgenes continues to grow. Of the 191.7 million hectares dedicated to growing genetically engineered crops in 2021, 42% were planted with crops with stacked traits, meaning multiple traits conferred by transgenes [1]. These mostly were obtained by crossing individual transgenics together. Going forward, multiple transgenes would be ideally placed in one locus to facilitate downstream breeding, but such molecular stacks are difficult to achieve while maintaining proper control of gene expression.

Like other organisms, plants are hypothesized to have insulators based on their evolutionarily conserved genome function and organization, and on gene expression patterns that hint of insulator function [2]. Nevertheless, plant insulators lack sequence conservation to known insulators and have been notoriously difficult to identify and isolate. Furthermore, insulators can be confused with silencers. Insulators must be placed between the enhancer and the promoter. In contrast, silencers are another type of element that retains function independently of its orientation or location [3]. Regardless, the lack of compact and dependable insulators is becoming recognized as a limitation for plant biotechnology applications when enhancers are present.

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Enhancers are cis-regulatory transcription elements that are bound by transcription factors to increase transcription of a gene and are position and orientation-independent. The presence of enhancers can trigger unwanted enhancer-promoter interactions, thereby disturbing the tissue-specificity and strength of promoters in transgenic plants [4]. Enhancers work to bridge distances by forming chromatin loops to bring the enhancer and promoter gene into proximity [5]. The commonly used plant promoter Cauliflower Mosaic Virus 35S promoter (CaMV 35S) is a prime example of this phenomenon (Figure 1); it has been shown to activate nearby tissue-specific promoters and cause ectopic expression with nearby transgenes, even in genes up to 4.3-kb way from the T-DNA integration site [6].

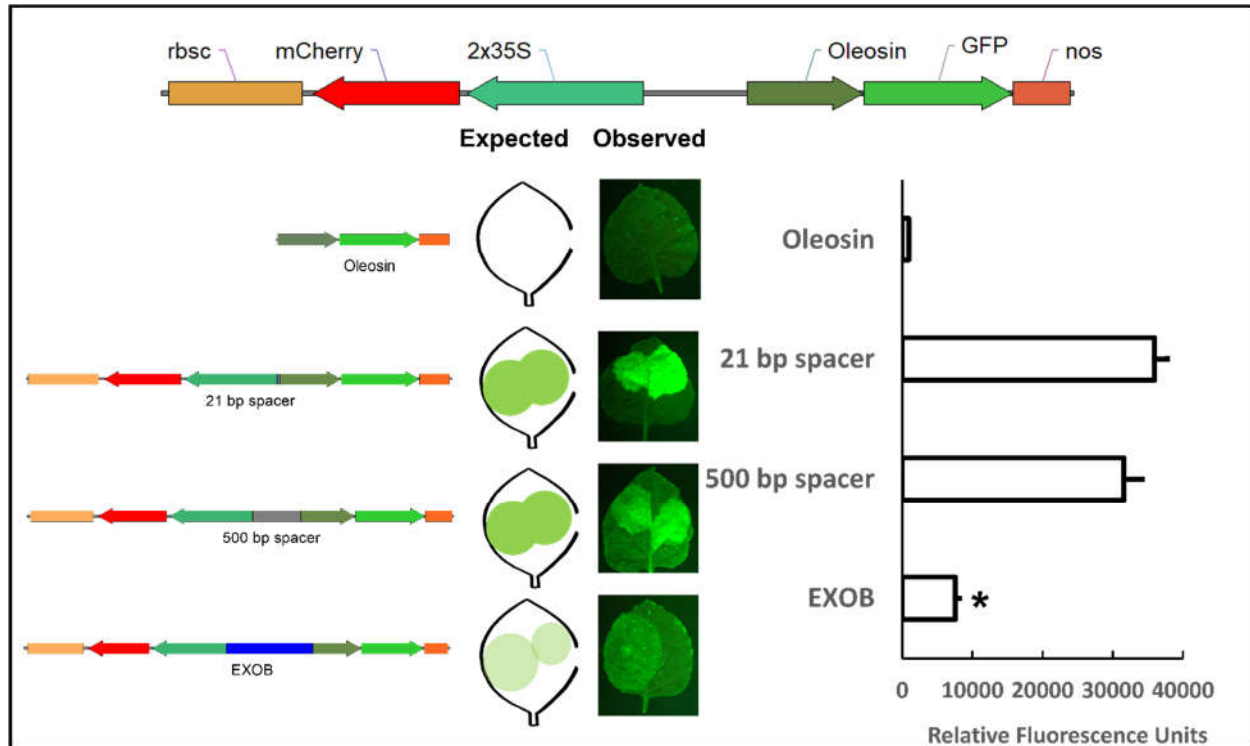


Fig 1. Test construct design, GFP fluorescence in infiltrated *Nicotiana benthamiana* leaves, and representative leaf images. rbsc: rubisco small subunit terminator from *Pisum sativum*, 2x35S: duplicated 35S promoter, Oleosin: *Glycine max* oleosin promoter, nos: Nopaline synthase terminator. Construct names denote the sequence between the 2x35S and Oleosin promoter. Lack of GFP expression is indicative of insulator activity. In this figure, placing the 35S:mCherry cassette in front of oleosin:GFP leads to ectopic expression of GFP. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Insulators have been well-characterized in animals and are classified into two groups: enhancer-blocking insulators, which interfere with enhancer-promoter interactions when placed between them [7], and scaffold/matrix attachment regions (S/MARs), which can protect a flanked transgene by preventing the spread of a heterochromatin state and the gene silencing it causes [8]. Numerous S/MARs have been isolated from plant species [9], but flanking a transgene with these S/MAR sequences does not consistently reduce transgene expression variability [4, 10], and in some cases, it even increases interference [9].

The 2-kb TBS (Transcription Booster Sequence) from *Petunia hybrida* was among the first plant insulator sequences reported based on its effectiveness in both *Arabidopsis* and tobacco [11, 12]. The same group serendipitously discovered EXOB, a 1-kb sequence from phage *Lambda* [11] and found that a 2.2-kb *gypsy*-like sequence from *Arabidopsis* with effective insulator activity [13]. Similarly, Gudynaite-Savitch et al, 2009 [14] also reported that an approximately 900-bp sequence from *Drosophila* (Fab-7 PRE) sequence had insulator function in *Arabidopsis*. However, to be useful in plant transformation, insulators must be as short as possible, as the large size of these insulators limits their usefulness in multigene cassettes by making the cassettes too unwieldy to be readily transformable.

Shorter insulators have been reported, including a 154-bp LTR sequence from the HIV-1 virus [10, 11], and NI29, a 16-bp fragment from *Arabidopsis* [12]. There is a 185-bp element within the HSP70 promoter that is critical for promoter activity but also has some insulator activity [15]. NI29, the 16-bp insulator from *Arabidopsis*, was an ideal and promising insulator because of its small size; however, it does not consistently block the influence of the CaMV 35S promoter, and in some cases, it actually increases ectopic expression [12, 14].

Two other short non-plant-derived insulators, BEAD1c (538 bp) from *Homo sapiens* and UASrpg (234 bp) from the fungus, *Ashbya gossypii*, have demonstrated insulator function in *Arabidopsis* [14]. These partially retain their insulator function when inverted [16], although their insulator ability depends on the genes involved. The use of these insulators in plants has not been studied beyond the initial report.

Sequencing and bioinformatics approaches that have been used to mine plant genomes for novel *cis*-regulatory elements [17, 18] can be extended to insulators. Towards that end, *Utricularia gibba*, or humped bladderwort (Figure 2), is a promising source of insulators. Bladderwort is an aquatic species of carnivorous weed found throughout North America, and it has one of the smallest and most compact genomes among angiosperms: flow cytometry places the genome at ~88 Mb [19] while a recent genome assembly is ~102 Mb in length and contains 29,666 genes [20]. The discrepancy could be due to intra-species variation or uncollapsed regions of the genome assembly.

For comparison, the genome of the model species *Arabidopsis* is 135 Mb long and has 33,252 genes [20]. The bladderwort genome contains a smaller-than-average amount of intergenic sequence relative to other sequenced angiosperm genomes. Despite this small amount of intergenic sequence, regulation of genes is tightly controlled, with many genes showing greater than 100-fold differences in expression levels despite being separated by fewer than 1 kb [20].

Bladderwort has comparatively small intergenic sequences, which comprise only 50% of its genome versus 85% in tomato and 95% in maize. From the published PacBio genome assembly, it is evident that 62% of gene pairs are less than 1 kb apart, and of these gene pairs, many show a > 50-fold difference in expression despite being adjacent [21]. All of these traits make bladderwort a promising candidate for mining novel and compact *cis*-regulatory elements.

The bladderwort genome was computationally mined for putative insulator elements. Bladderwort was re-sequenced, building upon the existing annotation [20]. Three-prime RNA-seq data were used to mine the intergenic regions associated with the presence of insulator

elements, producing a ranked list for the top 10 candidate sequences (Table S1). This list was based on several criteria: (1) the magnitude of expression differences between neighboring genes, (2) evidence of independent regulation, and (3) size of the intergenic region, with small regions preferred over large ones.

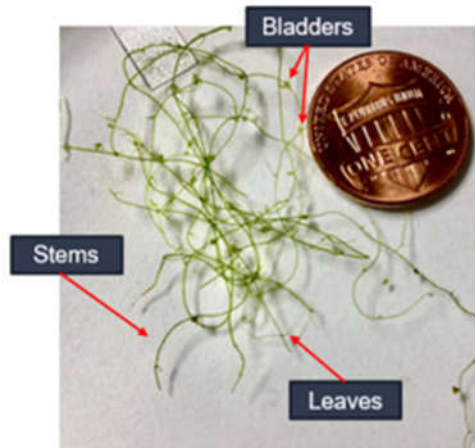


Fig 2. *Utricularia gibba* stems, bladder, leaves, and growth architecture.

Results

Genome assembly and annotation

U. gibba has two public genome sequences, an initial Illumina assembly [21] and a later improved assembly based on PacBio reads [20] from the same accession collected from Michoacán, Mexico. After several attempts to get a sample of this material failed, we obtained live *U. gibba* from a public resource and used Illumina sequencing to create a draft assembly of this specific accession, which we call “PP01”. Although our draft assembly is more fragmented than the published sequence, the final gene count and BUSCO score—a measure of identified conserved genes [22]—are essentially the same (Table S2). This indicates that our assembly covers most of the known gene space.

Identifying potential insulators

To identify potential insulators, we performed 3' RNA-seq on four different tissues: rhizoid (a root-like tissue), stem, leaf, and bladder. The resulting gene expression data were combined with the genome annotation to look for putative insulators between gene pairs. Our criteria for flagging intergenic regions as potential insulators depended on the gene orientation of the genes in the pair and expression correlation (or lack thereof) across tissues. We required both genes to have at least some expression to avoid false calls due to misannotations, and at least one gene to be in the top 50% of expressed genes overall to enrich for stronger insulators and avoid calling ones based only on stochastic noise. Putative insulators were flagged based on uncorrelated gene expression between the pair. In total we identified 43 putative insulators passing our thresholds, 16 of which showed conservation across asterids based on whole-genome alignment. We prioritized short elements (<1000 bp) over longer ones as our goal was to identify insulators useful for biotechnology, not to perform an exhaustive annotation of these elements in the genome.

The test system

To investigate the effectiveness of the 10 identified putative bladderwort insulators, we developed a series of test vectors (Table S3). Each vector contains two reporter genes cassettes: one with GFP driven by the *Glycine max* oleosin promoter, a tissue-specific promoter that is not expressed in *Nicotiana benthamiana* leaves; the second cassette is in the opposite orientation and consists of the duplicated CaMV 35S promoter (2x35S) that drives the expression of the mCherry reporter gene. In control vectors, a minimal amount of spacer DNA (21 bp) separates the two cassettes. The absence of insulator activity results in the enhancer in the 2x35S promoter causing ectopic expression of GFP in tobacco leaves [14] (Figure 1). In other vectors, the cassettes are separated by an approximately 500-bp spacer or by the sequence being tested for insulator activity. Effective insulators will stop enhancer interference from the 2x35S promoter and block GFP expression in leaves. The *U. gibba* insulators were also compared to the HIV-LTR, TBS, and EXOB sequences [11, 23], all of which have been reported to demonstrate insulator activity in plants. Two animal insulators that have been reported to work in plants (BEAD1c and UASrpg) [14, 16] were compared in our test system as well.

Spacer DNA does not consistently block enhancer interference from the 2x35S promoter

Enhancers are known to work at distances as far away as several Mb [24]. Yet, in some cases, ~3 kb between the 35S promoter and tissue-specific promoters can dampen ectopic interference [14, 25] in combination with some promoters, perhaps suggesting the presence of a silencer in the sequence. To test this, we compared the difference in ectopic GFP expression between the 21-bp spacer DNA construct and the 500-bp spacer DNA construct, with 500 representing a practical upper size limit. As evident from Figure 1, adding 500 bp between the cassettes did not lead to significant differences in GFP expression ($p=0.094$).

Bladderwort insulators

Three standout bladderwort insulators were identified that consistently resulted in little to no GFP expression across three separate trials; data from the final trial are shown in Figure 3. These are referred to as Ugi1, Ugi3, and Ugi4, with Ugi standing for *Utricularia gibba* insulator. Ugi1 showed GFP levels comparable to the EXOB insulator ($p=1.5E-08$). The insulator activity of Ugi4 is the strongest one we have observed so far ($p=2.7E-08$).

The function of some animal insulators and of the TBS insulator is orientation-dependent and therefore is more effective in one orientation than when reversed [26, 27]. To test if this is also the case with the identified bladderwort insulators, the reverse complements of Ugi1, Ugi3, and Ugi4 were evaluated. Insulator activity as shown by GFP expression was reduced for all three insulators, though the reverse complement of Ugi3 still reduced ectopic GFP expression relative to that of the control (Figure 3).

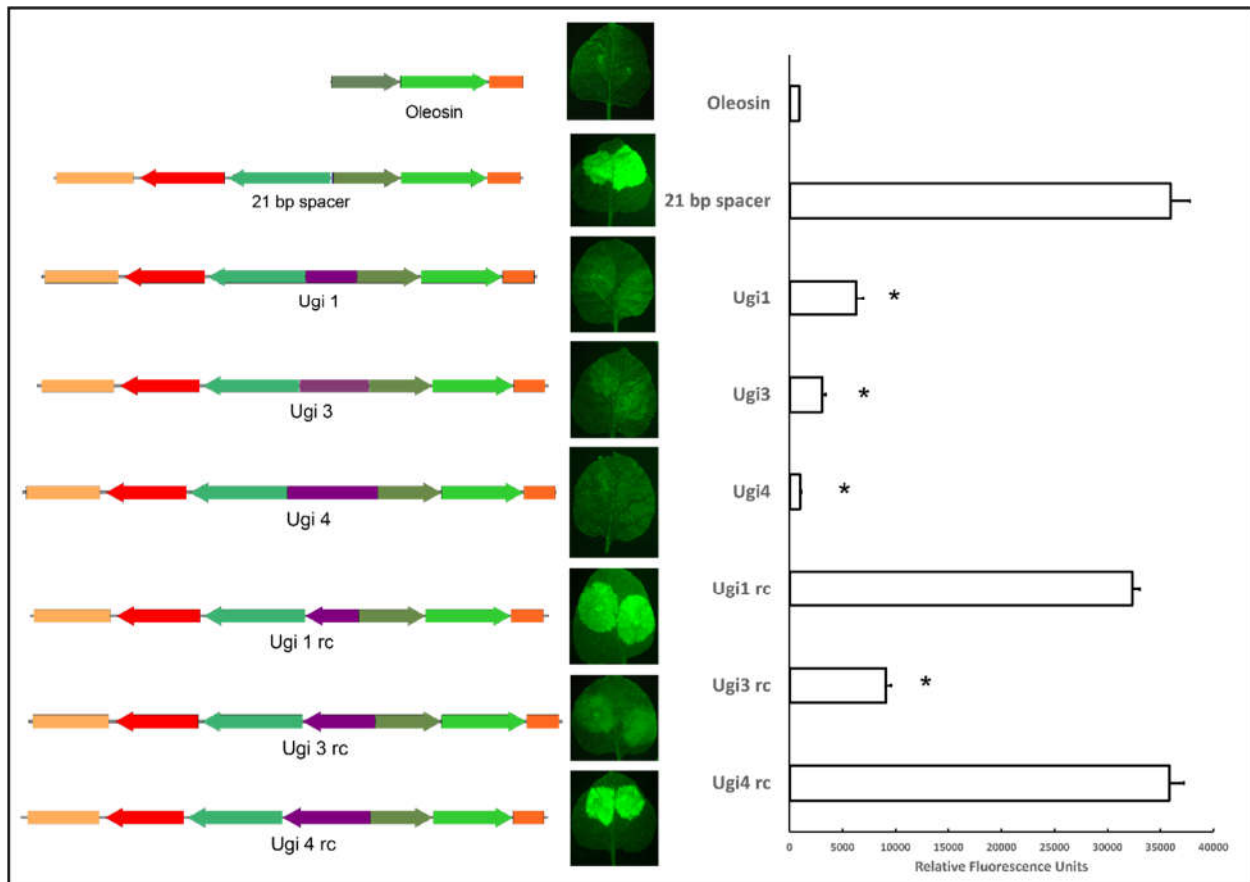


Fig 3. **Bladderwort insulators in forward and reverse (rc) orientation.** Components are as described for Figure 1. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Bladderwort insulators were compared with currently available insulators for plants (Figure 4). The TBS insulator showed high levels of ectopic GFP expression, meaning it has poor insulator activity in our assays. In contrast, EXOB is four times more effective than TBS, despite being half the size, which makes it comparable to Ugi 1. The HIV-LTR sequence also showed insulator activity, though not as effectively as that of EXOB or the Ugi insulators.

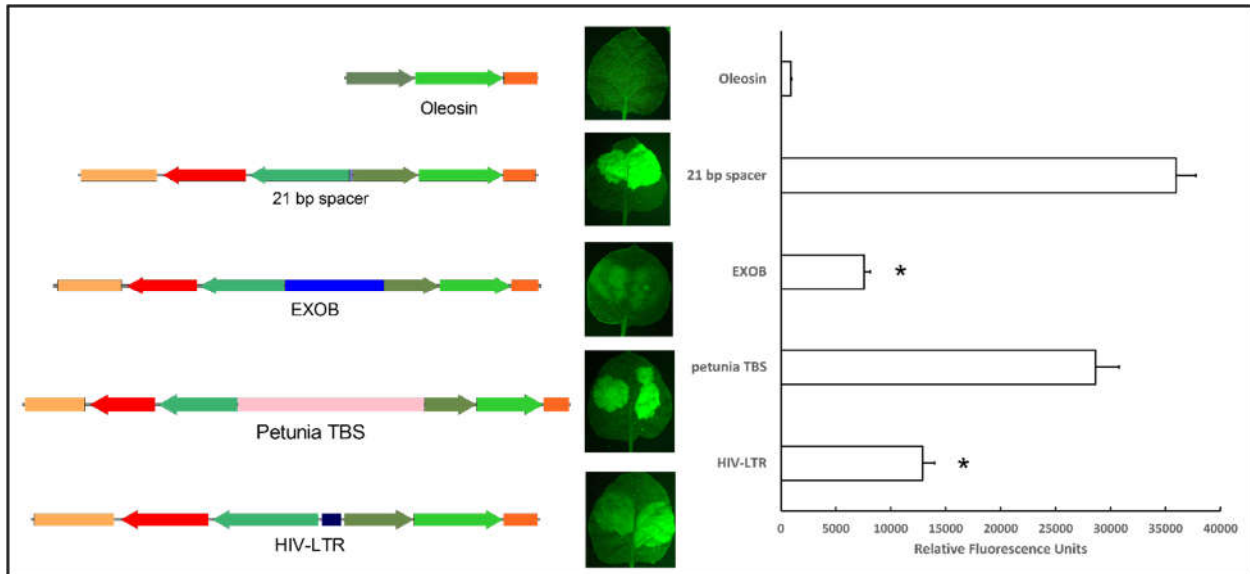


Fig 4. **Insulators previously reported to work in plants.** Components are as described for Figure 1. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean

BEAD1c showed a significantly lower level of GFP expression compared that of the 21-bp spacer control, but still more than double that of the EXOB insulator. GFP expression from the UASrpg construct showed no significant difference compared to that of the 21-bp control, so it has no insulator activity in our test system (Fig 5).

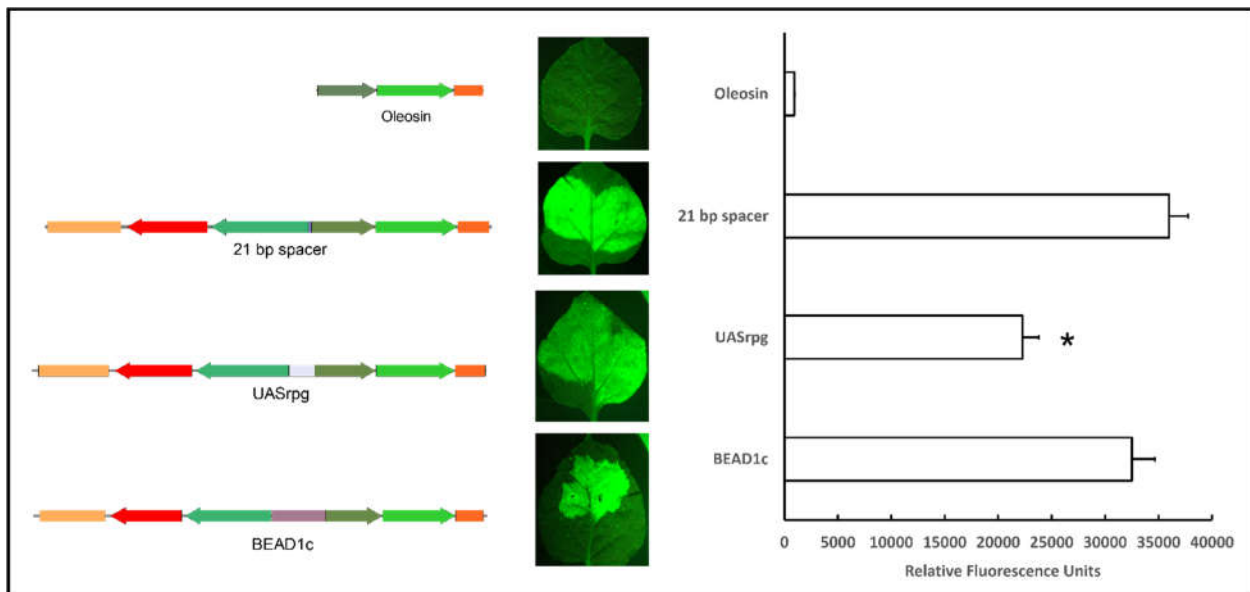


Fig 5. **Fungal and human insulators UASrpg and BEAD1c.** Components are as described for Figure 1. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

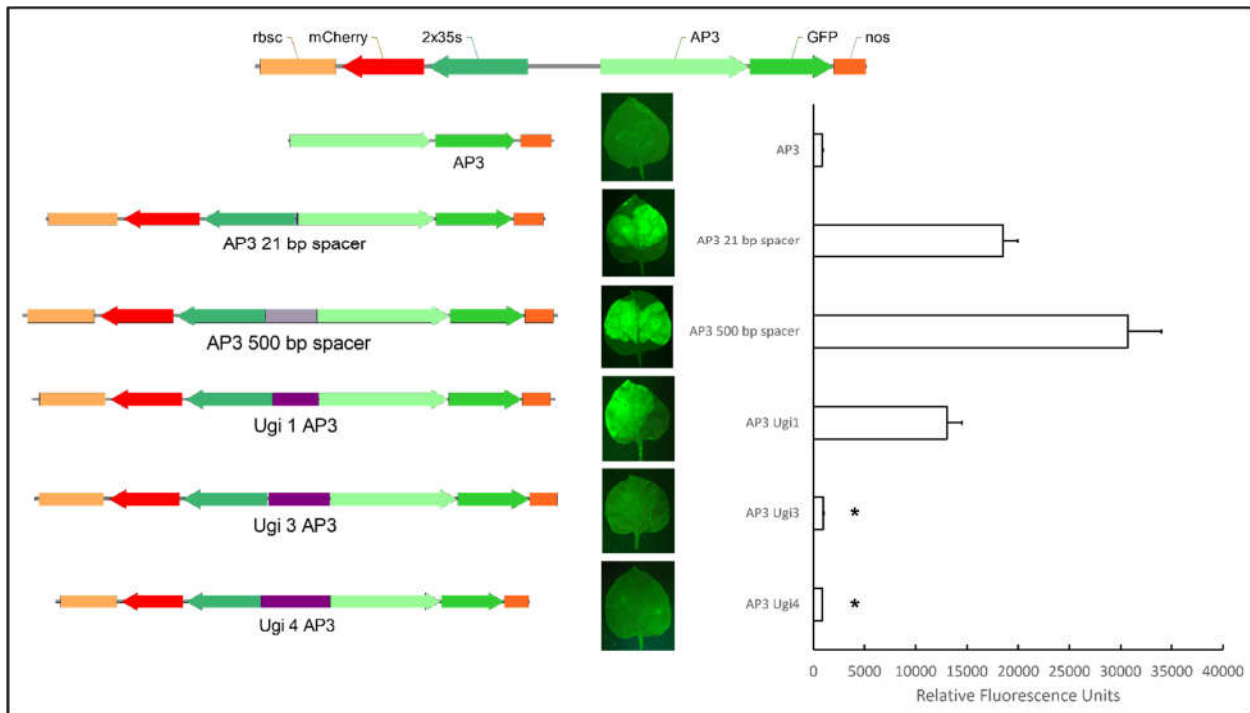


Fig 6. Bladderwort insulators with the flower-specific *Apetala 3* promoter. *rbsc*: rubisco small subunit terminator from *Pisum sativum*, *2x35S*: duplicated 35S promoter, *AP3*: *Arabidopsis thaliana* *Apetala 3* promoter, *nos*: Nopaline synthase terminator. *Indicates t-test significant difference from the control construct (*AP3* promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

BEAD1c and UASrpg are described as being context-dependent [28]. For insulators to be useful in biotechnology, they need to function in a variety of genic contexts. Hence, we tested our bladderwort insulators with a second tissue-specific promoter: the *APETALA3* (*AP3*) flower-specific promoter from *Arabidopsis* [29]. The *AP3* 500-bp spacer construct showed higher ectopic GFP expression than the *AP3* 21-bp spacer construct. *Ugi1* did not show significant differences in GFP expression compared to the 21-bp control ($p = .008$). For *Ugi3* and *Ugi4*, results from this assay very closely matched the results with the oleosin promoter, with respective p values $1.13E^{-06}$ and $1.07E^{-06}$ (Fig. 6).

The silencing ability of insulators in plants has not been sufficiently evaluated. All of the sequences evaluated in this study that showed insulator activity, including *Ugi1*, *Ugi2*, and *Ugi4*, significantly decreased not only GFP expression, but *mCherry* expression as well, with respective p values $1.92E^{-07}$, $4.32E^{-07}$, and $2.40E^{-07}$ (Fig. 7), pointing to the idea that these sequences may be partially silencing the flanking 35S promoter and may better be classified as silencers rather than insulators. While other studies have used a similar construct design to evaluate insulator activity [14], few have described or adequately tested for the effect that the intervening sequence has on the gene driven by 35S. Savitch et al. (2009) tested various sequences with the 35S promoter driving hygromycin phosphotransferase, which they refer to as *hptII* [14]. Transformed plants were selected on medium supplemented with hygromycin, but hygromycin resistance or *hptII* protein expression was not quantified. Other studies utilized a 35S:GFP cassette similar to our *2x35S*:*mCherry* cassette, but did not evaluate GFP expression in any form [12, 30]. Yet other studies using a 35S:GFP cassette confirmed GFP expression via

fluorescent imaging, but did not quantify fluorescence or expression [31]. Only one study evaluating the effect of a gypsy-like sequence from Arabidopsis as an insulator evaluated the gene driven by 35S through real-time RT-PCR and found no significant differences in expression, indicating that silencing was not occurring [13]. True enhancer-blocking insulators can block enhancer-promoter interference without affecting the expression flanking genes, so the evaluation of the gene driven by the enhancer-containing promoter is critical in identifying true insulators.

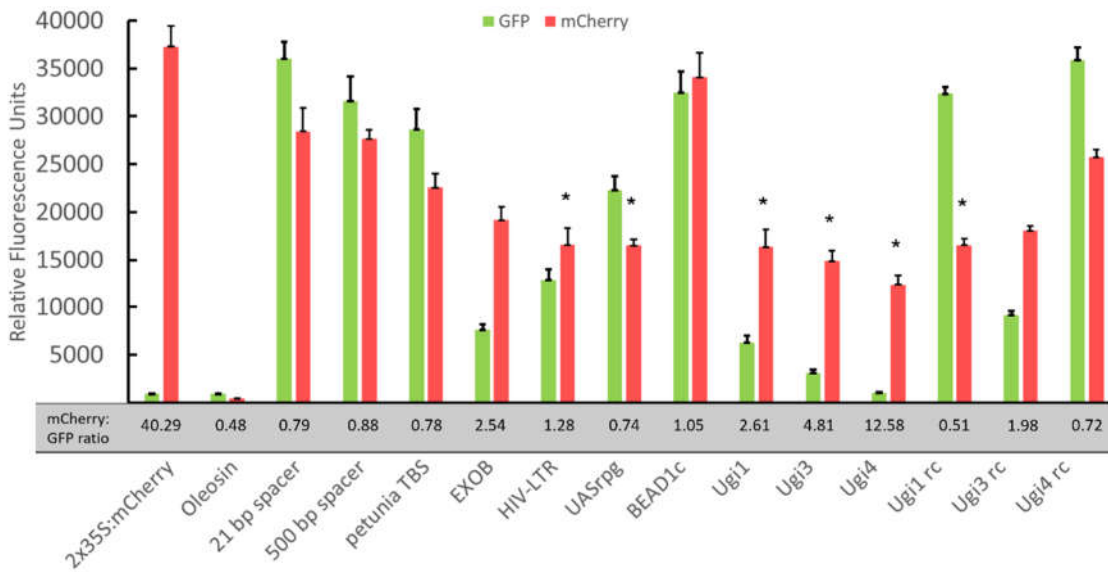


Figure 7. **Effect of putative insulator sequences on the expression of 2x35S:mCherry.** *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Discussion

Variable transgene expression has been an issue with plant transformation since the beginning, though the reason has not always been clear. Since the effect of genomic insertion site on transgene expression is minor among independent, single-copy transgenics [32], any differences in expression probably come from endogenous enhancers in the genome, rather than from complex T-DNA arrangements at the insertion site [6, 32].

In some cases, interference can be attenuated by spacer DNA. In our assays, 500 bp of spacer DNA did not significantly reduce ectopic GFP expression compared to only 21 bp of spacer DNA. In contrast, kilobases of spacer DNA [14] have reduced interference; however, the repeated use of such long pieces of spacer DNA is unrealistic in multigene constructs as transformation efficiency at both the bacterial and plant level is hindered by large cassette sizes [33]. This limitation on vector size dictates that genes be closely spaced to each other, increasing the chance of intergenic interference [34].

The alternative is to use insulators to overcome interference with gene expression, so long as the insulators do not overly inflate the size of the resulting vectors. To find appropriate insulators, this work used a system of vectors very similar to those used previously to identify insulator function [11, 14], but our work differs in several key aspects. First, we devised a bioinformatics screen based on gene expression, closeness of gene pairs, and conservation across asterids to identify sequences with putative insulator function. Secondly, agroinfiltration (the method used in the current study) provides feedback from millions of individually transformed cells, while stably transformed plants are derived from single transformed cells that, depending on the transgene insertion site, can exhibit transcriptional interference from endogenous enhancers. However, our results reflect expression from both integrated and non-integrated T-DNA at three days after infiltration. It is not known if insulators differently affect genes that are not yet integrated into the genome.

Of the 43 putative insulators found, the most promising 10 were chosen for *in planta* validation based on the filtering criteria: degree of expression difference between genes and the length of the putative insulator sequence. Within these candidates, four out of the 10 contain conserved sequences (30-60 bp) based on whole genome alignment with asterids. However, of these, only Ugi4 has significant insulator activity.

Looking at the top 10 identified putative bladderwort insulators from the list of 43, two (Ugi3, Ugi4) show significant insulator activity, reducing ectopic GFP expression significantly compared to the control 21-bp spacer construct, with Ugi4 nearly eliminating GFP expression. A third potential insulator, Ugi1, is effective with the oleosin promoter, but not the *apetala* promoter. The Ugi insulators have the added benefit of being shorter: 451, 619, and 790-bp long, respectively, for Ugi1, Ugi3, and Ugi4. All are smaller than the EXOB insulator, though still longer than desirable. Further analysis can determine which part of the sequence is needed for effective insulator activity in the Ugi insulators.

Collectively, these results indicate that these newly identified *U. gibba* sequences can function as insulators in the sense that they prevent the enhancer in the 2x35S promoter from leading to ectopic expression of GFP in the adjoining cassette. Based on the results in this test system, these Ugi insulators, along with EXOB, may currently be the most effective enhancer-blocking elements for use with plants. However, all these sequences also have silencing activity such that the expression of the 2x35S:mCherry construct is attenuated. They do stand apart in that they have more enhancer-blocking insulator activity than silencing activity than all other tested sequences except EXOB (Figure 7). Hence, they could still have utility in transgenic applications.

Prior to this work, only three plant-derived enhancer-blocking insulators had been reported. Of these, the N129 from *Arabidopsis* is reported to be undependable [14], and the petunia TBS and gypsy-like sequence from *Arabidopsis* are over 2-kb and therefore are too large to be practical in multigene-containing vectors. The petunia TBS is also among the least effective of the insulators tested here. EXOB is the more effective of the pre-existing insulators, while HIV-LTR is intermediate in effectiveness to TBS and EXOB. UASrpg is intermediate to TBS and HIV-LTR in terms of insulator activity.

Ultimately, it is still not clear what constitutes an insulator sequence. CTCF binding sites are components of insulators in animals and are numerous in the EXOB insulator, pointing to their conserved function in plants. However, CTCFs are unique to bilateran phyla [35] and plant sequences without insulation ability also have CTCF binding sites [11], so even though these sequences exist, they are likely not functional in the same way they are in animals and fungi. Likewise, the gypsy insulator from *Drosophila melanogaster* contains several Su(Hw) protein binding sites. *Arabidopsis* lacks the Su(Hw) proteins needed to bind to the gypsy insulator, but insulation was still observed by She et al. (2010) [36], even when Su(Hw) proteins were not co-expressed in the plant, suggesting that an *Arabidopsis* protein was binding to the gypsy insulator and providing insulator activity; however, another study was unable to replicate these results [16].

The observation that insulators previously reported to be effective in plants were not effective in our test system is consistent with the observation that some insulators are context-dependent and are more effective with some genes than others. Furthermore, the same element may function as an insulator, enhancer, or silencer depending on which transcription factors are expressed in any given tissue [37-39]. Therefore, it may be necessary to identify a library of gene- and tissue-specific insulator-like sequences for use in plant transformation.

Given the apparent lack of canonical insulator sequences in plants, plant sequences are better referred to as 'insulator-like.' Regardless, the Ugi sequences identified here can be used as insulators for plant transformation, despite their silencing activity, if moderate expression levels of the flanking gene are sufficient to meet the transformation objectives.

Methods

Plant material

U. gibba plugs were obtained from Meadowview Biological Research Station (<https://www.pitcherplant.org>) and were propagated in three separate tanks. Plugs were anchored in a layer of sand above a layer of peat moss under approximately six inches of municipal water. Illumination was provided by 50-watt LED grow lights (Morsen) were placed outside each tank on a timer of 16 hours on / 8 hours off. Water was changed and the tanks cleaned as needed.

Whole-genome sequencing

DNA from bulk bladderwort tissue was isolated using the Promega Wizard Genomic DNA Purification Kit. Libraries were prepped using a KAPA library prep kit (#KK8231) and sequenced on two Illumina MiSeq flowcells with paired-end 300-bp reads at the Georgia Genomics and Bioinformatics Core.

RNA sequencing

Live bladderwort was placed in ice water and dissected under the microscope to obtain 10 cm each of rhizoids and stems, 30 leaves, and 30 bladders from each of three biological replicates. RNA was isolated from these individual tissues and from whole plants using TRIzol/chloroform extraction (Invitrogen). Full-length RNA transcripts were prepared from bulk tissue using a stranded KAPA kit

(#KK8420) and sequenced on two Illumina MiSeq flowcells, producing paired-end 250-bp reads. Tissue-specific libraries of 3' RNA transcripts were prepared using a Lexogen QuantSeq 3' mRNASeq FWD kit and sequenced on a mid-output NextSeq 500 flowcell, producing single-end 150-bp reads.

Genome assembly and annotation

Raw paired-end genomic reads were merged and trimmed in Trimmomatic v0.36 using the “ILLUMINACLIP” option with default parameters [40], keeping only trimmed reads 36 bp or longer. Read quality was evaluated with FastQC v1.8.0 [41] and MultiQC v1.5 [42]. Genome assembly was performed with SPAdes v3.13.1 [43] with default parameters, and quality metrics determined with QUAST v5.0.2 [44]. Contigs originating from contaminant, mitochondrial, and chloroplast genomes were filtered out using Kraken v2.0.7 [45] with a database of bacterial, fungal, and plant sequences from NCBI plus published *U. gibba* genome sequences [46].

Annotation was performed in multiple rounds using Maker v2.31.10 [47]. First, basic annotation was performed using Trinity v2.6.6 [48] based on whole-transcript RNA-Seq and protein models from four other sequenced asterid genomes: *Daucus carota* (GCF_001625215.1), *Helianthus annuus* (GCF_002127325.1), *Nicotiana attenuate* (GCF_001879085.1), and *Solanum lycopersicum* (GCF_000188115.4). After the initial round of annotation, putative genes were pulled out and used to train a gene prediction model in Augustus v3.2.3 [49]. Gene models were refined in the next rounds of annotation using Augustus gene prediction models trained after each Maker run. Maker was run for three rounds, at which point the Annotation Edit Distance (AED) score distribution stopped improving. Finally, the published PacBio *U. gibba* assembly [20] was used to BLAST against the annotated genome to identify any proteins the pipeline had missed. Only BLAST results with at least 95% sequence similarity and 90% sequence length that did not overlap an existing Maker annotation were kept. Overlaps were found using BedTools intersect v2.29.2, [50].

Mining for regulatory regions using 3' RNA-Seq data

Raw 3' RNA-Seq reads were trimmed using Trimmomatic v0.36 using the “ILLUMINACLIP” option, with max adapter mismatch count equal to two, palindrome clip threshold equal to 30, and match accuracy to 10. Quality metrics were then visualized using FastQC v1.8.0 and MultiQC v1.5 [41, 42]. Reads were mapped to the genome using the STAR aligner v2.7.0 [51] and reads per gene were quantified using htseq-count v0.9.1 [52]. Once counts were obtained, they were normalized in DESeq2 using the standard “DESeq” function [53].

Pairs of genes were extracted and classified as either “divergent,” “convergent,” or “parallel” based on their relative orientations. Expression levels quantified as the relative expression difference between the two genes (“relative fold difference”). Intergenic regions were selected as potential insulators if the flanking genes (1) were both expressed, (2) were in the top 10% of gene pairs based on their relative fold difference, (3) were <1000 bp apart, and (4) the higher-expressed gene was in the top 50% of expressed genes in the dataset. Once candidate regions meeting these criteria were selected, they were prioritized by relative fold change, sequence length (shorter preferred over longer), and lack of correlation of neighboring gene expression across the tissue-specific datasets.

Intergenic sequence conservation and presence of putative insulator sequences

Conserved intergenic sequences were found using whole genome multiple alignments of different clades of angiosperms to the newly assembled *U. gibba* genome. The four clades comprising whole genome alignments were asterids (*Vaccinium corymbosum*, *Actinidia chinensis*, *Lactuca sativa*,

Helianthus annuus, *Cuscuta australis*, *Capsicum annuum*, *Solanum lycopersicum*, *Petunia axillaris*, *Coffea canephora*, *Utricularia gibba*, *Mimulus guttatus*, *Genlisea aurea*), rosids (*Kalanchoe fedtschenkoi*, *Carica papaya*, *Arabidopsis thaliana*, *Durio zibethinus*, *Cannabis sativa*, *Prunus persica*, *Glycine max*, *Medicago truncatula*, *Betula pendula*, *Citrullus lanatus*, *Manihot esculenta*, *Vitis vinifera*), and monocots (*Zostera marina*, *Spirodela polyrhiza*, *Ananas comosus*, *Setaria italica*, *Sorghum bicolor*, *Brachypodium distachyon*, *Oryza sativa*, *Asparagus officinalis*, *Phalaenopsis equestris*). Alignments of each clade to *Utricularia gibba* were completed according to the UCSC genome browser full genome alignment tutorial (http://genomewiki.ucsc.edu/index.php/Whole_genome_alignment_howto), but using LastZ v1.04.03 [54] instead of MultiZ for the initial alignment step. After alignments were completed and combined, PhastCons v1.5 [55] was used to generate genome-wide sets of conserved coordinates for each clade with options target-coverage = 0.125 and expectedlength = 20; all other options were set to default. Analysis of conserved region distribution in gene pairs was carried out with custom R scripts.

Entry vector assembly and transformation

Entry vectors for Golden Gate cloning were made by digesting the Greengate Kit [56] entry vector plasmids (PGGA000, PGGB000, PGGC000, PGGD000, PGGE000, PGGF000) with BSA1-HF V2 (New England Biolabs Inc.). Bladderwort sequences were synthesized (IDT, Twist, Genewiz) and amplified by PCR (Table S3) to create 20-bp of homology with the digested vector on each side. The reaction leaves 21 bp between cassettes. In addition, a 500-bp spacer DNA fragment (Table S4) was randomly generated with the Random DNA Sequence Generator (<http://www.faculty.ucr.edu/~mmaduro/random.htm>).

Gibson assembly was performed by combining 1 μL of digested vector, 1 μL of gel-purified PCR product, and 2 μL of NEBuilder Hifi DNA Assembly Mastermix 2x and incubating at 50°C for 15 minutes. *E. coli* transformation was performed with DH5 α chemically competent cells following the protocol from New England Biolabs. Transformants were screened for the insert sequence with PCR. Positive transformant colonies were cultured overnight and purified using the Epoch GenCatch Plasmid DNA Mini-prep kit. Completed entry vector plasmids were confirmed by Sanger sequencing (Genewiz).

Golden Gate assembly and transformation

Completed entry vector plasmid stocks were quantified by A260 absorbance and diluted to 100 ng μL^{-1} . Golden Gate cloning was performed as described by Decaestecker et al. [57]. The assembled constructs and their components are listed in Table S2. One μL of finished reaction product was transformed into 20 μL *E. coli* competent cells (genotype DH5 α) following the supplied protocol and plated on Luria Bertani (LB) agar plates supplemented with spectinomycin (100 $\mu\text{g mL}^{-1}$) to select for positive transformants. Single positive transformant colonies were cultured overnight and purified using the Epoch GenCatch Plasmid DNA Mini-prep kit. Completed entry vector plasmids were confirmed by Sanger sequencing. The destination vector used for every Golden Gate assembly reaction was pGGP-AG-nptII, created by inserting the Stubi3P:nptII:Stubi3T cassette from AddGene plasmid 59175 between the XbaI/KpnI sites of pGGP-A-G [57].

Agrobacterium electroporation

Twenty-five μL of electrocompetent *Agrobacterium tumefaciens* strain LBA4404 cells were electroporated in an electroporator (Bio-Rad Micropulser) according to the manufacturer's instructions with 1 μL of Golden Gate assembled plasmid prep.

***Nicotiana* agroinfiltration**

Nicotiana benthamiana TW17 plants were grown in 2.54-cm (2 inch) pots using SunGro potting mix. Plants were grown under a 12:12 hour light/dark photoperiod at 22°C and 24°C respectively. Agroinfiltration of 4-week-old plants was carried out as described by Felippes and Weigel 2010 [58]. Twenty mL of LB liquid medium with the appropriate antibiotics were inoculated with a single colony of *A. tumefaciens* and incubated for 48 hours shaking at 28°C. After incubation, cells were pelleted, supernatant was removed, and the cells were resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, 100 μM acetosyringone) and diluted to an optical density of 0.75 and incubated at room temperature for 3 hours. Three leaves in each of three plants were infiltrated per construct with a 1-mL needleless syringe. After infiltration, plants were kept for 3 days at 12-hr daylight, 24°C day, 22°C night temperatures. The Xite Fluorescent Flashlight system (Nightsea, Designation RB-GO) with excitation at 440-460 nm and emission at 500-560 nm was used to visualize GFP in leaves. Pictures were taken with a Canon EOS 60D camera fitted with a green camera lens (Tiffen Green #58), manual settings, a 6-second exposure time, and aperture 2.8.

Fluorescence microtiter plate spectroscopy

Fluorescence microtiter plate spectroscopy of intact *Nicotiana benthamiana* leaf discs was performed as described by [59]. Single leaf discs were obtained with a 6-mm diameter cork borer and floated adaxial side down in the wells of an opaque 96-well plate with 300 μL of water. Fluorescence detection was performed using a Synergy 2 reader with Gen5 version 3.09 software (BioTek Instruments Inc., Winooski, VT, USA) with excitation at 475 - 495 nm, and emission at 508-548 nm for GFP and excitation of 587 nm and emission of 610 nm for mCherry. For each construct, a total of nine leaf discs were sampled across three infiltrated plants. Three leaves were infiltrated per plant, and one leaf disc was taken from each.

Experimental design and statistical analysis

For each treatment there were three biological replicates (three plants) and three technical replicates (three leaf discs). Three leaves were infiltrated per plant, and one leaf disc was sampled from each leaf, resulting in nine leaf discs per treatment. Raw fluorescence reads were compared by analysis of variance (ANOVA) followed by two-sample t-tests between the Oleosin 21 bp spacer or AP3 21 bp spacer controls and the bladderwort insulator outputs to determine significant differences in GFP expression. All statistically significant results were judged based on a *p* value of ≤.001.

Materials availability

Ugi1, Ugi2, and Ugi3 are available as GenBank OK086967, OK086968, and OK086969, respectively. Plasmids with the Ugi sequences are also publicly available on AddGene (ID numbers 177194, 177195, 177196)]

All bioinformatic scripts for this analysis, including assembly and annotation, are available at <https://github.com/lkov0/bladderwort-analysis>. Raw DNA, RNA, and 3' RNA reads are available at the NCBI Sequence Read Archive (BioProject PRJNA595351).

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Author contributions

LK: Carried out bioinformatic analyses; wrote & edited manuscript

PRL: Designed and help build all the vectors for the project

JP: Performed molecular cloning and *in planta* assays; wrote & edited manuscript

WAP: Conceived the project & initial experimental design, supervised execution; edited manuscript

JGW: Designed experiments; supervised analyses; edited manuscript

Competing interests

The authors all declare to have no competing interests.

References

1. ISAAA, *Global status of commercialized biotech/GM crops: 2019 ISAAA Brief No. 55*. 2019: Ithaca, NY.
2. Kurbidaeva, A. and M. Purugganan, *Insulators in plants: progress and open questions*. *Genes*, 2021. **12**(9): p. 1422.
3. Segert, J.A., S.S. Gisselbrecht, and M.L. Bulyk, *Transcriptional silencers: Driving gene expression with the brakes on*. *Trends in Genetics*, 2021. **37**(6): p. 514-527.
4. Singer, S.D., K.D. Cox, and Z. Liu, *Enhancer-promoter interference and its prevention in transgenic plants*. *Plant Cell Rep*, 2011. **30**(5): p. 723-31.
5. Krivega, I. and A. Dean, *Enhancer and promoter interactions-long distance calls*. *Curr Opin Genet Dev*, 2012. **22**(2): p. 79-85.
6. Zheng, X., et al., *The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters*. *Plant Cell Reports*, 2007. **26**(8): p. 1195-1203.
7. Gaszner, M. and G. Felsenfeld, *Insulators: exploiting transcriptional and epigenetic mechanisms*. *Nat Rev Genet*, 2006. **7**(9): p. 703-13.
8. Yang, J. and V.G. Corces, *Chromatin insulators: a role in nuclear organization and gene expression*. *Adv Cancer Res*, 2011. **110**: p. 43-76.
9. Allen, G.C., et al., *Scaffold attachment regions increase reporter gene expression in stably transformed plant cells*. *The Plant Cell*, 1993. **5**(6): p. 603-613.
10. Shabarina, A.N. and M.V. Glazkov, *Nuclear envelope attachment sites of interphase chromosomes: Barrier elements but not insulators*. *Russian Journal of Genetics*, 2012. **48**(8): p. 864-867.

11. Hily, J.-M., et al., *A transformation booster sequence (TBS) from Petunia hybrida functions as an enhancer-blocking insulator in Arabidopsis thaliana*. Plant Cell Reports, 2009. **28**(7): p. 1095-1104.
12. Yang, Y., S.D. Singer, and Z. Liu, *Evaluation and comparison of the insulation efficiency of three enhancer-blocking insulators in plants*. Plant Cell, Tissue and Organ Culture (PCTOC), 2011. **105**(3): p. 405-414.
13. Singer, S.D. and K.D. Cox, *A gypsy-like sequence from Arabidopsis thaliana exhibits enhancer-blocking activity in transgenic plants*. Journal of Plant Biochemistry and Biotechnology, 2013. **22**(1): p. 35-42.
14. Gudynaite-Savitch, L., D.A. Johnson, and B.L.A. Miki, *Strategies to mitigate transgene-promoter interactions*. Plant Biotechnology Journal, 2009. **7**(5): p. 472-485.
15. Zhang, Y.M., et al., *Functional analysis of the HS185 regulatory element in the rice HSP70 promoter*. Mol Biol Rep, 2012. **39**(2): p. 1649-57.
16. Tran, A. and D.A. Johnson, *Mutational analysis identifies functional Rap1, Su(Hw), and CTCF insulator sites in Arabidopsis thaliana*. Plant Cell Rep, 2020. **39**(12): p. 1743-1753.
17. Korkuc, P., J.H. Schippers, and D. Walther, *Characterization and identification of cis-regulatory elements in Arabidopsis based on single-nucleotide polymorphism information*. Plant Physiol, 2014. **164**(1): p. 181-200.
18. Xiohan Yang, C.W., Susheng Gan, *Genome-wide analysis of the intergenic regions in Arabidopsis thaliana suggests the existence of bidirectional promoters and genetic insulators*. Current Topics in Plant Biology, 2012. **12**: p. 19.
19. Greilhuber, J., et al., *Smallest angiosperm genomes found in Lentibulariaceae, with chromosomes of bacterial size*. Plant Biology, 2006. **8**(6): p. 770-777.
20. Lan, T., et al., *Long-read sequencing uncovers the adaptive topography of a carnivorous plant genome*. Proceedings of the National Academy of Sciences, 2017. **114**(22): p. E4435-E4441.
21. Ibarra-Laclette, E., et al., *Architecture and evolution of a minute plant genome*. Nature, 2013. **498**(7452): p. 94-98.
22. Simão, F.A., et al., *BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs*. Bioinformatics, 2015. **31**(19): p. 3210-3212.
23. Padidam, M. and Y. Cao, *Elimination of transcriptional interference between tandem genes in plant cells*. Biotechniques, 2001. **31**(2): p. 328-30, 332-4.
24. Weber, B., et al., *Plant Enhancers: A Call for Discovery*. Trends in Plant Science, 2016. **21**(11): p. 974-987.
25. Jagannath, A., et al., *The use of a Spacer DNA fragment insulates the tissue-specific expression of a cytotoxic gene (barnase) and allows high-frequency generation of transgenic male sterile lines in Brassica juncea L*. Molecular Breeding, 2001. **8**(1): p. 11-23.
26. Hark, A.T., et al., *CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus*. Nature, 2000. **405**(6785): p. 486-489.
27. Bell, A.C. and G. Felsenfeld, *Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene*. Nature, 2000. **405**(6785): p. 482-5.
28. Tran, A. and D.A. Johnson, *Mutational analysis identifies functional Rap1, Su(Hw), and CTCF insulator sites in Arabidopsis thaliana*. Plant Cell Reports, 2020. **39**(12): p. 1743-1753.
29. Sundström, J.F., et al., *Direct regulation of the floral homeotic APETALA1 gene by APETALA3 and PISTILLATA in Arabidopsis*. Plant J, 2006. **46**(4): p. 593-600.
30. Singer, S.D., J.M. Hily, and K.D. Cox, *Analysis of the enhancer-blocking function of the TBS element from Petunia hybrida in transgenic Arabidopsis thaliana and Nicotiana tabacum*. Plant Cell Rep, 2011. **30**(11): p. 2013-25.

31. Singer, S.D., J.-M. Hily, and Z. Liu, *A 1-kb Bacteriophage Lambda Fragment Functions as an Insulator to Effectively Block Enhancer–Promoter Interactions in Arabidopsis thaliana*. *Plant Molecular Biology Reporter*, 2009. **28**(1): p. 69.
32. Betts, S.D., et al., *Uniform expression and relatively small position effects characterize sister transformants in maize and soybean*. *Frontiers in Plant Science*, 2019. **10**(1209).
33. Hamilton, C.M., *A binary-BAC system for plant transformation with high-molecular-weight DNA*. *Gene*, 1997. **200**(1): p. 107-116.
34. Komori T, U.J., Komari T, *Transformation and expression of foreign genes in higher plants, in Plant Transformation Technology Revolution in Last Three Decades - Vol 1: Historical Technology Developments in Plant Transformation*. 2011, Bentham Science Publishers. p. 55-75.
35. Heger, P., et al., *The chromatin insulator CTCF and the emergence of metazoan diversity*. 2012. **109**(43): p. 17507-17512.
36. She, W., et al., *The gypsy insulator of Drosophila melanogaster, together with its binding protein suppressor of hairy-wing, facilitate high and precise expression of transgenes in Arabidopsis thaliana*. *Genetics*, 2010. **185**(4): p. 1141.
37. Pang, B. and M.P. Snyder, *Systematic identification of silencers in human cells*. *Nature Genetics*, 2020. **52**(3): p. 254-263.
38. Kolovos, P., et al., *Enhancers and silencers: an integrated and simple model for their function*. *Epigenetics & Chromatin*, 2012. **5**(1): p. 1.
39. Doni Jayavelu, N., et al., *Candidate silencer elements for the human and mouse genomes*. *Nature Communications*, 2020. **11**(1): p. 1061.
40. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data*. *Bioinformatics*, 2014. **30**(15): p. 2114-20.
41. Andrews, S., *FastQC: A quality control tool for high throughput sequence data*. 2010.
42. Ewels, P., et al., *MultiQC: summarize analysis results for multiple tools and samples in a single report*. *Bioinformatics*, 2016. **32**(19): p. 3047-3048.
43. Bankevich, A., et al., *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing*. *J Comput Biol*, 2012. **19**(5): p. 455-77.
44. Gurevich, A., et al., *QUAST: quality assessment tool for genome assemblies*. *Bioinformatics*, 2013. **29**(8): p. 1072-5.
45. Wood, D.E., J. Lu, and B. Langmead, *Improved metagenomic analysis with Kraken 2*. *Genome Biol*, 2019. **20**(1): p. 257.
46. Wood, D.E. and S.L. Salzberg, *Kraken: ultrafast metagenomic sequence classification using exact alignments*. *Genome Biology*, 2014. **15**(3): p. R46.
47. Holt, C. and M. Yandell, *MAKER2: an annotation pipeline and genome-database management tool for second-generation genome projects*. *BMC Bioinformatics*, 2011. **12**(1): p. 491.
48. Haas, B.J., et al., *De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis*. *Nat Protoc*, 2013. **8**(8): p. 1494-512.
49. Stanke, M., et al., *AUGUSTUS: a web server for gene finding in eukaryotes*. *Nucleic Acids Research*, 2004. **32**(suppl_2): p. W309-W312.
50. Quinlan, A.R. and I.M. Hall, *BEDTools: a flexible suite of utilities for comparing genomic features*. *Bioinformatics*, 2010. **26**(6): p. 841-842.
51. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner*. *Bioinformatics*, 2013. **29**(1): p. 15-21.
52. Anders, S., P.T. Pyl, and W. Huber, *HTSeq—a Python framework to work with high-throughput sequencing data*. *Bioinformatics*, 2014. **31**(2): p. 166-169.
53. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2*. *Genome Biology*, 2014. **15**(12): p. 550.

54. Harris, R.S., *Improved pairwise alignment of genomic DNA*. 2007, Pennsylvania State University.
55. Siepel, A., et al., *Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes*. *Genome Res*, 2005. **15**(8): p. 1034-50.
56. Lampropoulos, A., et al., *GreenGate - A novel, versatile, and efficient cloning system for plant transgenesis*. *PLOS ONE*, 2013. **8**(12): p. e83043.
57. Decaestecker, W., et al., *CRISPR-TSKO: A technique for efficient mutagenesis in specific cell types, tissues, or organs in Arabidopsis*. *The Plant Cell*, 2019. **31**(12): p. 2868-2887.
58. de Felippes, F.F. and D. Weigel, *Transient assays for the analysis of miRNA processing and function*, in *Plant MicroRNAs: Methods and Protocols*, B.C. Meyers and P.J. Green, Editors. 2010, Humana Press: Totowa, NJ. p. 255-264.
59. MacDonald, J., et al., *Co-expression with the type 3 secretion chaperone CesT from enterohemorrhagic E. coli increases accumulation of recombinant Tir in plant chloroplasts*. *Frontiers in Plant Science*, 2017. **8**(283).

Figure legends

Fig 1. **Test construct design, GFP fluorescence in infiltrated *Nicotiana benthamiana* leaves, and representative leaf images.** rbsc: rubisco small subunit terminator from *Pisum sativum*, 2x35S: duplicated 35S promoter, Oleosin: *Glycine max* oleosin promoter, nos: Nopaline synthase terminator. Construct names denote the sequence between the 2x35S and Oleosin promoter. Lack of GFP expression is indicative of insulator activity. In this figure, placing the 35S:mCherry cassette in front of oleosin:GFP leads to ectopic expression of GFP. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Fig 2. ***Utricularia gibba* stems, bladder, leaves, and growth architecture.**

Fig 3. **Bladderwort insulators in forward and reverse (rc) orientation.** Components are as described for Figure 1. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Fig 4. **Insulators previously reported to work in plants.** Components are as described for Figure 1. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean

Fig 5. **Fungal and human insulators UASrpg and BEAD1c.** Components are as described for Figure 1. *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Fig 6. **Bladderwort insulators with the flower-specific *Apetala 3* promoter.** rbsc: rubisco small subunit terminator from *Pisum sativum*, 2x35S: duplicated 35S promoter, AP3: *Arabidopsis thaliana* *Apetala 3* promoter, nos: Nopaline synthase terminator. *Indicates t-test significant difference from the control construct (AP3 promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Figure 7. **Effect of putative insulator sequences on the expression of 2x35S:mCherry.** *Indicates t-test significant difference from the control construct (Oleosin promoter with 21 bp between cassettes) $p < 0.001$. Error bars represent standard error of the mean.

Supplementary material

Table S1: Bladderwort insulator candidates

Bladderwort insulator	Length (bp)
Ugi1	451
Ugi2	23
Ugi3	619
Ugi4	790
Ugi6	124
Ugi7	194
Ugi11	342
Ugi14	74
Ugi15	258
Ugi20	24

Table S2: Genome assembly metrics

	Reference Genome [20]	PP01 draft assembly
Length	102Mb	101Mb
# Contigs	581	13033
# Genes	29,666	27,166
BUSCO %	88%	84%

Table S3: Vector components

Plasmid	AB	BC	CD	DE	EF	FG	AG
Oleosin	<i>Glycine max</i> oleosin promoter	pGGB003 24 bp spacer DNA	GFP	pGGD002 24 bp spacer DNA	Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII

					pGGD002	<i>Pisum sativum</i> rubisco small subunit terminator	24 bp spacer DNA	pGGP-AG_np tII
2x35S:mCherry	duplicate d 35S promoter	pGGB003 24 bp spacer DNA	pGGC015 mCherry					
21 bp spacer	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	21 bp spacer DNA	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
500 bp spacer	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	500 bp spacer DNA	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
EXOB	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	EXOB insulator	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
petunia TBS	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	<i>Petunia hybrida</i> transformation booster sequence	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
HIV-LTR	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	long terminal repeat from the HIV-1 virus	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
Ugi1	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	<i>Utricularia gibba</i> insulator 1	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
Ugi3	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	<i>Utricularia gibba</i> insulator 3	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
Ugi4	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	<i>Utricularia gibba</i> insulator 4	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	
Ugi1 rc	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2x35S promoter	<i>Utricularia gibba</i> insulator 1 reverse complement	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII	

			<i>Utricularia gibba</i>			24	
		<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	insulator 2 reverse complement	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	bp spacer DNA	pGGP-AG_np tII
Ugi3 rc	21 bp spacer DNA						
			<i>Utricularia gibba</i>			24	
		<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	insulator 4 reverse complement	<i>Glycine max</i> oleosin promoter	GFP:Nopaline synthase terminator	bp spacer DNA	pGGP-AG_np tII
Ugi4 rc	21 bp spacer DNA						
		<i>Arabidopsis thaliana</i> Apetala 3 promoter		pGGD002 24 bp spacer DNA	Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII
AP3		pGGB003 24 bp spacer DNA	<i>GFP</i>				
				<i>Arabidopsis thaliana</i> Apetala 3 promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII
AP3 21 bp spacer	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	21 bp spacer DNA				
				<i>Arabidopsis thaliana</i> Apetala 3 promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII
AP3 500 bp spacer	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	500 bp spacer DNA				
				<i>Arabidopsis thaliana</i> Apetala 3 promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII
AP3 Ugi1	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	<i>Utricularia gibba</i> insulator 1				
				<i>Arabidopsis thaliana</i> Apetala 3 promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII
AP3 Ugi3	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	<i>Utricularia gibba</i> insulator 3				
				<i>Arabidopsis thaliana</i> Apetala 3 promoter	GFP:Nopaline synthase terminator	24 bp spacer DNA	pGGP-AG_np tII
AP3 Ugi4	21 bp spacer DNA	<i>Pisum sativum</i> rubisco small subunit terminator:mCherry:2 x35S promoter	<i>Utricularia gibba</i> insulator 4				

Table S3: Entry vector primer sequences

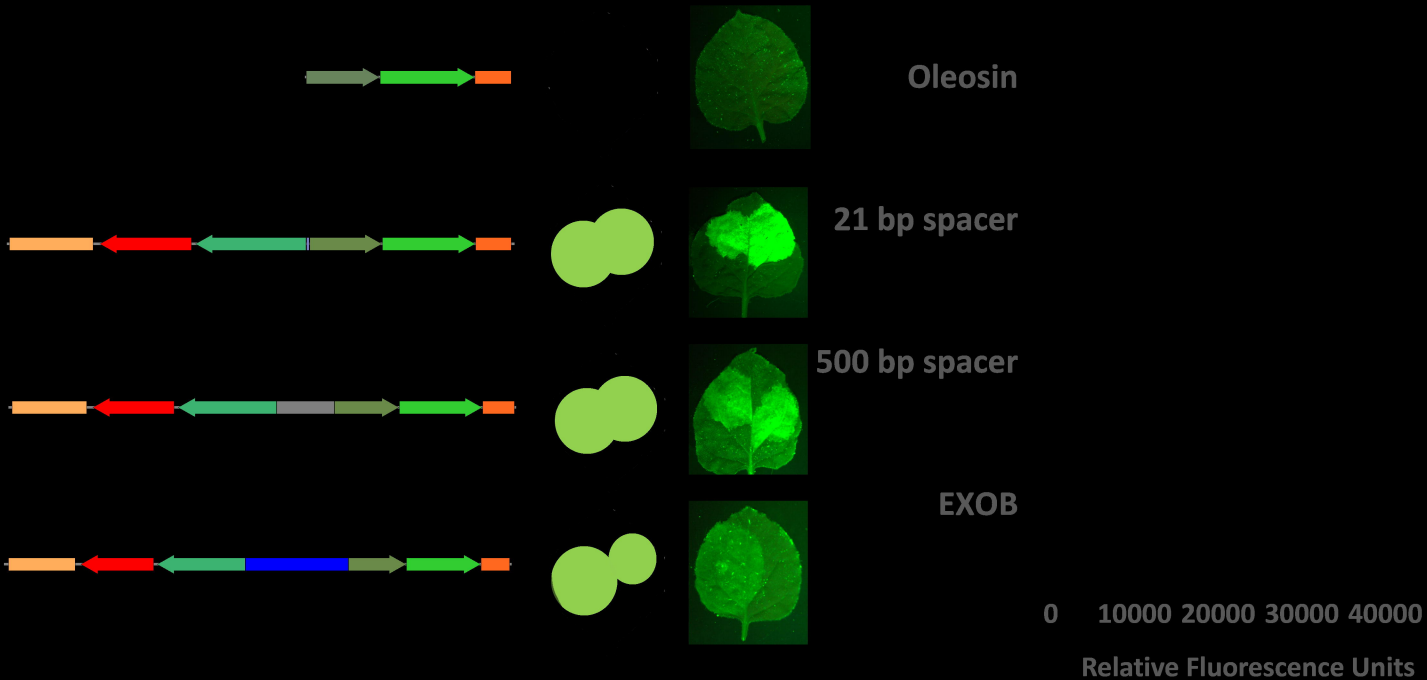
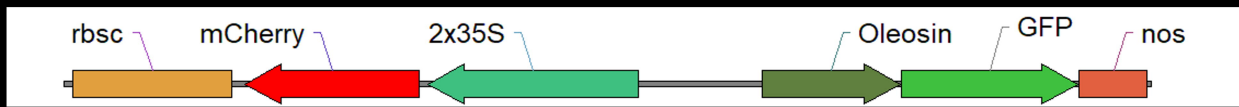
	Primers for PCR
AB-F	5'- AGAAGTGAAGCTTGGTCTCAACCT – insert forward
AB-R	5'- AGGGCGAGAATTCGGTCTCATGTT – insert reverse
BC-F	5'- AGAAGTGAAGCTTGGTCTCAAACA – insert forward
BC-R	5'- AGGGCGAGAATTCGGTCTCAAGCC – insert reverse
CD-F	5'- AGAAGTGAAGCTTGGTCTCAGGCT – insert forward

CD-R	5'- AGGGCGAGAATTCGGTCTCACTGA – insert reverse
DE-F	5'- AGAAGTGAAGCTTGGTCTCATCAG insert forward
DE-R	5'- AGGGCGAGAATTCGGTCTCAGCAG - insert reverse
EF-F	5'- AGAAGTGAAGCTTGGTCTCACTGC – insert forward
EF-R	5'- AGGGCGAGAATTCGGTCTCATAGT – insert reverse
FG-F	5'- AGAAGTGAAGCTTGGTCTCAACTA – insert forward
FG-R	5'- AGGGCGAGAATTCGGTCTCAATAC – insert reverse

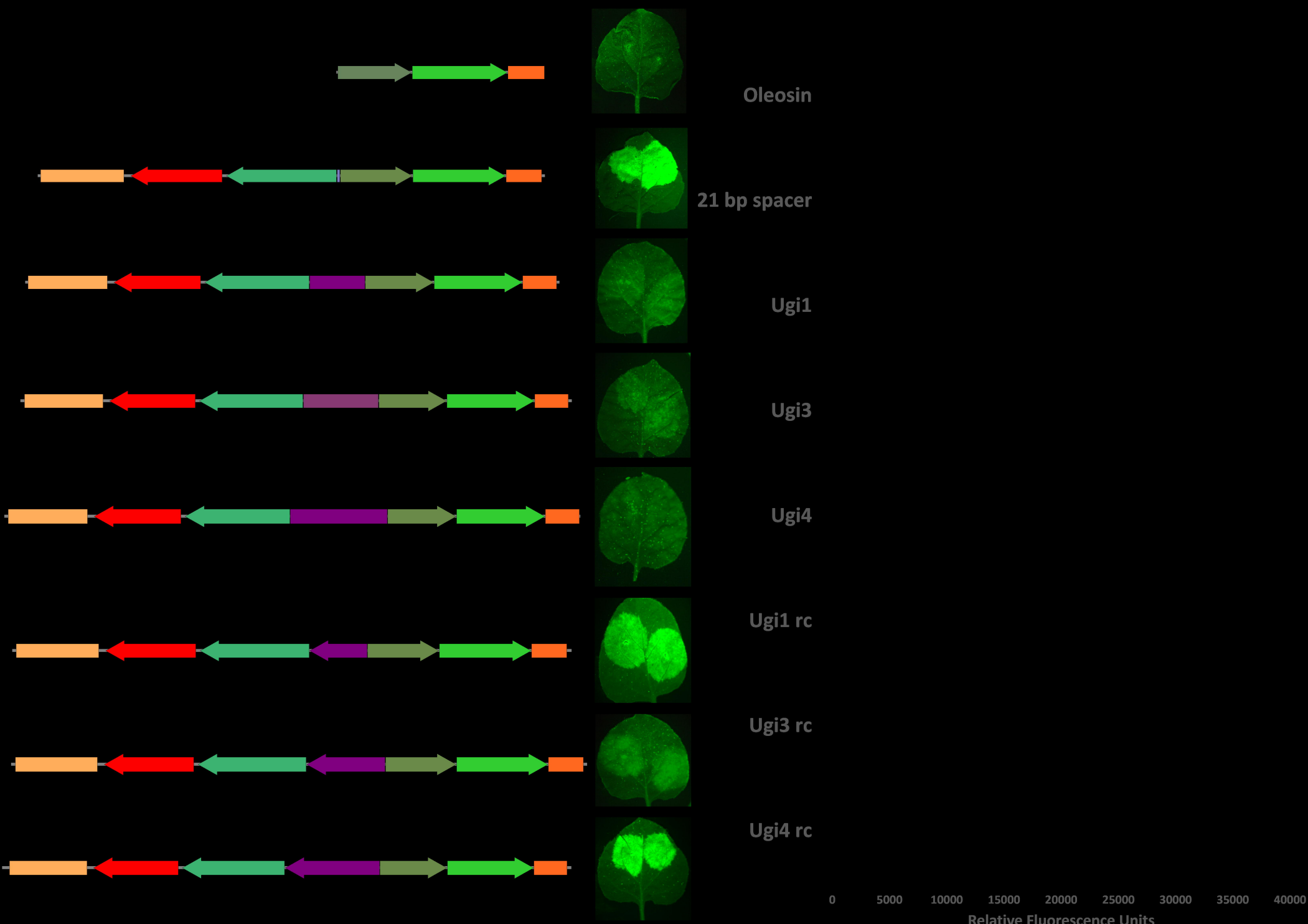
Table S4: Spacer DNA sequences *

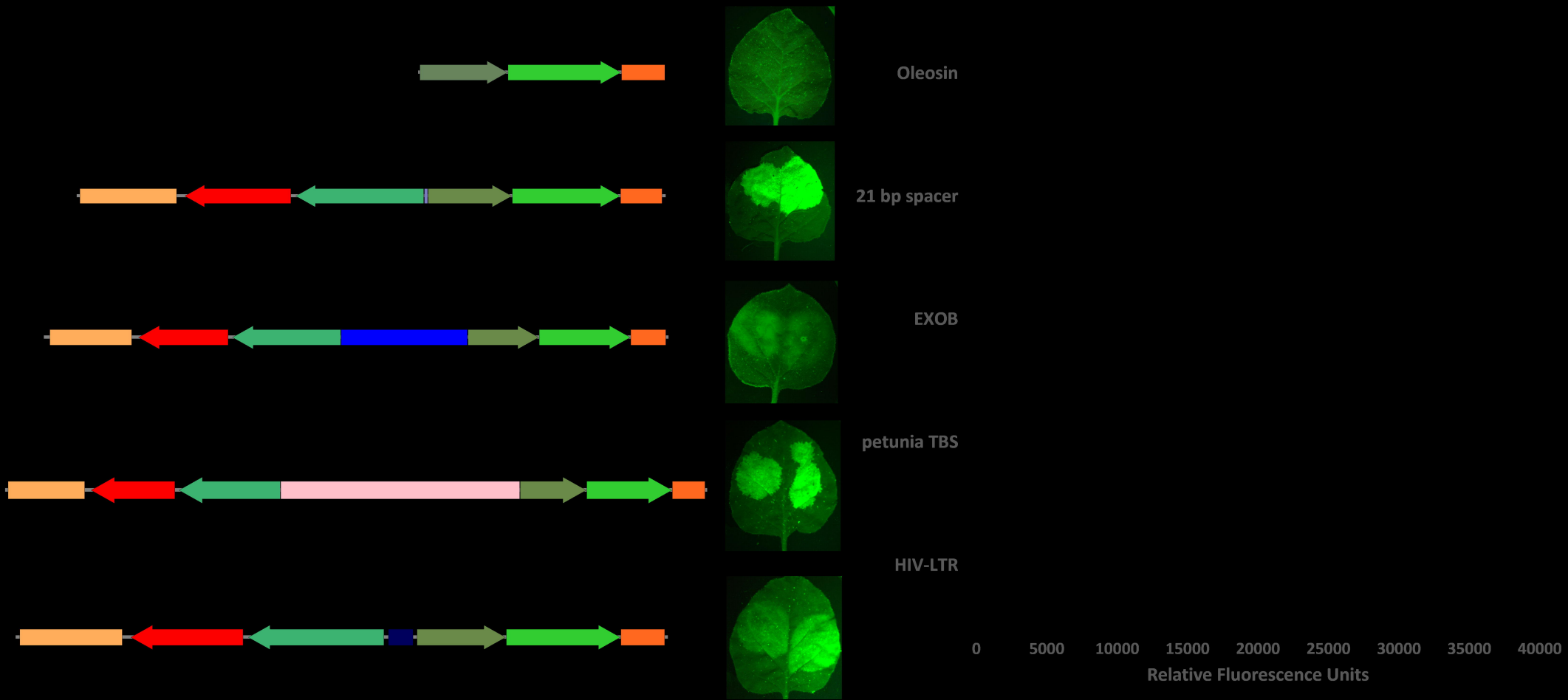
Spacer DNA	Sequence
21 bp spacer (AB)	TCTTTAAATCGAGGAAACAGA
21 bp spacer (CD)	TAGATACCTTTACTCATCCTG
24 bp spacer (FG)	CGCACCATCCATTGTCGTGGTGAC
500 bp spacer (CD)	GCGTTGGAACGTCTTAACTCTGGCAGGCAATTAAGGGAACGTATATATAACGCAAAAAAACTGGAAAATTGGCGAG ATACTAGACGTATACTGTCCGCCTTGCAGACGAAACCAGTCGGAGATTACCGAGCCTTCTATCACGTCGGCGACCACTA AAC

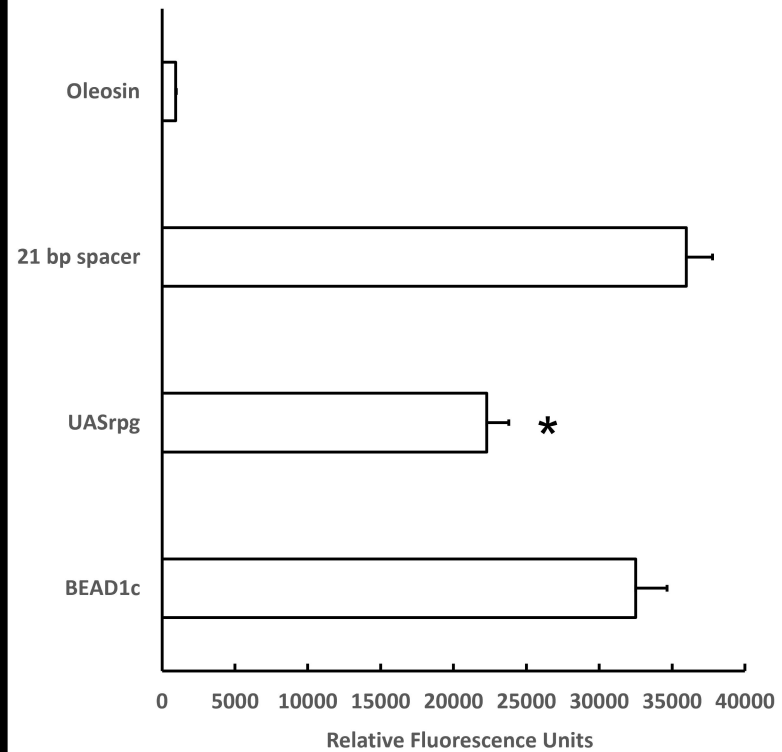
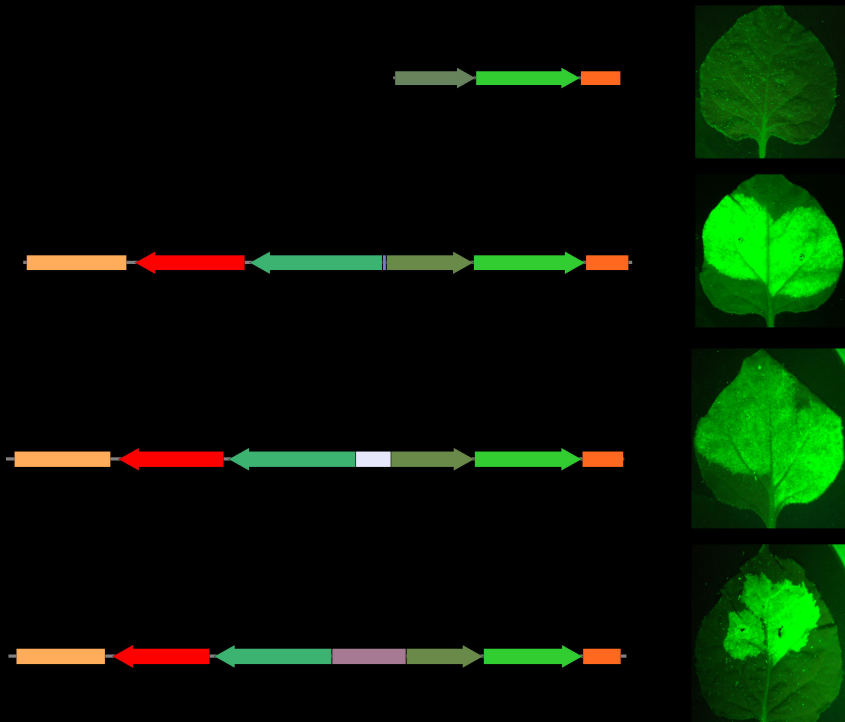
*The above sequence was randomly generated with <http://www.faculty.ucr.edu/~mmaduro/random.htm>. Translational start sites were removed.

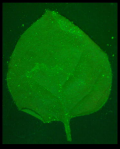




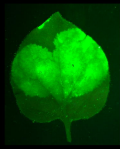




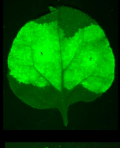




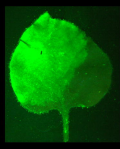
AP3



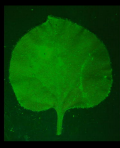
AP3 21 bp spacer



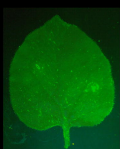
AP3 500 bp spacer



AP3 Ugi1



AP3 Ugi3



AP3 Ugi4

